THE ROLE OF IMMUNE EFFECTORS IN MONOCROTALINE PYRROLE-INDUCED PULMONARY INJURY

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ABSTRACT

THE ROLE OF IMMUNE EFFECTORS IN MONOCROTALINE PYRROLE-INDUCED PULMONARY INJURY

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Monocrotaline (MCT) is a pyrrolizidine alkaloid found in the plant Crotalaria spectabilis which causes pulmonary hypertension in rats. Pulmonary lesions caused by MCT in rats are similar to lesions found in humans having primary pulmonary hypertension. Thus, the MCT-treated rat is a good animal model for primary pulmonary hypertension in humans. MCT is metabolically activated to the proximate toxicant, monocrotaline pyrrole (MCTP), by the mixed function oxidases in the liver. MCTP then travels to the lungs via the circulation where damage occurs. The mechanisms by which MCTP causes the damage are unknown.

Initial studies were undertaken to evaluate the time-course of injury in rats after a single injection of chemically synthesized MCTP. The onset of injury due to MCTP is delayed 4-7 days after injection, which suggests that MCTP acts via indirect mechanisms. The time-course of the injury and histologic lesions in the lungs are consistent with the possibility that immune-mediated mechanisms may be involved in the pathogenesis. Accordingly, the role of immune effector systems in pulmonary injury due to MCTP were evaluated.

Rats co-treated with the immunosuppressant agents antilymphocyte serum or cyclosporin A and MCTP were not protected from injury due to MCTP. Adoptive transfer of lymphocytes from MCTP-treated donors did not alter the

severity or the time-course of injury due to MCTP in recipients of cells. Thus, cell-mediated immune mechanisms are not involved in the pathogenesis of MCTP-induced pulmonary injury.

The role of the complement system was evaluated by measuring serum hemolytic complement activity in rats after MCTP, and by depleting serum copmlement in MCTP-treated rats. Hemolytic complement activity is not changed in vivo after MCTP, and complement depletion does not protect against injury due to MCTP indicating that the complement system is not a mediator of pulmonary damage due to MCTP.

The possibility that toxic oxygen metabolites may contribute to the injury caused by MCTP was evaluated by co-treating rats with MCTP and desferroxamine mesylate, dimethylsulfoxide or catalase. These agents did not protect lungs from injury due to MCTP indicating that toxic oxygen metabolites are not involved as important mediators of the injury.

The effect of diethylcarbamazine (DEC) on MCTP-induced injury also was tested. DEC delayed the onset of injury due to MCTP, but did not protect lungs from injury due to MCTP.

To Mom, Dad, Terry and Cathy with many thanks

for tremendous help, support and love

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INTRODUCTION

I. PYRROLIZIDINE ALKALOIDS (PZA):

A. General

Monocrotaline (MCT) is a member of a class of compounds called the pyrrolizidine alkaloids (PZA). The PZA are toxic chemicals found in many varieties of plants that are widely spread geographically, occurring on all continents and found in many families. One or more species in at least 41 genera contain PZA (Bull et al., 1968). These PZA-containing plants cause tremendous economic losses through livestock poisoning and also are responsible for human illness and mortality.

There have been approximately 150 PZA identified. These occur as the free alkaloid and also as alkaloidal N-oxides. The base structure of the PZA is two fused, five-membered rings with a nitrogen atom in the center (Chemical Abstracts designation: hexahydro 1H-pyrrolizine) (Huxtable, 1979) (Figure 1). Different substitutions on the ring structure at positions 1 and 7 make up the many known PZA. There also are many PZA that have not been completely identified structurally and it is likely many PZA wil be discovered in the future (Huxtable, 1979).

B. Human and Animal Intoxication with PZA

The PZA are a major public health, veterinary and economic problem (Huxtable, 1979). Human poisoning has occurred in all areas of the world, especially in developing third world countries where grain crops have been contaminated with Senecio and Heliotropium families. Mass outbreaks of PZA

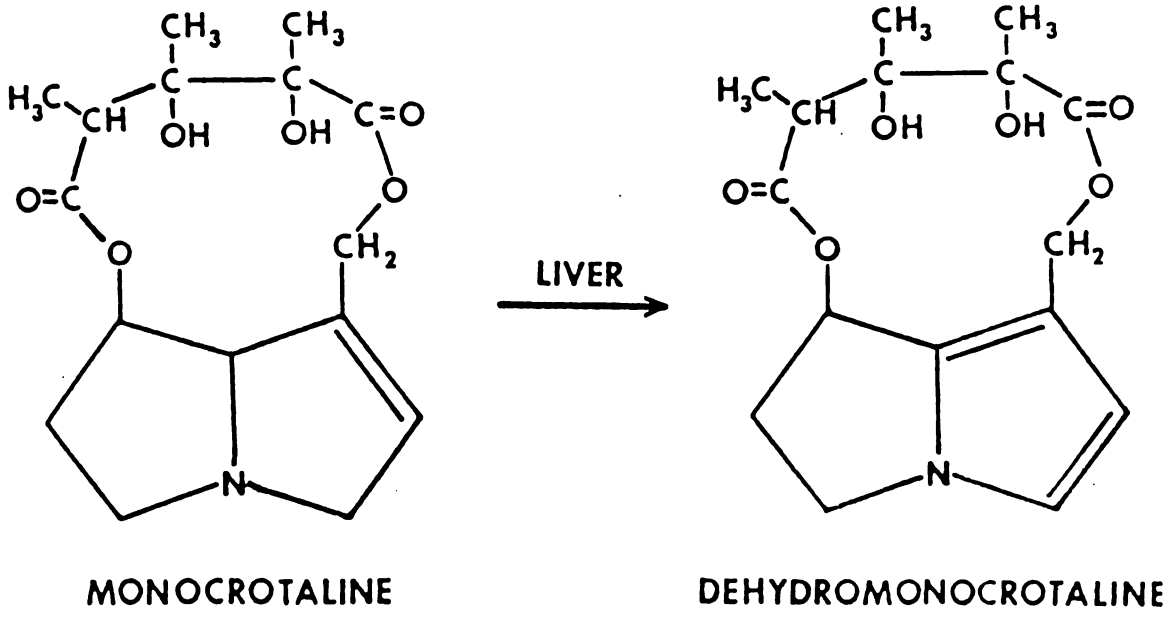


Figure 1

Figure 1. Structures of monocrotaline and its toxic hepatic metabolite, dehydromonocrotaline or monocrotaline pyrrole.

intoxication have occurred in Afghanistan (Mohabbat et al., 1976) and India (Tandon et al., 1976) due to contamination of grain made into flour for bread.

Humans have been poisoned after consuming PZA present in various herbal medications and "natural teas". In Jamaica, veno-occlusive disease of the liver is endemic due to the consumption of teas prepared from leaves of wild scrub (Kay and Heath, 1969; Huxtable, 1979). The offending plants are usually Senecio or Crotalaria genera (Hill et al., 1951; Bras et al., 1954; Stuart and Bras, 1957). The problem has been so serious that an educational campaign was instituted to stop consumption of Crotalaria teas in the West Indies (Kay and Heath, 1969).

In the United States, PZA poisoning has been reported in Arizona (Stillman et al., 1977; Fox et al., 1978) after people consumed a widely used and commercially marketed herbal tea called gordoloba yerba. The potential for human poisoning also exists after consumption of honey from bees or milk from cows and goats that have had access to plants containing PZA (Dickenson et al., 1976; Dienzer et al., 1977).

The primary target organ in humans poisoned with PZA is the liver (Kasturi et al., 1979). The onset of symptoms due to PZA intoxication is often delayed considerably, making it difficult to relate consumption of a PZA with its toxic effects. Initial alterations in the liver after PZA ingestion include massive centrolobular necrosis, hemorrhage and portal hypertension. Subsequently, destruction of hepatocytes with fibrosis and collagen deposition occurs. Severe cirrhosis is the usual final result. Hepatic venous angiographic studies show that patients poisoned by Crotalaria-derived PZA have severe injury of the hepatic circulation with loss of venous branching, tapering and sinusoidal filling. In severe cases, the venous circulation is nearly obliterated. Hepatic blood flow is

decreased and portal venous pressure is increased. Hepatic function is compromised. In humans, cardiopulmonary injury or altered cardiopulmonary hemodynamics has not been reported after Crotalaria poisoning (Kasturi et al., 1979) or after poisoning due to other PZA (Stillman et al., 1977; Fox et al., 1978).

Since it is not possible to measure PZA metabolites in the blood, diagnosis of PZA poisoning is made on circumstantial evidence and observation of characteristic liver lesions. Since the onset of clinical signs and symptoms are often delayed, the relationship between PZA consumption and liver disease may go unnoticed, and thus it is likely that more PZA poisoning occurs than is recognized.

Animals grazing on fields infested with PZA-containing plants develop a toxic syndrome known by a large number of colorful names (Bull, 1968; McLean, 1970). In certain parts of the USA, a significant proportion of livestock become chronically ill or die as the result of liver disease due to grazing on PZA-containing plants. Losses to cattlemen and other livestock owners are estimated to be in the tens of millions of dollars each year (Huxtable, 1979). The injury is primarily hepatic, but the CNS is often affected, especially in horses. Cardiac, pulmonary, renal, thyroid and pancreatic lesions also have been reported (Bull et al., 1968).

II. MONOCROTALINE

A. General

Monocrotaline (MCT) is a PZA found primarily in plants that are members of the genus Crotalaria (Bull et al., 1968). The major source for MCT in the United States is from the seeds of Crotalaria spectabilis (IARC). C. spectabilis is indigenous to India, but is now widely scattered through the tropics and subtropics of both hemispheres. It was introduced into Florida by the Florida

Agricultural Experimental Station during 1921 to be used as a leguminous cover crop for the return of nitrogen to the soil between plantings and to protect the soil from erosion. C. spectabilis has grown wild and is now found in many southern and southeastern states (Kay and Heath, 1969). Other sources of MCT are the seeds of C. retusa, which has been used as a dye plant and edible vegetable in east Africa (Dalziel, 1948; Watt and Breyer-Brandwijk, 1932), and C. recta, which is used against childhood malaria in Tanzania (Schoenthal and Cody, 1968). MCT also occurs in a wide variety of other Crotalaria species (listed, IARC).

The structure of MCT was elucidated by Adams and Rodgers (1939) and proved to be the monocrotalic acid ester of retronecine (Figure 1). Pure MCT is a colorless, crystalline powder with a bitter taste. It has a melting point of 202-203°C and has been identified using infrared, NMR and mass spectrometry (Culvenor and Dal Bon, 1964; Bull et al., 1968). Other physical characteristics are described elsewhere (IARC).

B. Metabolism and Bioactivation

MCT in its native form is very stable and not toxic. For injury to occur, MCT and other PZA require metabolic activation. Many investigators believe the metabolite responsible for toxicity is a reactive pyrrole. There is considerable evidence that supports this contention. Pyrroles of metabolic origin are covalently bound to injured tissues (Mattocks, 1968; Allen et al., 1972) and are found in the urine of animals treated with PZA in vivo (Mattocks, 1968). Pyrrole concentration increases in liver tissues within minutes after PZA administration and it persists for approximately 48 hours after treatment (Mattocks and White, 1971; Allen et al., 1972). There is a positive correlation between the amount of pyrrole found in tissues and tissue injury (Mattocks, 1972; Chesney et al., 1974) and the relative toxicity of a given PZA correlates with the amount of pyrrole

produced in vivo or in vitro (Mattocks and White, 1971; Mattocks, 1972). When chemically synthesized monocrotaline pyrrole (MCTP) is given i.v. in the tail vein, pulmonary lesions similar to those that occur after MCT develop in the lung (Butler, 1970; Butler et al., 1970; Chesney et al., 1974; Lalich et al., 1977). When MCTP is given via the mesenteric veins, liver injury occurs (Butler, 1970).

Experimental data indicate that the reactive pyrrole metabolites are produced in the liver by the hepatic mixed function oxidase enzymes (MFO) and are carried via the circulation to organs where damage is caused (Mattocks, 1968). When rats are treated with inducers or inhibitors of hepatic MFO activity in vivo, toxicity and tissue concentrations of pyrroles are increased and decreased, respectively (Allen et al., 1972; Mattocks and White, 1971; Mattocks, 1972; Tuchweber et al., 1974). Liver slices produce pyrroles when exposed to PZA in vitro (Mattocks, 1968) but lung slices and lung microsomes do not produce MCTP from MCT in vitro (Mattocks, 1968; Mattocks and White, 1971). Thus, after being generated in the liver, MCTP passes to the lungs where binding at nucleophilic sites of pulmonary macromolecules occurs. This binding of MCTP to the tissues then is likely responsible for causing damage in vivo.

In addition to metabolic dehydrogenation to the proximate toxicant, MCTP, MCT may undergo other metabolic changes. Hydrolysis by hepatic esterases decrease PZA toxicity but apparently is a minor metabolic pathway for the PZA (Mattocks, 1968). N-oxidation also occurs in the microsomal fraction of hepatocytes in a pathway separate from that which produces MCTP (Mattocks and White, 1971). There is no apparent conversion of N-oxides to pyrroles and since the N-oxides are highly water soluble, renal excretion of the N-oxides is rapid. Parenterally administered N-oxide is not toxic (Mattocks, 1971). Dehydroretronecine (DHR) is another pyrrole metabolite of MCT. It is the major detectable metabolite found in rats treated with MCT (Hsu et al., 1973). When DHR is given

to rats in one large dose (100 mg/kg, sc) or at weekly intervals (50-70 mg/kg, sc) for 6 weeks, no lung injury is caused. When given chronically (4 mg/kg/day, sc) for 2-3 weeks, decreased body weight, increased lung weight, right ventricular hypertrophy and decreased 5HT uptake by isolated perfused lung is caused (Huxtable et al., 1978). Thus, if DHR plays a role in causing lung injury, its effects are apparently minor compared to those of MCTP.

C. Pharmacokinetics

Definitive pharmacokinetic studies have not been performed since radiolabeled MCT with high specific activity is not available. After injection of MCT, pyrrole levels, as measured by the Ehrlich assay (Mattocks and White, 1970), are detected in tissues within minutes and reach a peak 25-90 minutes after treatment. Thereafter, the tissue pyrrole concentration decreases to low levels by 48 hours (Allen et al., 1972; Mattocks, 1972). Hayashi et al. (1966) reported that 60-70% of a ^{3}H-MCT dose appears in the urine (with intact PZA ring) and the rest of the label appears in the bile (without an intact pyrrolizidine ring structure).

D. Effect of Diet on MCT-induced Pneumotoxicity

Hayashi et al. (1979) reported that diet restriction to about 50% of ad libitum intake protects against the development of lung injury and right ventricular hypertrophy after MCT. Mortality due to MCT also is markedly reduced by diet restriction. Rats given a restricted diet for 30 days after a single injection of MCT and then fed ad libitum develop labored breathing, lung lesions and begin to die. These results suggest that MCT causes long-lasting alterations in the lungs that result in development of characteristic pulmonary lesions when the nutritional status is adequate.

Newberne et al. (1971) reported that rats fed a low lipotrope diet (lacking in choline, methionine and vitamin B12) are protected from hepatic injury

due to MCT, but not due to MCTP. There is less conversion of MCT to pyrroles in liver tissue and decreased activity of MFO-associated metabolism in rats on the low lipotrope diet. The results suggest that the diet restriction prevents the conversion of MCT to the hepatotoxic metabolite MCTP and may explain, in part, why lung injury is decreased in diet-restricted, MCT-treated rats. However, diet restriction partially protects against lung injury due to MCTP, thus ruling out inhibition of metabolic activation as the sole protective mechanism (Ganey et al., 1985). The protective mechanisms involved are not known, but may include depression of cell growth and hypertrophy due to lack of necessary nutrients, alterations in key enzyme pathways (e.g., polyamine synthesis) or depression of immune system and/or inflammatory processes.

E. Biologic Effects

1. Species affected

MCT-induced intoxication was first described in 1884 as a syndrome in horses called "crotalism" or Missouri River bottom disease. Since then, many diseases have been ascribed to ingestion of MCT in a wide number of species, including man (Kasturi et al., 1979), horses (Rose et al., 1957), poultry (Thomas, 1934; Allen et al., 1960, 1963; Simpson et al., 1963), pigs (Emmel et al., 1935), cattle (Becker et al., 1935; Sanders et al., 1936), non-human primates (Allen et al., 1965; Roczniak et al., 1978), rats (Schoenthal and Head, 1955; Roth, 1981), rabbits (Gardiner et al., 1965), mice (Miranda et al., 1981), dogs (Miller et al., 1981) and goats (Dickenson et al., 1980). Guinea pigs (Chesney and Allen, 1973), gerbils and hamsters (Cheeke and Pierson-Goeger, 1983) are resistant to the toxic effects of MCT.

2. Hepatic toxicity

The liver is the most commonly identified target organ in humans and livestock exposed to PZA. Administration of PZA causes hepatic venoocclusive disease in horses (Hill and Martin, 1958), cows (Bras et al., 1957), monkeys (Allen and Carstens, 1968) and rats (McLean et al., 1964). Megalocytosis, centrolobular necrosis, fatty degeneration and hyperemia are the major histological changes in the liver of rats due to PZA (Schoenthal and Head, 1955; Miranda et al., 1980). Release of serum glutamic pyruvate transaminase from damaged hepatocytes occurs after MCT (Roth et al., 1981).

PZA also cause decreased hepatic function. Protein synthesis is altered with changes in production of clotting factors (Rose et al., 1945) and albumin (Miranda et al., 1980). Indocyanin green transport into the biliary tree is decreased in rats given MCT (Roth et al., 1981).

3. Renal damage due to MCT

Gross changes in kidneys after MCT include a change in color to light green or brown with mottling, development of an irregular capsular surface and petechial hemorrhage (Hayashi and Lalich, 1967; Carstens and Allen, 1970). There are numerous microscopic changes in the kidney glomeruli and arterial vasculature (Hayashi and Lalich, 1967; Carstens and Allen, 1970; Kurozumi et al., 1983).

Functional impairment of the kidneys also is caused by MCT. Blood urea nitrogen (Roth et al., 1981) and serum creatinine (Kurozumi et al., 1983) concentrations are increased after MCT, suggesting that glomerular filtration rate is decreased. Accumulation of para-aminohippuric acid by kidney slices is decreased whereas accumulation of tetraethylammonium is increased, suggesting that MCT also causes altered renal tubular function (Roth et al., 1981).

4. Carcinogenesis

Hepatotoxic pyrrolizidine alkaloids (PZA) are carcinogens of varying potencies. The carcinogenic effects of the PZA were first reported by Cook et al. (1950). The carcinogenicity of MCT was demonstrated when Newberne and Rodgers (1973) reported that MCT causes liver tumors.

When MCT is injected, a variety of widely distributed tumors including pulmonary adenocarcinoma, hepatocellular carcinoma, acute myelogenous leukemia, rhabdomyosarcoma, adrenal adenoma, and renal adenoma develop. Alternatively, dehydroretronecine, a metabolite of MCT, causes rhabdomyosarcomas at the injection sites, but few tumors elsewhere (Allen et al., 1975; Schumaker et al., 1976). Topical application of MCTP results in malignant tumor induction at the dermal application sites when the skin is co-treated with the promoter, croton oil (Mattocks and Cabral, 1982). MCTP also interferes with hepatocellular mitosis and DNA synthesis in vivo (Hsu et al., 1973b). Since MCT causes tumors at widely distributed sites, whereas MCT metabolites cause tumors at the site of administration, it suggests that the metabolites of MCT rather than MCT itself are the proximate carcinogens.

5. Cardiac effects

Right ventricular hypertrophy (RVH) has been demonstrated by a great many investigators after MCT or MCTP exposure. Hemodynamic studies indicate that RVH is preceded by increased pulmonary arterial pressure. Thus, the RVH likely results from increased work the right heart must provide to sustain pulmonary blood flow through the damaged lungs (Meyrick et al., 1980; Ghodzi and Will, 1981; Bruner et al., 1983; Lafranconi et al., 1984).

Histologic changes in right ventricular myocardium are many and include myocardial cytolysis, intracellular edema, fibrosis and cellular hypertrophy (Kajihara, 1970; Raczniak et al., 1978).

Biochemical composition of the right ventricle changes after MCT and these changes include increased total protein and increased collagen content. There is no change in right ventricular lipid content. RNA synthesis rate is increased in the right ventricle after MCT and the ratio of DNA:RNA is decreased (Lafranconi et al., 1984).

6. Pulmonary pathology due to monocrotaline

Much work has been done to identify the lesions associated with injury due to MCT and MCTP. A multitude of protocols using different modes of administration have been employed in these studies. Pulmonary injury can be caused in the rat by prolonged administration of ground C. spectabilis seeds in the diet, by administering MCT in the drinking water, by giving single or multiple injections of MCT or by giving a single injection of MCTP via the tail vein. Thus, comparison of results between different studies is often difficult.

a. Gross changes after MCT: The effects of MCT and MCTP differ depending on the dose and route of administration. When high doses of MCT are given either orally or parenterally, severe liver damage occurs resulting in death due to hepatic failure. Smaller doses of MCT cause transient, non-fatal hepatic injury characterized by an increase in serum glutamate pyruvate transaminase activity (Hilliker et al., 1982). After recovery from the initial hepatic damage, delayed pulmonary vascular injury, pulmonary hypertension and right ventricular hypertrophy develops (Hilliker et al., 1982). When MCTP is given to rats i.v., damage occurs in the first vascular bed the MCTP enters. MCTP given via the mesenteric veins causes hepatic injury, whereas administration via the tail vein results in pulmonary vascular injury (Butler, 1970; Butler et al., 1970). High doses of MCTP (15-30 mg/kg) result in severe, acute pulmonary injury with massive pleural effusions and death within hours after treatment (Plestina and Stoner, 1972; our own unpublished observations). In contrast, lower doses of

MCTP (3-5 mg/kg) result in development of delayed pulmonary vascular injury and pulmonary hypertension similar to MCT-induced lung injury.

Rats given moderate doses of MCT or MCTP have no overt signs of toxicity until several days after treatment. The first signs include failure to gain weight, ruffled hair coat and increased respiratory rate. As pulmonary lesions progress, rats become anorectic and lose weight; are listless; may have diarrhea; are overtly dyspnic, cyanotic and occasionally have epistaxis (Schoenthal and Head, 1955; Lalich and Erhart, 1962; Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Hislop and Reid, 1974; Bruner, unpublished observations).

Postmortem examination reveals bulky, congested and edematous lungs (Lalich and Merkow, 1961; Merkow and Kleinerman, 1966; Schoenthal and Head, 1955) often having subpleural petechial hemorrhage or large patches of dark brown discoloration that may involve entire lobes. Lung weight is increased due to edema, cell hyperplasia and hypertrophy (Lafranconi and Huxtable, 1984). Often there is pleural effusion (Chesney et al., 1974; Hislop and Reid, 1974; Schoenthal and Head, 1955).

Other gross changes include enlargement of the heart due to development of right ventricular hypertrophy (RVH). There are reports that the thymus is smaller than normal in MCTP, but this is not a consistent finding (Hislop and Reid, 1974; Schoenthal and Head, 1955). Other organs affected by MCT include the liver, which undergoes fibrotic changes, and the kidneys which appear discolored.

b. Microscopic lesions: Although minor histologic changes such as mild interstitial alveolar edema, elastolysis and thrombi in small vessels occur within 4-24 hours after MCT (Valdivia et al., 1967), major pulmonary vascular and alveolar injury due to MCT or MCTP takes several days to develop.

1) Endothelial cell lesions. Beginning approximately 2-7 days after MCT or MCTP, endothelial cell injury is manifest. Initially, intraalveolar endothelial cells swell, sending cytoplasmic projections into the vessel lumen, causing vessel occlusion (Merkow and Kleinerman, 1966; Valdivia et al., 1967; Butler, 1970; Chesney et al., 1974; Lalich et al., 1977). The endothelial cell vesicles may rupture, leading to release of endothelial cell contents into the lumen and to disruption of the endothelial cell surface (Merkow and Kleinerman, 1966; Allen and Carstens, 1970). Fibrin and platelet-containing thrombi form at the site of endothelial cell rupture and denudation. Vascular permeability increases, leading to leakage of luminal materials into the interstitial spaces (Merkow and Kleinerman, 1966; Heath and Smith, 1978), thus causing edema.

Endothelial cells in the larger arterial vessels also are injured; platelet and fibrin-containing thrombi are present on the intimal surface leading to decreased luminal diameter (Turner and Lalich, 1965; Allen and Carstens, 1970; Plestina and Stoner, 1972). Endothelial cells of venules and larger pulmonary veins also swell, causing occlusion of the post capillary vessels which may contribute to increased pulmonary vascular resistance (Smith and Heath, 1978). Thus, endothelial cell injury and platelet and fibrin-containing thrombus formation occurs at all levels of the pulmonary vasculature. Since the earliest changes in lungs from MCT and MCTP-treated rats occur at the endothelial cell it suggests that the primary target of MCT or MCTP is the pulmonary endothelial cell (Valdivia et al., 1967; Butler, 1970; Chesney et al., 1974; Lalich et al., 1977; Meyrick and Reid, 1979).

2) Vascular smooth muscle remodelling. In association with endothelial cell injury, structural changes develop in the pulmonary vasculature. Muscle in the pulmonary trunk thickens (Heath and Kay, 1970) and increased collagen is formed in the pulmonary artery (Kameji et al., 1980). In large

pulmonary arteries, morphometric studies indicate that there is thickening of the medial layer of smooth muscle cells. This is due to both hypertrophy and hyperplasia of circularly oriented medial smooth muscle (Kay and Heath, 1966; Kay et al., 1967; Smith et al., 1970; Hislop and Reid, 1974; Ghodzi and Will, 1981). The medial smooth muscle cells are enlarged, have altered myofilaments and contain abundant cytoplasmic organelles (Allen and Carstens, 1970; Chesney et al., 1974; Heath and Smith, 1978).

The normal pulmonary arteriole in the rat is less than 20 μM in diameter and consists of endothelial cells resting on a single elastic lamina. There is no muscle in the normal pulmonary arterioles except at the origin from a muscular pulmonary artery (Smith et al., 1970). Pulmonary arterioles from MCT and MCTP-treated rats have extension of smooth muscle into arterioles where smooth muscle is usually not present. This muscle layer is often very thick and lies between internal and external elastic lamina (Kay et al., 1969; Smith et al., 1970; Hislop and Reid, 1974; Meyrick and Reid, 1979). This extension of smooth muscle cells peripherally and vessel wall thickening may lead to decreased lumen diameter and altered vasoactivity. Such arteriolar changes may contribute to increased pulmonary vascular resistance and pulmonary hypertension after MCT.

In pulmonary veins, there is protrusion of endothelial cells into the lumen due to evagination of underlying smooth muscle cells (Smith and Heath, 1978). A priori, it has been suggested the evagination of smooth muscle cells represents prolonged constriction of pulmonary veins induced by MCT and that this constriction may be responsible for causing pulmonary hypertension (Smith and Heath, 1978). Additionally, the lumens of the veins are occluded by the enlargement of intimal fibromuscular pads after MCT (Kay and Heath, 1966).

3) Vascular inflammatory changes. Associated with MCT treatment is inflammation of the pulmonary vasculature. In larger pulmonary arteries where there are damaged endothelial cells, fragmentation of the internal elastic lamina occurs (Allen and Carstens, 1970). There is fibrin deposition along the denuded surface and under the remaining endothelial cells. There is edema surrounding medial smooth muscle cells and in the adventitia with abundant fibrin, erythrocyte and leukocyte infiltration (Lalich and Merkow, 1962; Allen and Carstens, 1970). In severely damaged vessels, most usual morphologic features are lost with remaining structures composed of isolated endothelial cells, randomly dispersed smooth muscle cells, collections of RBC and leukocytes and abundant fibrin (Turner and Lalich, 1965; Allen and Carstens, 1970; Hislop and Ried, 1974).

A common morphologic change associated with MCT-induced vascular injury is necrotizing vasculitis characterized by the deposition of a PAS positive, diastase resistant material in vessel walls and lumens. The amorphous material is also found in smaller arterioles surrounding endothelial cells, smooth muscle cells and adventitial cells (Merkow and Kleinerman, 1966). The amorphous material is thought to be fibrin forced into vessel walls from the lumen via breaks in the endothelium (Heath and Smith, 1978). In some areas of the injured arteries, muscle cells undergo necrosis and degeneration with associated infiltration of neutrophils into the damaged tissue (Heath and Smith, 1978). In addition to fibrin accumulation, muscle cells are separated and made less distinct by accumulation of ground substance, thought to be sulphated mucopolysaccharide similar to basement membrane material (Heath and Smith, 1978).

Although it is not completely clear, there appears to be decreased numbers of blood vessels at the level of the alveoli in lungs from rats treated with MCT. Contrast radiographic studies on once frozen lung tissue

suggest that there are decreased numbers of small arterial vessels present in MCT-injured lungs (Meyrick and Reid, 1979). There also are increased numbers of "ghost arteries" in injured lungs which are thought to be the remnants of obliterated blood vessels (Hislop and Reid, 1974). The total number of arteries per alveolus is decreased in lungs, also suggesting that there are decreased numbers of small pulmonary arterioles in lungs from MCT-treated rats (Meyrick and Reid, 1979). However, alveolar size and cross sectional area increases in lungs after MCT (Kay et al., 1982). When the total number of small pulmonary blood vessels less than 50 μm in diameter lying distal to the respiratory bronchioles is normalized to total lung cross-sectional area, the total number of small arterioles is not decreased due to MCT (Kay et al., 1982). Thus, while the radiographic evidence of decreased vascular filling suggests that there is severe vascular damage and perhaps decreased numbers of vessels present, the morphometric data yield different results depending on the method used to evaluate the changes. More work will be required to determine if vessel number and vascular cross-sectional area are decreased due to MCT.

4) Pulmonary interstitial lesions. Mild interstitial edema is an early change that occurs after MCT (Valdivia et al., 1967). The severity of the damage is progressive such that from 7-21 days after MCT, interstitial edema causes significant thickening of the alveolar wall. Perivascular edema also is evident (Valdivia et al., 1967). Swelling of interstitial cells develops due to formation of cytoplasmic projections, with swelling and dilation of endoplasmic reticulum. Alveolar wall thickness also is increased by accumulation of cells within the interstitial space (Valdivia et al., 1967; Butler, 1970). During the later stages of the injury, alveolar type I cell swelling, proliferation of fibrous connective tissue and increased thickness of the basement membrane add to the

widening of the blood-air barrier. These changes likely contribute to the hypoxia that occurs at this time (Meyrick et al., 1980).

5) Alveolar damage after MCT or MCTP. In the alveoli, there is accumulation of large granular pneumocytes (Chesney et al., 1974; Lalich et al., 1977), alveolar macrophages (Sugita et al., 1983), neutrophils (Chesney et al., 1970; Stemmer et al., 1985; Dahm et al., unpublished observations), hemorrhage (Butler et al., 1970; Chesney et al., 1970), fibrin (Kay et al., 1969; Smith and Heath, 1978), edema (Butler et al., 1970; Smith et al., 1970; Sugita et al., 1983) and amorphous cellular debris (Kay et al., 1969). The accumulation of materials is more pronounced during the later stages of the injury and likely interferes with gas exchange.

c. Effect of MCT on pulmonary mechanics and airway function: After rats are treated with MCT (single dose, 105 mg/kg, s.c.), there are numerous changes in pulmonary airway function. At 20 days after MCT, these alterations include decreased total lung capacity, decreased residual volume, decreased tidal volume, and increased relaxation volume. MCT also causes increased respiratory rate, increased airway resistance, decreased dynamic compliance and decreased quasistatic compliance. Gas exchange is compromised. These alterations in pulmonary mechanical, ventilatory and gas exchange parameters are indicative of severe lung damage associated with decreases in lung elasticity and gas exchange capacity (Gillespie et al., 1985). Whether the alterations in pulmonary function are related to pulmonary airway inflammation or to pulmonary vascular injury is not known. Since many of the mechanical and airway alterations caused by MCT in rats are similar to the alterations accompanying pulmonary hypertension in humans, the MCT-treated rat also may be an appropriate model for evaluating the relationships between pulmonary airway and pulmonary vascular disease (Gillespie et al., 1985).

d. Hemodynamic alterations due to MCT: Hemodynamic, blood gas and histologic alterations in lungs of rats fed ground C. spectabilis seeds in the diet have been studied (Meyrick et al., 1980). No significant changes in blood gas composition, pH, hematocrit, arterial oxygen saturation or blood HCO_3^- content occur until the animals are near death. The first histologic changes that occur after the start of dietary MCT are in the pulmonary vasculature and are marked by appearance of new muscle and increased medial thickness of arteries less than 200 μm in diameter. These changes are first significant at 14 days. At about this time, cardiac index and pulmonary arterial pressure increase. Subsequently, medial thickness increases in larger arteries and vascular lumen diameter, measured in arteriograms, decreases. The magnitude of the increased pressure is correlated with the extent of medial thickness in arteries and other morphometric parameters of vascular injury (Meyrick et al., 1980).

These results suggest that hypoxia is not a cause of increased pulmonary arterial pressure since arterial oxygen content is not decreased until long after pulmonary hypertension is evident. Meyrick et al. (1980) also suggested that vasoconstriction does not appear to be the initial cause of the pulmonary hypertension since cardiac index is increased and pulmonary vascular resistance is decreased when pulmonary hypertension is first evident. This conclusion is based on the assumption that if vasoconstriction were involved, then increased pulmonary vascular resistance and decreased cardiac index would have been observed. One problem with this conclusion, however, is that rats treated with MCT did not gain weight as fast as controls. Cardiac index was normalized to body weight, and thus, values obtained for cardiac index may have been artifactually increased. Similarly, calculations of pulmonary vascular resistance were also normalized to body weight and thus may have been artifactually low, making the conclusion incorrect.

These data were the first describing the association between morphometric vascular changes and hemodynamic alterations that occur after feeding MCT. The results indicate that significant changes in pulmonary vascular structure occur about the same time as increased pulmonary arterial pressure, but it was not possible to distinguish which comes first.

In a similar study, Ghodzi and Will (1981) evaluated the hemodynamic and structural changes in lungs of rats after one dose of MCT. The findings were similar to those of Meyrick et al. (1980), except that medial thickness of small pulmonary vessels was increased one week after MCT followed by increased pulmonary arterial pressure and RVH at 2 weeks. Since medial thickening occurred prior to the increases in pulmonary arterial pressure, Ghodzi and Will (1981) concluded that injury occurs first at the level of the vasculature and that vascular remodelling with increased pulmonary arterial pressure is secondary to initial endothelial cell injury.

Kay et al. (1982) evaluated morphometric and hemodynamic changes in rats given a single injection of MCT. There were no significant changes in the lungs of treated rats at 1, 3, and 5 days after MCT. The first significant change was medial hypertrophy of muscular pulmonary arteries at 7 days. At 10 days after MCT, there was significantly increased pulmonary arterial pressure and extension of smooth muscle into peripheral arteries. At 12 days, there was RVH. Thus, it is apparent that changes in the vasculature precede the development of increased pulmonary arterial pressure and that RVH is secondary to the increased pressure.

McNabb and Baldwin (1984) evaluated hemodynamic changes in MCT-treated rats during exercise. After 35-37 days of feeding ground C. spectabilis seeds in the diet, MCT-treated rats have increased heart rate, increased pulmonary arterial pressure, blood gas abnormalities and changes in

cardiac work indices. During exercise, MCT-treated rats are not able to keep the same pace as control rats, and pulmonary arterial pressure is not maintained through the exercise period. Pulmonary arterial pressure remains below pre-exercise levels for at least 10 minutes during recovery, whereas controls return quickly to baseline. Thus, at the later stages of MCT-induced pulmonary injury, rats attain lower peak exercise intensity and have other hemodynamic changes most likely due to abnormal right ventricular function.

In summary, concurrent morphologic, morphometric and hemodynamic data suggest that the primary target of MCT metabolites in the lung are endothelial cells. Depending on the method of administration, major vascular lesions accompanied by increased pulmonary arterial pressure develop starting 7-14 days after treatment. Subsequently, elevated pulmonary arterial pressure is maintained and pulmonary vascular lesions progress, ultimately resulting in right ventricular failure. Most of the morphometric studies done to date have evaluated changes at only weekly intervals after starting treatment. Consequently, detailed knowledge relating the time-course of endothelial cell and vascular remodelling to concurrent changes in hemodynamic parameters is still lacking. Accordingly, more work needs to be directed toward detailed, ultrastructural evaluation of the early alterations that MCT exposure causes in endothelial cells. Furthermore, more information on how endothelial cell damage and altered function may affect surrounding pulmonary vascular structures is needed. Detailed morphometric and hemodynamic studies done at more frequent intervals may help to elucidate more precisely the time-course of structural and associated hemodynamic change in the lungs.

e. Biochemical changes due to MCT or MCTP: The pulmonary endothelium has a complex array of enzymes, receptors and transport structures on the luminal surface directly accessible to solutes and colloids

circulating in the blood (Ryan and Ryan, 1984). Interaction of blood substances with endothelial cells allows for regulation and control of the concentration of many substances in the blood. Thus, endothelial cells may remove certain agents from the blood and be responsible for the generation and release of hormones and other substances into the general circulation. Since the pulmonary circulation receives all of the cardiac output and sends its venous output to the systemic circulation, reactions between blood solutes and pulmonary endothelium are important for the proper function of many target tissues throughout the body (Ryan and Ryan, 1984).

1) Angiotensin converting enzyme (ACE). ACE is a dipeptidyl carboxypeptidase which converts angiotensin I to angiotensin II (AII) and inactivates bradykinin (Dorer et al., 1974). The enzyme is located on the luminal surface of the pulmonary endothelial cell in small invaginations of the cell membrane called calveolae (Ryan and Ryan, 1984). Since ACE is responsible for generation of the potent vasoconstrictor AII and inactivation of the vasodilator, bradykinin, alterations in ACE activity due to MCT-mediated injury may play a role in the pathogenesis of the pulmonary hypertension. Thus, the role of ACE and AII activity after MCT treatment have been examined by a number of investigators.

Huxtable and coworkers (1978) measured ACE activity in isolated, perfused lungs of rats exposed to MCT in drinking water for 14 and 21 days. Rats developed pulmonary vascular injury due to MCT but had no change in ACE activity/whole lung. The lungs were perfused at room temperature which may have artifactually decreased ACE activity.

Molteni et al. (1984) measured ACE activity in homogenized lung tissue and serum and found ACE activity (U/mg protein) varied, depending on when the tissue samples were tested. ACE activity in homogenized

lung tissue is increased 1 week after MCT, not different from controls at 2 and 4 weeks and decreased at 6 and 12 weeks after MCT. Serum ACE activity does not change due to MCT. Concurrent electron microscopic studies demonstrated progressive pulmonary vascular injury starts 1 week after MCT. PGI_2 synthesis, another endothelial cell function, was increased at 12 weeks after MCT in homogenized lung. Molteni et al. (1984) concluded that there is altered endothelial cell function associated with MCT treatment, and the decrease in ACE activity associated with increased PGI_2 production may be a compensatory response favoring decreased pulmonary arterial pressure in the face of MCT-induced pulmonary hypertension.

Kay and colleagues evaluated ACE activity in lung homogenates of rats treated with a single dose of MCT given subcutaneously. Rats killed 21 days after MCT have decreased ACE activity (Kay et al., 1982; Keane et al., 1982) and the magnitude of the decrease in ACE activity is directly correlated with the severity of RVH and the magnitude of pulmonary arterial pressure (Keane et al., 1982). Concurrent morphometric and hemodynamic studies show that pulmonary vascular lesions occur prior to development of pulmonary hypertension and that changes in ACE activity occur simultaneously with the development of the increased pressure. Thus, the authors suggested that decreased ACE activity may be due to the pulmonary hypertension and that these changes may be a protective mechanism to limit the elevation in pulmonary arterial pressure. Hayashi et al. (1984) confirmed the findings of Kay et al. (1982) and Keane et al. (1982) in a similar study.

A cautionary note related to these conclusions is that use of whole lung homogenates to measure ACE activity may not necessarily reflect physiologic ACE activity. Changes in blood flow through parts of the pulmonary capillary beds due to injury may alter the exposure of blood to the

enzyme. Such changes in vivo would not be reflected by measuring tissue homogenate enzyme activity. Also, lung homogenates may contain proteases other than ACE which can cleave the hippuryl-histidyl-leucine substrate giving rise to inaccurate quantification of ACE activity (Bakhle, 1976; Lafranconi and Huxtable, 1983).

MCTP may alter the response of the pulmonary vasculature to the pressor actions of AII, and thus may play a role in the development or maintenance of pulmonary hypertension. This possibility has been suggested by studies in the isolated, perfused lung (Hilliker and Roth, 1985). Isolated lungs from rats treated with MCTP 14 days earlier have a greater pressor response to AII infused into the pulmonary artery than do corresponding controls. The mechanism by which the increased responsiveness arises is not known, but the result suggests that lungs from MCTP-treated rats are more responsive to AII in vivo. Whether or not the changed responsiveness occurs as the result of the increased pulmonary arterial pressure or is the cause of it is not known. Kay and Keane (1984) reported that immuno-reactive AII blood concentrations are decreased in rats treated with MCT. Thus, it is possible that the increased responsiveness is due to altered sensitivity of pulmonary AII receptors secondary to decreased circulating levels of AII. This possibility has not yet been explored.

2) Polyamines in MCT pneumotoxicity. Olson et al. (1984a) proposed that polyamines may have a role in the cell proliferation and cellular remodelling that occurs in lungs of rats treated with MCT. The importance of this pathway is that the polyamine metabolites, putrescine, spermidine and spermine are believed essential for cell growth and proliferation (Williams-Ashman and Canellakis, 1979; Heby, 1981; Pegg and McCann, 1982). After MCT, the activity of ornithine decarboxylase (ODC) , which is the rate-limiting and control enzyme in the polyamines pathway, is increased for 7 days

(Olson et al., 1984a). The activity of adenosylmethionine decarboxylase, the enzyme that converts putrescine to spermidine and spermidine to spermine is increased in lungs at day one, at 10-14 days and at 21 days after MCT (Olson et al., 1984b). Pulmonary concentrations of putrescine, spermidine and spermine are increased 7 through 10 days after MCT (Olson et al., 1984b). Thus, increases in enzyme activity and polyamine levels occur at a time before RVH or increased pulmonary arterial pressure are observed, suggesting that these compounds may be involved in the injury due to MCTP.

Alpha-difluoromethylornithine (DFMO), a specific, irreversible inhibitor of ODC prevents development of increased pulmonary arterial pressure, RVH, pulmonary edema and increased medial thickness in arteries of MCT-treated rats (Olson et al., 1984b). DFMO also prevents MCT-induced increases in pulmonary putrescine and spermidine but does not block MCT-induced increases in pulmonary spermine. These results suggest that polyamines may play a role in early changes after MCT, perhaps those involved with cellular proliferation that leads to vascular remodelling and pulmonary hypertension.

A problem that remains with the results of experiments with DFMO is that the specificity of the treatment has not been confirmed. Pulmonary ODC activity after DFMO/MCT has not been reported. Since spermine concentration increases in lungs of MCT/DFMO-treated rats, it is possible that mechanisms other than ODC inhibition may be responsible for the protective effect of DFMO. DFMO does not alter the production of Ehrlich positive metabolites by liver after MCT (personal communication). However, whether or not DFMO changes MCT metabolism so that less toxic pyrroles (such as dehydroretronecine) are synthesized by the liver or whether less pyrrole binds in the lungs after DFMO has not been tested. These results are nevertheless interesting, and suggest polyamines may be important in MCT-induced pulmonary

injury. Further study of the role for polyamines is warranted since these mediators may play a significant role in vascular remodelling after endothelial cell injury.

3) Serotonin (5HT) in MCT toxicity. Another function of pulmonary endothelium is removal of vasoactive amines from the circulation. 5HT is removed by pulmonary endothelial cells by an active, carrier-mediated process (Junod, 1972; Iwasawa et al., 1973; Pickett et al., 1975). After uptake, 5HT is metabolized by monoamine oxidase to 5-hydroxyindoleacetic acid. Thus, the pulmonary endothelium may play an important role in controlling circulating levels of free 5HT.

MCT causes endothelial cell injury resulting in decreased 5HT clearance by lungs (Gillis et al., 1978; Huxtable et al., 1978; Hilliker et al., 1982). 5HT clearance also is decreased after MCTP (Hilliker et al., unpublished observations). After both MCT and MCTP, there is a delay of several days before decreased clearance of 5HT is significant (Hilliker et al., 1982, unpublished obervations). When MCT (Gillis et al., 1978) or MCTP (Hilliker and Roth, 1985) is infused directly into isolated perfused lungs, 5HT clearance is not decreased; and lung slices do not have decreased ability to take up 5HT when exposed to MCT, MCTP or liver slice-generated MCT metabolites in vitro (Hilliker et al., 1983). This suggests that MCT and MCTP do not act directly on the 5HT uptake mechanism, but rather that other secondary processes are responsible for damage to the removal system. Whether the secondary mechanisms involve alteration of endothelial cell biochemical processes, platelet-mediated injury, neutrophil-mediated injury, or damage via other inflammatory mechanisms is not known.

5HT is a pulmonary vasoconstrictor (Bergofsky, 1980; Van Neuten, 1983) and stimulator of platelet aggregation in some species. Since

removal of 5HT from the circulation is decreased after MCT or MCTP (Hilliker et al., 1982; Hilliker et al., unpublished observations), 5HT may mediate part of the pulmonary hypertension by direct pulmonary vasoconstriction, or by causing aggregation of platelets within the lung microvasculature, leading to occlusion of the microvascular bed.

There are several sources for increased 5HT in lungs of MCT-treated rats. Mast cells are rich in 5HT and proliferate in lungs after MCT (Takeoka et al., 1962; Kay et al., 1967). Also, platelet-containing thrombi form within the pulmonary vasculature after MCT and MCTP. Several authors have suggested that since platelets contain large amounts of 5HT, these aggregates may release 5HT stores, perhaps resulting in pulmonary vasoconstriction (Tucker et al., 1983; Lafranconi and Huxtable, 1984) and additional accumulation of platelet thrombi in the lungs (Valdivia et al., 1967; Chesney et al., 1974; Lalich et al., 1977). Thus, if uptake of blood-borne 5HT is impaired, increased concentrations of 5HT may result in microvascular obstruction and pulmonary hypertension.

5HT concentration in plasma and circulating platelets from MCT-treated rats has been evaluated. There are no differences in 5HT content of platelets or plasma levels due to MCT (Kay et al., 1967).

Depletion of 5HT by treatment with p-chlorophenylalanine (PCPA) has been used by several investigators to evaluate the role of 5HT in MCT-induced pneumotoxicity. In vivo, PCPA is an irreversible inhibitor of tryptophan hydroxylase (Jequer et al., 1967), the rate-limting enzyme for 5HT synthesis. Thus, treatment with PCPA results in prolonged depletion of 5HT in vivo. Carillo and Aviado (1969) first tested the effect of PCPA on MCT-induced pulmonary injury. Cotreatment of PCPA with MCT resulted in lower pulmonary arterial pressure compared to MCT/saline-treated controls. 5HT content of lungs

from PCPA-treated rats was decreased compared to controls, but 5HT depletion was not complete.

Tucker et al. (1983) extended the work of Carrillo and Aviado (1969) by treating rats with PCPA and a single dose of MCT. PCPA treatment alone caused rats to lose weight. RVH was prevented in rats that received PCPA/MCT, but co-treatment with PCPA did not protect against MCT-induced muscularization of the pulmonary vasculature as measured by changes in medial thickness, medial area:lumen ratio or development of vascular smooth muscle cell hypertrophy. These results suggest that 5HT is not involved with the development of lung lesions per se, but may be involved in development of pulmonary hypertension and right ventricular hypertrophy.

Kay et al. (1985) confirmed and extended somewhat the findings of Tucker et al. (1983). PCPA co-treatment with MCT-protected rats against fibrin exudation into aveoli and resulted in accumulation of fewer inflammatory cells in the lungs. PCPA co-treatment also resulted in decreased RVH, lower right ventricular blood pressure and a smaller increase in medial muscle thickness compared to MCT/saline-treated controls. PCPA did not protect against the extension of smooth muscle into smaller pulmonary arterial vessels. Thus, consistent with earlier findings, PCPA treatment had an effect on the development of pulmonary hypertension and right ventricular hypertrophy, but did not prevent pulmonary vascular alterations.

Although the results of the PCPA/MCT studies suggest 5HT may be involved in the pathogenesis of MCT-induced pulmonary injury, interpretation of the results should be made with caution. Confirmation of the effect of PCPA as a 5HT depletor was not done by Tucker et al. (1983) or by Kay et al. (1985). Thus, it is not known to what extent 5HT stores were depleted in PCPA-treated animals, making it impossible to confirm that the protective effect

of PCPA was due to 5HT depletion or due to some other non-specific effect. PCPA treatment caused significant weight loss in the rats (Tucker et al., 1983). This is important since diet restriction results in protection from injury due to MCT and MCTP by unknown mechanisms. PCPA also may have decreased metabolic activation of MCT in the liver. Whether or not PCPA affects MCT metabolism was not determined.

4) Protein, DNA and RNA content in MCT-treated lungs. After MCT and MCTP, both wet and dry lung weight increase (Hilliker et al., 1982; Bruner et al., 1983; Lafranconi et al., 1984). This is due to edema, hypertrophy and hyperplasia and accumulation of inflammatory cells in the lungs. Lungs from rats given MCT chronically in drinking water have greater than 50% more protein content than do controls at 21 days after MCT. The new protein is cytoplasmic protein derived from intrapulmonary sources (Lafranconi et al., 1984). There is no significant parenchymal fibrosis up to 21 days after continuous MCT in drinking water since pulmonary hydroxyproline content and total collagen content in homogenized whole lung is not increased after MCT (Lafranconi et al., 1984). There is, however, increased collagen content in the trunk of pulmonary artery of rats treated with a single injection of MCT (Kameji et al., 1980). Thus, there may be increased collagen synthesis in the pulmonary vasculature after MCT even though it cannot be detected by measuring collagen content of whole lung homogenates. Lungs from MCT-treated rats also contain more lipid and RNA than do controls and the DNA:RNA concentration ratios are decreased, suggesting that cellular hypertrophy is more important than hyperplasia for the increased lung mass (Lafranconi et al., 1984).

Meyrick and Reid (1982) measured incorporation of ^{3}H-thymidine into the DNA of different cell types in the lungs of rats fed MCT chronically to quantify the rate of cell division. ^{3}H-Thymidine incorporation is

increased in fibroblasts, smooth muscle cells and endothelial cells of the hilar pulmonary arteries and intra-acinar vessels. In the alveolar walls, incorporation of ^{3}H-thymidine is increased primarily in endothelial cells.

The time course of changes in ^{3}H-thymidine incorporation differs depending on the cell type studied and its location. In the hilar pulmonary arteries, incorporation of ^{3}H-thymidine in fibroblasts, smooth muscle cells and endothelial cells is biphasic, occurring at 3-7 days and again at 21-28 days (Meyrick and Reid, 1982). In intra-acinar areas of the lung, ^{3}H-thymidine incorporation in arterial and venous endothelial cells occurs at 7-21 days and in smooth muscle cells at 35 days. In alveolar areas, ^{3}H-thymidine incorporation is increased in endothelial cells 14 days after MCTP. These results along with histologic and hemodynamic studies suggest that injury due to MCT occurs in phases, the first being early initial injury to endothelial cells and vasculature represented by changes in vascular structure and changes in DNA incorporation. The second phase involves a more intense incorporation of ^{3}H-thymidine into all cell types, associated with medial hypertrophy, new muscle extension into arterioles, increased pulmonary arterial pressure and RVH (Meyrick et al., 1980; Meyrick and Reid, 1982; Lafranconi et al., 1984).

5) Lavage fluid lactate dehydrogenase (LDH). Another biochemical change that occurs after MCT or MCTP is release of the enzyme LDH into the airway (Roth, 1981; Bruner et al., 1983). LDH is a cytosolic enzyme that is released when cells are injured. The activity of LDH in the pulmonary alveolar lavage fluid of normal, untreated rats is low. After MCT or MCTP, lavage fluid LDH activity is increased and thus, is a good marker of lung injury after treatment (Roth, 1981; Bruner et al., 1983).

6) Lavage fluid protein. Lavage fluid protein concentration also is increased after MCT or MCTP (Roth, 1981; Bruner et al., 1983).

Thus, measuring protein concentration in the pulmonary lavage fluid is another method by which lung injury can be assessed.

f. Electrophysiologic changes due to MCT: Suzuki and Twarog (1982) studied the electrophysiologic changes in smooth muscle cells (SMC) from the main pulmonary artery (MPA) and small pulmonary artery (SPA) in lungs of rats treated with MCT or from rats exposed to alveolar hypoxia. After MCT, resting membrane potential (RMP) of SMC in MPA and SPA changed simultaneously with changes in vessel wall thickness. RMP of cells in MPA decreases whereas RMP in SPA cells increases with increased wall thickness. The cause of the membrane potential changes in smooth muscle cells is increased Cl^- flux from the cells of the MPA and increased activity of the Na^+/K^+ pump in the membrane of cells from SPA. The increase in SPA Na^+/K^+ pump activity may be associated with cellular hypertrophy, since increased Na^+/K^+ pump activity occurs during hypertrophy of vascular SMC in spontaneously hypertensive rats (Hermsmeyer, 1976; Webb and Bohr, 1979). Also, these results suggest that there is a divergence in responses of SMC to injury after MCT depending on the location of the SMC in the pulmonary vasculature.

There are several major problems with the design of this study and the way the data were analyzed. First, the numbers of animals evaluated at any time point are very low. In the MCT-treated groups, some conclusions are drawn from results obtained from only one rat. Also, data obtained from MCT-treated rats were combined with data from rats exposed to hypoxia. Since the vascular responses in MCT-induced pulmonary hypertension are different from those occurring after hypoxia (Hislop and Reid, 1974; Meyrick et al., 1980), combining data from the two treatment regimens may not be appropriate. Finally, statistical analysis of the data was not done. In spite of these shortcomings, the results suggest that there are identifiable electrophysio-

logic alterations occurring in conjunction with hemodynamic and morphologic changes after MCT. More thorough study of these changes is warranted and may lead to a better understanding of the pathogenesis of the smooth muscle cell damage and vascular responses after MCT.

g. Role of platelets in MCT pulmonary hypertension: The appearance of platelet-containing thrombi within the vessels of lungs in MCT and MCTP-treated rats suggests that platelets may play a role in development of pulmonary lesions and pulmonary hypertension (Valdivia et al., 1967; Hilliker et al., 1982). Platelets may play a role in the development of injury and pulmonary hypertension by releasing a variety of vasoactive substances including 5HT, histamine, adenine nucleotides and prostaglandins. Platelets also may release mitogenic factors such as platelet-derived growth factor (PGDF) that could contribute to vascular remodelling. Platelet aggregation and formation of microemboli in the pulmonary vasculature also may be important in causing increased pulmonary arterial pressure. Acute increases in pulmonary vascular resistance after pulmonary embolization are reduced in animals made thrombocytopenic, suggesting that platelets may be important in altering pulmonary hemodynamics in various pathologic conditions (Cade, 1975; Mlczoch et al., 1977). Accordingly, the role of the platelet in MCT-induced pulmonary injury has been evaluated.

MCT causes decreased circulating platelet counts starting approximately 2 days after treatment. Platelet counts decrease to approximately 45% of control values through 5 days, begin to recover by 10 days and are 47% greater than control counts at 14 days after MCTP (Hilliker et al., 1982). There also are platelet-containing thrombi within the lungs after MCT (Valdivia et al., 1967). Thus, these findings suggest platelets sequestered in the microvasculature may play a role in the injury (Hilliker et al., 1982).

To test whether platelets are involved in the pathogenesis of MCTP-induced pulmonary damage, the effects of MCTP in rats made thrombocytopenic with goat anti-rat platelet serum was examined (Hilliker et al., 1984). Since it is not technically possible to maintain thrombocytopenia for more than 3 days using this method, rats were made thrombocytopenic prior to treatment with MCTP (days 0-2), at 3-5 days after MCTP or at 6-8 days after MCTP. These times were chosen because they correspond with initiation of injury due to MCTP, the time when major lung injury first occurs after MCTP and the time when pulmonary hypertension is first manifest, respectively. The rats were killed 14 days after MCTP and cardiopulmonary injury was assessed. Rats made thrombocytopenic at 3-5 days and at 6-8 days after MCTP are protected from development of right ventricular hypertrophy. These rats, however, were not protected from lung injury (Hilliker et al., 1984). These results suggest that platelets do not mediate the lung injury but are needed for the development of pulmonary hypertension. Alternatively, the antibody-induced thrombocytopenia may have only delayed the effects of MCTP for 2-3 days. This would not have been detected since the effects of MCTP on indices of lung injury is greatest at 7-10 days after treatment whereas RVH is a late event in the pathogenesis (see below). Thus, if thrombocytopenia delayed the injury process by 2-3 days, the indices of pulmonary injury could reach maximum values by 14 days without concurrent development of RVH. Evaluation of lung injury in thrombocytopenic rats killed at 7 days after MCTP would help determine if thrombocytopenia does delay the lung injury due to MCTP.

The role of the platelet in lung injury also has been tested by evaluating the effects of MCTP in Fawn-hooded (FH) rats (Hilliker et al., 1983). FH rats have a platelet defect characterized by a decreased ability of their platelets to take up and release 5HT. The platelets also have decreased

stored adenine nucleotides and 5HT in the dense granules. When stimulated, platelets from FH rats release less of these mediators than do normal platelets (Raymond and Dodds, 1975) and have abnormal aggregation responses in vitro (Wey et al., 1982). FH rats, however, respond typically to MCTP. These results suggest that the ability of platelets to release and or accumulate 5HT and ADP may not be essential for the pathogenesis of MCTP-induced lung injury (Hilliker et al., 1983).

The response of platelets harvested from MCTP-treated rats to various aggregating agents has been evaluated in vitro (Hilliker et al., 1983). Platelets harvested at 1 and 4 days after MCTP aggregate normally to ADP, dog collagen or arachidonic acid. Platelets harvested 7 days after MCTP respond less to ADP. At 14 days after MCTP, platelets respond less to ADP, collagen and arachidonic acid. Since platelet function is dependent upon environment, and since the altered aggregation responses were seen at a time when only a small portion of the platelets, if any, would have been exposed to MCTP -- these results suggest that MCTP treatment may alter one or more plasma components that affect platelet function, thereby resulting in decreased responsiveness. The identity of these factors is not yet known. These results also suggest that the role of the platelet in MCTP pathogenesis is not due to a hyperresponsiveness of the platelet to aggregating agents.

In summary, the platelet may be involved in the development of pulmonary hypertension after MCTP. The mechanisms by which the platelets exert their effects, if any, are is still unknown and are currently under study.

h. Drug treatments and MCT pneumotoxicity: Several studies have been undertaken to evaluate the protective effect of various drugs in rats treated with MCT or MCTP. Kay et al. (1976) evaluated two muscle

relaxants to test whether vasoconstriction is involved in the development of pulmonary hypertension. MCT-treated rats received the smooth muscle relaxant cinnarzine or the skeletal muscle relaxant zoxazolamine. Zoxazolamine mediates its effect via action on spinal cord neurons. Only zoxazolamine, which also is an inducer of certain hepatic microsomal enzymes (Conney, 1967), protected against the toxic effects of MCT. Whether this effect was due to muscle relaxation or to some other non-specific effect of zoxazolamine, such as competitive inhibition of MCT metabolism, is not known. No other studies using this drug have been done to define further its protective mechanism.

Huxtable et al. (1977) tested the effect of the beta-adrenergic antagonist propranolol on MCT pulmonary injury. DL-propranolol protected against the MCT-induced development of RVH and increased pulmonary arterial pressure. The protective effect of DL-propranolol may be due to its beta-blocking activity or to its local anesthetic properties. Alternatively, since propranolol is metabolized by the mixed-function oxidase enzymes, co-treatment may have decreased the metabolic activation of MCT. Thus, the effect of DL-propranolol on MCT-induced lung injury may have been due to decreased activation of MCT to MCTP.

Tanabe et al. (1981) tested the effect of prednisolone on MCT-induced pulmonary injury. Prednisolone co-treatment did not protect against MCT-induced weight loss, mortality, elevated pulmonary arterial pressure or RVH. Characteristic pulmonary lesions appeared in rats treated with MCT/prednisolone although prednisoline decreased the severity slightly.

The effects of drugs that alter platelet function were tested in vivo to determine if co-treatment would protect against MCT-induced injury (Hilliker and Roth, 1984). Hydralazine, a vasodilator and platelet prostaglandin synthesis inhibitor (Greenwald et al., 1978), decreased development of

RVH and increased lavage fluid total protein concentration due to MCTP. Sulfinpyrazone, an inhibitor of platelet prostaglandin biosynthesis, prevented development of RVH without affecting development of pulmonary injury due to MCTP. Dexamethasone, a corticosteroid derivative that has antiinflammatory properties and is an inhibitor of phospholipase, decreased RVH due to MCTP. Each drug tested has a common action which is to inhibit prostaglandin synthesis. Hydralazine and sulfinpyrazone tend to inhibit platelet thromboxane synthesis more than vascular PGI_2 producton (Greenwald et al., 1978; Livio et al., 1980; Srivastava and Awasthi, 1982). Dexamethasone decreases synthesis of all eicosanoids by preventing release of arachidonic acid from membrane phospholipids via inhibition of phospholipase (Flower, 1978). These results, therefore, suggest that prostaglandins and platelets may play a role in the injury due to MCTP. Alternatively, these drugs have other effects including decreased weight gain, vasodilation, antiinflammatory and immunosuppressive properties that also may account for their protective effects.

III. PULMONARY HYPERTENSION

A. Chronic Pulmonary Hypertension in Man

Although chronic pulmonary hypertension is usually secondary to another disease process, there exists a group of human patients in whom the cause of elevated pulmonary arterial pressure cannot be determined. In such cases, the diagnosis of primary pulmonary hypertension (PPH) is made.

PPH is not common, but the difficulty in diagnosis, lack of effective therapy and high mortality make it a serious problem (Voelkel and Reeves, 1979). Presenting symptoms are non-specific and occur late in the time-course of the disease. By the time diagnosis is made, major pulmonary vascular alterations and right ventricular hypertrophy (RVH) have developed, making it difficult to relate

any causal factors to the onset of the early vascular lesions and pulmonary hypertenion. Therefore, little is known about the initiating events and pathophysiological processes that lead to PPH.

B. MCT as a Model for Human Pulmonary Hypertension

There are many similarities between MCT-induced pulmonary hypertension and PPH. Both have vasculitis, intimal proliferation and fibrosis, endothelial cell swelling, platelet- and fibrin-containing thrombi, capillary obstruction and decreased lumen diameter. Vascular changes include increased medial thickness and extension of smooth muscle into normally non-muscular pulmonary arterioles. Also, patients with PPH have decreased ability to remove biogenic amines from the circulation (Sole et al., 1979). Plexiform lesions, characteristic of human PPH, have been reported in rats treated with MCT (Watanabe and Ogata, 1976). Because of these similarities, the MCT-treated rat is an excellent animal model for the study of PPH.

C. Possible Role of the Immune System in the Cardiopulmonary Effects of MCTP

The mechanism by which delayed cardiopulmonary effects result from a single injection of unstable MCTP is unknown. The delay in major effects is consistent, however, with an immune response. For example, MCTP covalently bound to pulmonary endothelium might act as a hapten or might cause alterations in membrane structure that expose endogenous antigenic determinants which are normally sequestered. An immune response may then be mounted against this altered tissue, resulting in the inflammation and other vascular changes.

Carpenter et al. (1976) have listed major categories of histologic changes that are considered to be markers for the involvement of humoral immunity in the rejection of tissue allografts. These are (1) immunoglobulin deposits, (2) vasculitis or fibrinoid necrosis, (3) PMN infiltration, (4) accumulation of platelets and fibrin in vessels, and (5) mononuclear cell infiltration. It is of

interest that all of these are hallmarks of MCTP-induced histopathology. The last four characteristics have been described by numerous investigators following administration of MCT or MCTP to rats. With regard to the first criterion, the deposition of IgG in tissue after MCTP does occur (Bruner et al., 1982).

There also are striking similarities between the lesions observed in the lungs of rats treated wtih MCT or MCTP and those observed in tissues during rejection after transplantation. Pederson and Morris (1974a) found that major changes in primary kidney graft hemodynamics started 3-5 days after transplant. Major abnormalities in graft vascular permeability occurred by 5 days after transplant. The progression of damage continued until the organs were rejected. Similarly, Forbes et al. (1983) found that diffuse microvascular endothelial cell injury is an early characteristic of first-set rat cardiac allograft rejection. Microvascular lesions are not seen up to three days after transplant. By 4-5 days, endothelial cells are swollen and changes in their membrane permeability are observed. Likewise, after MCT or MCTP is given to rats, the endothelial cells are among the first cells altered, and the endothelial cell changes are very similar to the alterations described by Forbes in rejecting hearts.

The immune system may mediate pulmonary damage by one or more of several mechanisms. A cell-mediated immune response, initiated by T lymphocytes and not involving antibody, may cause the damage. When sensitized lymphocytes are exposed to pulmonary antigens that may arise after MCTP treatment they could be stimulated to produce factors that increase vascular permeability and attract macrophages and monocytes to the site of the antigen. The macrophages might then liberate lysosomal enzymes and toxic oxygen metabolites that lead to tissue injury. Lymphocytes may also release factors that are directly toxic to pulmonary cells.

A humoral mediated immune mechanism might also be involved in MCTP cardiopulmonary injury. Antibody directed against MCTP acting as a hapten or against newly exposed antigens may develop. Binding of this antibody to antigens may result in fixation of complement (C), leading to direct cell lysis and the generation of anaphylatoxins that contract smooth muscle and increase vascular permability. Also, chemotactic factors that attract neutrophils into the site of injury are generated as the result of C fixation.

Neutrophils (PMNs) are phagocytes associated with and essential for acute inflammatory reactions due to immune reactions and non-immunologic injury in tissues. In addition to playing an essential role in killing bacteria and removing debris by phagocytosis, these cells produce tissue injury via the release of oxygen-derived free radicals, proteolytic enzymes, arachidonic acid metabolites and platelet-activating substances (Janoff et al., 1968; Henson, 1972; Senior et al., 1977; Fantone and Ward, 1982; Repine et al., 1982). The PMN has been implicated as a mediator of lung injury in the adult respiratory distress syndrome (Repine et al., 1982), immune complex injury of the lung (Johnson and Ward, 1974), endotoxin-mediated lung damage (Heflin and Brigham, 1981), complications of acute pancreatitis (Barrie et al., 1982) and chemical intoxication of the lung (Yamada et al., 1982).

In MCT and MCTP pneumotoxicity, the PMN is prominent in the damaged lung tissue. PMNs are found in the alveoli, in perivascular areas and attached to the endothelial surface of blood vessels (Merkow and Kleinerman, 1966). Increased numbers of neutrophils are found in bronchopulmonary lavage fluid from rats treated with MCT (Stenmark et al., 1985). Since they are abundantly present, it is possible that these cells play an important role in producing the endothelial cell damage and vasculitis.

D. The Role of Oxygen Radicals in MCTP-induced Lung Injury

One of the mechanisms by which phagocytic cells such as the PMN may produce tissue injury is by production and release of toxic oxygen metabolites. Studies in vitro have indicated that oxygen metabolites released from activated neutrophils and macrophages are toxic to a wide variety of eukaryotic cells including erythrocytes, fibroblasts, tumor cells, leukocytes, platelets, spermatazoa and endothelial cells (Fantone and Ward, 1982). The oxygen metabolites implicated include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), myeloperoxidase metabolites such as hypochlorous acid, hydroxyl radical ($OH^\cdot$) and singlet oxygen (Fantone and Ward, 1982). The particular metabolite involved depends on many factors including the type of effector cells, target cells and activating stimulus. Once O_2^- and H_2O_2 are formed it is thought that they may lead to the production of hydroxyl radical ($OH^\cdot$) via the Fenton reaction as follows:

$$O_2^- + F^{+++} \longrightarrow Fe^{++} + O_2$$

$$H_2O_2 + Fe^{++} \longrightarrow Fe^{+++} + OH^- + OH^\cdot$$

In this reaction, oxidized trace metal (e.g., Fe^{+++}) is thought to react with O_2^-, producing reduced metal and O_2. The reduced metal can then react with H_2O_2 leading to regeneration of the oxidized metal and forming the $OH^\cdot$ which is highly reactive and can cause severe tissue damage (Ward et al., 1983b). It is of interst that in at least one clinical case, primary pulmonary hypertension was associated with increased iron absorption and storage (Molden and Abraham, 1982).

In the lung, there is evidence that local production of reactive oxygen metabolites by neutrophils may be responsible for adult respiratory distress syndromes observed clinically (Repine et al., 1982). In an animal model of pulmonary injury, the intravenous administration of cobra venom factor into rats

results in intrapulmonary sequestration of PMNs, which is associated with increased vascular permeability and focal intra-alveolar hemorrhage (Till et al., 1982). These changes are prevented by prior treatment with catalase or by neutrophil depletion, supporting the hypothesis that neutrophils cause vascular injury by the release of H_2O_2. Superoxide anion and H_2O_2 also produce injury during antigen-antibody reactions in the lung (Johnson and Ward, 1981).

Reactive oxygen species may cause injury by a number of mechanisms. Oxygen radicals may be directly cytotoxic. Also, toxic oxygen metabolites alter cell membrane by causing cross-linking of proteins, cleavage of polypeptide chains and lipids and by causing lipid peroxidation. Other effects include inactivation of α_1 antiprotease, potentiation of leukocyte proteases and activation of complement. Thus, reactive oxygen metabolites from activated neutrophils may be important in causing injury due to MCTP.

IV. SPECIFIC AIMS

Treatment of rats with MCT or its pyrrole metabolite (MCTP) causes pulmonary vascular injury and physiological alterations that result in pulmonary hypertension and right ventricular hypertrophy. The nature and progression of lesions observed in MCTP-treated rats are similar to those in humans suffering from primary pulmonary hypertension. Therefore, the MCTP-treated rat is an animal model that can be used for the study of this human disease. Experimental data indicate that the onset of MCTP pneumotoxicity is delayed four to seven days after a single dose, suggesting that MCTP acts indirectly to produce pulmonary vascular injury. Histologic lesions in lungs from MCTP-treated rats are similar to lesions occurring in immune reactions. Thus, immune-mediated mechanisms may be important in the development of MCTP-induced lung injury and pulmonary hypertension. Accordingly, the focus of these studies was to

examine the role of immune mechanisms in cardiopulmonary injury caused by MCTP. Experiments were undertaken to:

1. Characterize the pneumotoxic effects of MCTP by:
   a) Defining the time course of injury after MCTP.
   b) Testing the effect of a mixed function oxidase inducer and inhibitor on MCTP-induced pulmonary injury.
   c) Comparing the pneumotoxic effects of equivalent doses of MCT, MCT N-oxide and MCTP.
   d) Characterizing the stability of MCTP in aqueous vehicles.
2. Determine if a cell-mediated immune response is involved in the cardiopulmonary effects of MCTP by:
   a. Determining if the lymphocyte is integral to the production of MCTP pneumotoxicity by determining if cardiopulmonary effects of MCTP are altered in rats co-treated with anti-lymphocyte serum.
   b. Co-treating rats with MCTP and the immunosuppressant cyclosporin A.
   c. Examining the ability of lymphocytes adoptively transferred from MCTP-treated animals to produce lung injury or to alter the time-course and/or severity of MCTP-induced damage.
3. Assess the role of complement in mediating lung injury that occurs after the administration of MCTP by depleting animals of complement after treatment with MCTP and by measuring complement activity in the serum of MCTP-treated rats.
4. Determine if administration of enzymes that inactivate toxic oxygen metabolites, which are produced by activated neutrophils, protect against the damage caused by MCTP.

5. Determine if free radical scavengers protect against MCTP-induced injury *in vivo*.

6. Determine if leukotrienes may be involved in the injury due to MCTP by co-treating rats with MCTP and the leukotriene synthesis inhibitor diethylcarbamazine.

The long-term goal of this research is to understand the role of effector mechanisms of the immune system in the development of chronic pulmonary vascular injury and pulmonary hypertension. Such an understanding may lead to useful measures to prevent or to treat human chronic pulmonary hypertension and right heart failure.

MATERIALS AND METHODS

A. Animals

Respiratory disease free, male, Sprague-Dawley rats (CF:CD(SD)BR) or Fisher F-344 (CDF(F-344)/CRLBR) rats (Charles River Laboratories, Portage, MI or Kingston, NY), weighing 150-290 gm were used in these studies. F-344 rats are an inbred strain of rats which readily accept grafts from other F-344 donors (communication with Charles River Labs., our own unpublished observations). Thus, organs or cells transferred between F-344 rats are not rejected by F-344 recipients. The animals were housed in plastic cages on corn cob bedding under conditions of controlled temperature. The cages were kept in an animal isolator such that the rats breathed only HEPA-filtered air. The animals were maintained on a light:dark cycle (12:12 hours) and were allowed food (Wayne Lab Blox) and tap water ad libitum.

B. Treatment with MCT or MCTP

1. Single injection of MCT

MCT was dissolved in 0.2 M HCl and then the pH was readjusted to 7.0 using 2 M NaOH. A final concentration of 60 mg/ml was obtained by adding distilled water. This MCT preparation was given by subcutaneous injection at a dose of 105 mg/kg. Controls received an equivalent volume of 0.9% saline, s.c.

2. Treatment with MCTP

Rats were treated with a single injection of MCTP in the tail vein. MCTP was prepared in N,N-dimethylformamide (DMF) vehicle just prior to use. Controls received an equivalent volume of DMF vehicle. The MCTP and DMF were administered using an infusion apparatus that easily permitted administration of a 0.8 ml saline flush following DMF or MCTP.

C. Synthesis of MCTP

MCTP was synthesized from MCT via an N-oxide intermediate as described by Mattocks (1969). The MCTP isolated from the synthesis procedure has Ehrlich activity (Mattocks and White, 1971) and a structure consistent with MCTP as determined by mass spectrometry (Mattocks, 1969; Culvenor et al., 1970). MCTP was stored in the dark at -20°C in glass vials under nitrogen gas.

D. Cell Counting

White blood cell and platelet counts were determined in heparinized whole blood samples removed from the abdominal aorta, vena cava or from the tail. When blood samples were taken from the vena cava or aorta, rats were given 500 U of sodium heparin which was allowed to circulate for 60 seconds before the blood was drawn. When blood was taken from the tail, approximately 0.5 ml of blood was allowed to drip into a small, conical microcentrifuge tube that contained approximately 20 µl of 3% trisodium EDTA in 0.9% saline. For counting blood leukocytes, twenty-microliter volumes of blood were diluted in ammonium oxalate buffer using a Unopette system. White blood cells, lymphocytes in suspension, peritoneal exudate cells and platelets were counted in a modified Neubauer hemacytometer. Differential cell counts were obtained from Wright's stained smears by counting and identifying 100-200 cells. Absolute

counts in a given blood sample or cell suspension were determined by multiplying the fraction of each cell type found in the differential count by the total cell count in the blood sample or suspension.

E. Assessment of Cardiopulmonary Injury

1. Right ventricular hypertrophy (RVH)

RVH was measured as the ratio of right ventricle (RV) weight to the weight of the left ventricle plus septum (LV+S) as described by Fulton et al. (1952). The heart was blotted to remove excess blood, and the atria were trimmed off and discarded. The right ventricle was then cut away, leaving the left ventricle plus septum intact. Each piece was weighed to the nearest milligram and the weight ratio was calculated.

2. Electrocardiogram (ECG)

A six lead ECG was recorded in anesthetized rats using a Grass 7 PGA ECG preamplifier in a Grass model 7 polygraph. One electrode each was placed in the right and left shoulder and in the right and left inguinal region. The ECG was recorded with the rat placed in the ventral position (Fraser et al., 1967). The mean electrical axis of the ECG was determined to the nearest 30 degrees from the tracings (Fraser et al., 1967).

3. Pulmonary arterial pressure

After the ECG was recorded, the pulmonary arterial pressure was determined using a modification of the method of Hayes and Will (1978). The pressure was measured using a Statham PI 23 ID pressure transducer and Grass model 7 polygraph. A 24 gauge lightweight-wall Teflon catheter, in the shape of a shepherd's hook was fitted into an introducer cannula made of PE-160 polyethylene tubing. At that time, 500 U/kg of sodium heparin was injected into each rat via the catheter system. The catheter set was then advanced into the right

ventricle, and the introducer cannula was removed. The distal tip of the Teflon catheter was then carefully manipulated into the pulmonary artery and the pressure was recorded. The location of the catheter in the pulmonary artery was confirmed by visual inspection.

4. Bronchopulmonary lavage

The trachea was cannulated with a blunted 18 gauge disposable hypodermic needle. A ligature was placed around the trachea and cannula to hold the cannula in place. The abdomen and thorax were then opened and the lungs were carefully dissected free. A known volume of room temperature saline (0.9%) was instilled into the airway and then removed. The airway was then lavaged a second time with the same amount of saline. The volume of saline instilled was determined by multiplying the mean body weight (in kg) of both treated and control animals by 23 ml/kg (Mauderly, 1977). The lavage fluid was then spun in a centrifuge at 600 g for 10 min, and the activity of lactate dehydrogenase (LDH) in the cell-free supernatant fluids was assayed spectrophotometrically using the method of Bergmeyer and Bernt (1974). The LDH activity was quantified by measuring the conversion of the cofactor NADH to NAD as pyruvate substrate is converted to lactate. The remaining pellet containing cells from the pulmonary airway was resuspended in a known volume of saline, diluted in the Unopette system, and the cells were counted using a hemacytometer.

Lavage fluid protein concentration was determined using the method of Lowry et al. (1959). Bovine serum albumin was used as the standard.

5. Pulmonary sequestration of radiolabelled protein as a marker of lung injury

Pulmonary injury was assessed by measuring the retention of ^{125}I-labelled bovine serum albumin (^{125}I-BSA) in the lungs using a modification of the method of Johnson and Ward (1974). Rats were given an i.v. injection of 0.2 ml of ^{125}I-BSA (1.0 mg/ml) containing 400,000 cpm of radioactivity. Four hours later,

the rats were anesthetized with pentobarbital and were given 500 U sodium heparin in 0.5 ml saline via the posterior vena cava. One minute later, 1.0 ml of blood was removed and placed in a test tube for determination of radioactivity (Tracor 1185 series gamma counter, Chicago, IL).

A saline-filled catheter (PE 190, Clay Adams, Parsippany, NJ) was tied into the pulmonary artery. A cannula also was placed in the trachea. The lungs, cannulae and trachea were removed from the thorax, and the left atrial appendage was cut. The pulmonary vasculature was gently perfused with 10 ml saline via the pulmonary arterial cannula while the lungs were periodically ventilated with small volumes of air.

In studies where lavage fluid LDH activity was not measured, the lungs were trimmed from the connective tissue, washed with saline, blotted dry and placed in tubes for counting radioactivity. An index of lung injury was calculated as follows:

$$\text{Lund Injury Index} = \frac{\text{(lung cpm)}}{\text{cpm in 1.0 ml blood}}$$

In studies where lavage fluid LDH activity was measured, the airway was lavaged with saline after the vasculature was cleared with saline. The lung injury index was calculated as above except that radioactivity removed from the lungs in the lavage fluid was added to the total radioactivity in the lungs before the calculation was made.

6. Lung weight

Wet lung weight was determined by subtracting the weight of the lungs plus connective tissue, which was measured prior to pulmonary alveolar lavage and vascular perfusion, from the weight of the connective tissue remaining after the lungs were trimmed away.

F. Time-course of Injury After A Single Injection of MCTP

1. MCTP treatment

Rats were treated with a single injection of MCTP at a dose of 5 mg/kg in the tail vein or with a similar volume of DMF vehicle. The rats were then killed at 3, 5, 7, 10 and 14 days after treatment and the various indexes of injury were measured.

2. Assessment of cardiopulmonary injury

On the day of killing, the rats were weighed and anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and the following indexes of injury were measured as described above: body weight gain, ECG, pulmonary arterial pressure, white blood cell count and platelet count, bronchopulmonary lavage fluid LDH activity and protein concentration and RV/(LV+S).

3. Sequestration of ^{125}I-BSA in the vasculature after MCTP

In a separate group of rats, the time-course of ^{125}I-BSA sequestration in lungs after MCTP was determined. Rats were given MCTP (4 mg/kg, i.v.) or DMF. Groups of rats were killed 30, 60, 90, and 240 minutes after MCTP and on days 3, 5, 7, 10 and 14 after treatment. On the day of killing sequestration of ^{125}I-BSA in the lungs was measured as outlined above.

G. Effect of Pentobarbital and SKF-525A on Cardiopulmonary Injury Induced by MCT or MCTP

These studies were undertaken to evaluate the effect of a mixed function oxidase (MFO) inducer and inhibitor on pulmonary injury due to MCTP. Induction or inhibition of MFO activity increases or decreases MCT-induced pulmonary injury, respectively. To confirm this effect, rats were given phenobarbital (PB) (75 mg/kg, i.p.) or an equivalent volume of saline daily for 4 days prior to a single dose of MCT (105 mg/kg, s.c.) or saline vehicle. Rats were killed 7 days after MCT and lung injury was assessed. To confirm the efficacy of an MFO inhibitor

on MCT-pneumotoxicity, rats received SKF-525A (75 mg/kg, i.p.) or saline vehicle one hour prior to a single dose of MCT (105 mg/kg, s.c.) or saline vehicle. Rats were killed 7 days after treatment with MCT or SAL and lung injury was assessed. Similar studies were done using MCTP instead of MCT. Rats received MCTP (3.5 mg/kg, i.v.) or an equivalent volume of DMF vehicle and PB or SKF-525A according to the same schedule as described for MCT-treated rats.

H. Relative Efficacy of MCT, MCT N-oxide and MCTP

These studies were undertaken to compare the effect of equivalent doses of MCT, MCT N-oxide and MCTP on pulmonary injury. Rats were given MCT, MCT N-oxide or MCTP (5 mg/kg, i.v.) or an equivalent volume of DMF vehicle. Solutions of MCT, MCT N-oxide or MCTP were prepared in DMF (10 mg/ml). The rats were killed 14 days after treatment and cardiopulmonary injury was assessed.

I. Toxicity of MCTP Prepared in Aqueous Vehicle

To test the toxicity of chemically synthesized MCTP in aqueous solutions, MCTP was prepared using either serum or saline as the vehicle. The solutions were prepared by first mixing MCTP in DMF (32 mg/ml). A small volume of this solution was then mixed with saline or serum to give a solution containing 8 mg/ml MCTP. When MCTP was added to saline, an orange-brown, floculent precipitate formed. This solution was sonicated and then injected into rats at a dose of 0.5 ml/kg, i.v. When MCTP was added to fresh rat serum, no precipitate formed, but the plasma turned orange in color over approximately one minute. This solution was given at a dose of 0.5 ml/kg, i.v. Controls received MCTP in DMF vehicle (4 mg/kg, i.v.). The rats were killed 14 days after treatment, and cardiopulmonary injury was assessed. Pyrrole in the plasma was measured using a modified Ehrlich reaction (Mattocks and White, 1970).

J. Color Change in Serum After MCTP Addition

A red-orange color change was observed when MCTP was added to serum and likely represents the binding of MCTP to serum proteins or formation of polymer that occurs when MCTP is added to aqueous or acid vehicles (Mattocks, 1969). To determine the rate at which this color change occurs, MCTP was added to serum and allowed to stand at room temperature for 30 minutes. The difference spectrum was then measured with serum blank in the reference cuvette to ascertain the wavelength of maximum light absorbance (Amax). In a subsequent experiment, the rate of color change was determined by measuring change in absorbance versus time starting immediately after MCTP was added to serum using a Beckman UV 5260 recording spectrophotometer. A serum blank was similarly used in this experiment.

K. The Role of Cell-mediated Immunity in MCTP-induced Pulmonary Injury

1. Antilymphocyte serum (ALS)

a. Efficacy: ALS has been used to suppress immune system function in laboratory animals (Lance et al., 1973). The efficacy of ALS as an immunosuppressant was confirmed by determining the ability of ALS to prolong skin graft survival in treated rats. Three F-344 rats received an intraperitoneal injection of 1.25 ml/100 gm of ALS (M.R. Bioproducts, Walkersville, MD) starting one day before receiving skin grafts. Subsequently, the same ALS doses were given on days 1, 3, 5 and 7 after the grafting procedure. Three F-344 controls received equivalent doses of rabbit control serum (CS) and skin grafts according to the same schedule. Each rat received two skin grafts on day zero which were placed on the lateral thoracic wall using the method of Billingham and Medawar (1951). Patches of tail skin served as grafts. Each rat received a xenograft from a Sprague-Dawley donor and an allograft from its own tail. The allograft served

as an internal control. Six days after grafting, bandages were removed and the grafts were evaluated on a daily basis until the xenografts were rejected.

b. Effects of ALS on MCTP-induced pneumotoxicity: Rats were treated with MCTP and ALS to determine if immunosuppression by ALS co-treatment would protect rats against MCTP-induced pulmonary injury. Four groups of rats received either MCTP (3.5 mg/kg, i.v.)/ALS, MCTP/Rabbit CS, DMF/ALS or DMF/CS. The ALS was given one day before MCTP and on days 1, 3 and 5 after MCTP at a dose of 1.25 ml/100 gm, i.p. The extent of pulmonary injury was assessed seven days after MCTP.

2. Effect of Cyclosporin A (CyA) on MCTP-induced pneumotoxicity

The immunosuppressant CyA also was tested for its ability to protect against MCTP-induced pulmonary injury. Sprague-Dawley rats weighing 200-225 gm were given either CyA (10 mg/kg) and MCTP (4 mg/kg, i.v.), olive oil (OI) vehicle and MCTP, CyA and DMF or OI and DMF. CyA was given at a dose of 10 mg/kg s.c. every day starting 2 days before MCTP. Rats were killed 14 days after MCTP and lung injury was assessed. In a separate study of similar design, rats were treated with 20 mg/kg CyA. CyA doses in this range have been effective in suppressing organ graft rejection (Kawahara et al., 1980; Morris et al., 1983; Fritz et al., 1984; Hall et al., 1984; Kirkman et al., 1984) and in protecting from injury in rat models of autoimmune diseases (Nussenblatt et al., 1980; Thompson, 1983).

3. Adoptive transfer studies

If MCTP toxicity is mediated by immune mechanisms, then adoptive transfer of sensitized lymphocytes from MCTP-treated donor rats may alter the onset and severity of pulmonary injury in MCTP-treated recipients. To test this possibility, splenocytes or lymphocytes harvested from the lung-associated lymph nodes of MCTP-treated rats were adoptively transferred into recipient F-344 rats.

Several series of adoptive transfer studies were undertaken to test whether transfer of lymphocytes would alter MCTP-induced pulmonary injury in rats receiving the cells. In series 1, lymphocyte donors were treated with MCTP (4 mg/kg, i.v.) and killed 7 days later. Lung-associated lymph nodes and spleens were isolated, removed and minced into small pieces. Lymphocytes were isolated by gentle disruption in a conical glass tube with a loose fitting pestle. The pooled cells were washed 2 times in Hank's Balanced Salt Solution (HBSS), counted and viability determined using trypan blue dye exclusion. Cell suspensions contained 95-98% viable cells. Smears of cells were made on glass slides and stained with Wright's stain for differential counts.

Transfer of $5x10^7$ lymphocytes from CCl_4-treated donor mice caused liver injury in recipients that had not been given CCl_4 previously (Scheiffarth et al., 1967). The hepatic lesions in the recipients were attributed to damage mediated by the lymphocytes from CCl_4-treated donors that were sensitized against hepatocyte antigens. Accordingly, $5x10^7$ lymph node-derived lymphocytes from MCTP-treated donor rats were given to F-344 recipients in a volume of 2.0 ml HBSS, i.p., to determine if adoptive transfer of these cells would alter MCTP-induced lung injury. Control rats received an equivalent volume of HBSS without cells. The injections were given i.p., since i.v. administration of cells can cause non-specific pulmonary vascular injury (Bice et al., 1982). Twenty-four hours after receiving cells or HBSS, all rats received MCTP (4 mg/kg, i.v.). Three days after MCTP, the recipients were killed and lung injury was measured. Three days was chosen as the time point to assess lung damage since the onset of pulmonary injury after MCTP, as measured by increased LDH in the bronchopulmonary lavage fluid, occurs starting 4 days after MCTP (Bruner et al., 1983). Thus, if transferred lymphocytes caused a decrease in the time of onset of injury,

then it would be detected by measuring increased lavage fluid LDH activity in rats 3 days after MCTP.

A second group of recipients received $5x10^7$ splenic lymphocytes, i.p., from the series 1 donors. Controls were given an equivalent volume of HBSS, i.p. Twenty-four hours after receiving cells or vehicle, recipients were given a single injection of MCTP (4 mg/kg, i.v.) The rats were killed 3 days later and lung injury was assessed.

Boyer et al. (1981) reported that mineral oil-elicited peritoneal exudate cells (PEC) from rats immunized against a tumor cell line were capable of adoptively transferring immunity against the tumor cells into naive recipients. Accordingly, a similar protocol was used to assess the ability of PEC from MCTP-treated donors to alter the pneumotoxicity of MCTP in recipients of PEC. Thus, in series 2, donor rats were given 5 ml mineral oil 9 days after receiving MCTP (4 mg/kg, i.v.). Five days after receiving the mineral oil, the donors were killed and peritoneal exudate cells containing large numbers of lymphocytes were harvested by lavaging the peritoneum three times with 35 ml volumes of HBSS (Boyer et al., 1981). These cells then were washed in HBSS several times, counted and viability assessed. Differential counts were performed on Wright's stained smears. The recipients were given $1x10^8$ PEC, i.p. Controls received an equivalent volume of HBSS, i.p. Seven days later, the recipients received MCTP (4 mg/kg, i.v.). Groups of rats were killed 3 or 5 days after MCTP and pulmonary injury was assessed.

In series 3, PEC were obtained from donors exactly as in series 2. Recipients were given $1x10^8$ cells, i.p., and controls received an equivalent volume of HBSS, i.p. Twenty-four hours later, the recipients of the PEC or HBSS vehicle were given MCTP (4 mg/kg, i.v.). The recipients were killed either 3 or 5 days after MCTP.

In series 4, lung-associated lymph nodes were obtained from donor rats 28 days after receiving MCTP (3.0 mg/kg, i.v.). The lymphocytes were harvested and isolated as described above. Recipients were given $1x10^8$ cells, i.p., and controls received injections of HBSS. Seven days later, the recipients were given MCTP (4.0 mg/kg, i.v.). The MCTP-treated recipients were killed either 3 or 5 days after MCTP.

Pulmonary injury was assessed in each MCTP-treated lymphocyte donor. If lung injury did not occur in a particular donor, then the lymphocytes from that donor were not transferred into recipients.

L. The Role of Complement in MCTP-induced Pulmonary Injury

1. Treatment of rats with MCTP in vivo

All rats treated with MCTP in vivo received a dose of 3.5 mg/kg, i.v. Controls received an equivalent volume of DMF.

2. Assessment of complement activity

Serum complement consists of a group of serum proteins that act in an ordered sequence in response to certain activation stimuli. These proteins exert their effects primarily on cell membranes causing lysis in some cells and functional aberrations in others. The activity of complement in a serum sample can be measured using the hemolytic complement assay. Complement is activated when complement proteins encounter antibody bound to antigen. When antibody-coated cells are exposed to serum, complement is activated and the activation products can cause lysis of the cells. Since red blood cell (RBC) lysis is simple to measure, complement activity is assessed by exposing antibody-coated (sensitized) RBC to serial dilutions of a test serum sample and then measuring

hemolysis. Thus, hemolysis of sensitized RBC indicates the presence of complement and lack of hemolysis indicates absence of complement from a serum sample.

One hundred percent lysis of RBC is approached asymptotically as increasing concentrations of complement are added to a hemolysis test system. Accordingly, the hemolytic unit of complement activity which lyses 50% of sensitized RBC under conditions that are arbitrarily standardized with respect to the concentration of sensitized RBC, the concentration and type of sensitizing antibody, the ionic strength and pH of the buffer system and concentrations of Mg^{++} and Ca^{++}, and the temperature (Eisen, 1980).

Serum complement activity in the present study was measured by a hemolytic assay using antibody-coated sheep red blood cells (Colorado Serum Co., Denver CO) (Ward and Cochran, 1965). Sheep red blood cells (SRBC) were washed three times in a triethanolamine buffer solution (TBS). The TBS stock solution was prepared by adding the following to 500 ml of double-distilled water: 43.9 gm NaCl, 14.0 ml triethanolamine (Sigma Chemical, St. Louis, MO), 6.8 ml of 12 N HCl, 0.75 ml of 1 M $CaCl_2$ and 5 ml of 1 M $MgCl_2$. A 1:10 dilution of the TBS stock solution was prepared each day and adjusted to a pH of 7.35. After the last wash and centrifugation, the SRBC were resuspended in twice the volume of the pellet with 1:10 TBS to produce a 50% SRBC suspension. Approximately 0.68 ml of the 50% SRBC suspension was added to 17 ml of 1:10 TBS. 0.3 ml of the diluted RBC was then added to 1.7 ml of distilled water. After the SRBC were lysed, the sample was read on a spectrophotometer at 550 nm. If necessary, the diluted RBC suspension was adjusted to an OD reading between 0.52 and 0.56 units by adding either TBS or 50% RBC suspension.

The working solution of rabbit anti-SRBC serum was prepared by adding 0.5 ml of a 1:100 dilution of stock rabbit anti-SRBC serum (Colorado

Serum Co., Denver, CO) to 17 ml of 1:10 TBS. Equivalent volumes of diluted RBC (prepared as above) were then combined with the working solution of antibody. The new suspension was allowed to incubate at room temperature for 20 minutes with occasional stirring. After the incubation, these sensitized SRBC were used in the CH50 assay.

The hemolytic complement activity in a serum sample was measured by preparing the following dilutions of the test serum sample: 1:40, 1:60, 1:80, 1:120, 1:160 and 1:240. One ml of each dilution was placed in a separate test tube. Subsequently, 0.5 ml of the sensitized SRBC suspension was added to each tube. Three sets of control tubes were prepared. Set A was prepared by adding 0.5 ml of sensitized SRBC to 1 ml of 1:10 TBS. Set B contained 0.5 ml of sensitized SRBC and 1 ml of water and set C contained 0.25 ml of sensitized SRBC and 1.25 ml of water. All tubes were incubated for 60 min at 37°C with gentle shaking to allow complement-mediated hemolysis to occur. The tubes were then centrifuged to remove intact SRBC and the supernatant in each was measured spectrophotometrically (540 nm) to quantify hemoglobin release due to hemolysis. The mean absorbance value obtained from control set A was subtracted from the OD reading of each supernatant to account for spontaneous hemolysis. The absorbance values from control sets B and C were used as references for 100% and 50% hemolysis, respectively. The absorbance (in OD units) from the two tubes having absorbance readings just greater than and just less than the absorbance value obtained from the 50% hemolysis control tubes (set C) were identified. These two points were then plotted on a graph of absorbance versus serum sample dilution. The serum sample dilution corresponding to 50% hemolysis was interpolated from the two points. The inverse of this dilution was taken as the complement activity (in CH50 units) in the serum sample tested.

After the repeatability assay procedure was established, a computer program was written to calculate the CH50 activity.

Since complement activation may occur in vivo without causing a change in total serum hemolytic activity (Hammerschmidt et al., 1980), nephelometric measurement of neutrophil aggregation also was used to detect activation of complement in vivo and in vitro. The procedure used was a modification of the method of Hammserchmidt et al. (1980). Rat neutrophils were obtained by peritoneal lavage from rats that had received an intraperitoneal injection of 0.1% glycogen solution (35 ml) 4 hours earlier. Erythrocytes were lysed using an ammonium chloride lysing solution and the neutrophils were washed 3 times in HBSS. After the last wash, the cells were suspended in HBSS containing 0.05% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) at a concentration of $2x10^7$ cells/ml. Viability was assessed using trypan blue dye exclusion and quantification of cell types in the suspension was done by counting cells in Wright's stained smears. These suspensions contained approximately 95% viable neutrophils.

All neutrophils were exposed to 50 µl of cytochalasin B (CB) solution prior to aggregation (Hammerschmidt et al. (1980). The CB solution was prepared by adding 1 mg CB (Sigma Chemical, St. Louis, MO) to 20 µl dimethylsulfoxie (DMSO). Then ten ml of phosphate buffered saline (PBS) (isotonic, pH 7.4) was added and mixed thoroughly. Ten minutes later, another 10 ml of PBS was added, the solution mixed and then centrifuged. The solution was stored in the freezer at -70°C until use.

All serum samples were collected and stored frozen at -70°C until CH50 activity or neutrophil aggregation was evaluated. All samples from a single study were evaluated on the same day under the same conditions.

To measure neutrophil aggregation, 0.5 ml of the cell suspension was loaded into a siliconized aggregometer curvette and placed in a platelet aggregometer (Payton model 300B). The cells were stirred at 90 rpm at 37°C. After a 2-minute equiibration period, 50 µl of serum sample or zymosan-activated rat serum (ZAS) was added and the aggregation response was recorded on a chart recorder. ZAS was prepared by exposing fresh rat serum to boiled, washed zymosan (Sigma Chemical Co., St. Louis, MO) at a concentration of 2 mg/ml for 30 min at 37°C. The aggregometer/recorder system was calibrated such that the transmittance obtained from a fresh neutrophil suspension diluted 50% resulted in a 100% deflection of the chart recorder pen (Hammerschmidt et al., 1980). Neutrophils exposed to the ZAS aggregated in a manner similar to that previously reported (Craddock et al., 1977c; Hammerschmidt et al., 1980).

3. Effects of MCTP on serum complement in vivo

Serum complement activity was measured in rats treated with MCTP. Just prior to receiving MCTP or DMF vehicle, a blood sample was taken from the tail vein. Subsequently, blood samples were taken at 1, 2, 4, and 7 hours after treatment. In a second group of rats, a blood sample was taken prior to MCTP or DMF and then blood samples were taken at 1, 3, 5, 7, 10 and 14 days after treatment. Total hemolytic complement activity and the ability of the serum to cause neutrophil aggregation was evaluated.

4. Bronchopulmonary lavage fluid and neutrophil aggregation

To determine if there is aggregation activity in the bronchopulmonary lavage fluid of rats treated with MCTP, 50 µl samples of lavage fluid from DMF- and MCTP-treated rats were added to 0.5 ml of PMN suspension and the aggregation response was recorded. Subsequently, 50 µl of ZAS was added as a positive control.

5. Complement depletion in MCTP-treated rats

The effect of complement depletion on MCTP-induced pulmonary injury was evaluated by co-treating rats with purified cobra venom factor (CVF) (a gift from Dr. Gerd Till, The University of Michigan, Ann Arbor). CVF was isolated from crude, lyophylized cobra (Naja naja) venom by ion exchange chromatography and gel filtration (Pepys, 1976). Preliminary studies indicated that 3 doses of CVF (20 units/dose, i.p.), given once every 12 hours followed by 3 more doses given once every 24 hours, resulted in complement depletion for 5 days. Serum complement activity then returned to normal levels in spite of continued CVF treatment. Accordingly, rats were given CVF starting 2.5 days after a single dose of MCTP or DMF (i.e., before the onset of lung injury). Controls received saline using the same protocol as for CVF. Thus, treatment groups received either DMF/saline (SAL), DMF/CVF, MCTP/SAL or MCTP/DMF. Blood samples were obtained from each rat via the tail vein prior to receiving the first dose of CVF or SAL and then every 24 hours thereafter. The serum was collected for determination of CVF efficacy. All rats were killed 7 days after receiving MCTP or DMF, and lung injury was assessed.

The efficacy of CVF as a complement depletor was evaluated by measuring total serum hemolytic complement activity and depletion of C3. Elimination of serum C3 was verified using the Ochturlony immunodiffusion method. The center well of an Ochturlony plate was charged with goat anti-rat C3 (Cappel-Worthington Laboratories, Malvern, PA) and the outer wells were filled with serum samples obtained from the rats. C3 depletion was confirmed when no precipitate was visible between wells containing serum from CVF-treated rats and the anti-rat C3.

6. Effect of MCTP on serum complement activity in vitro

To test the effect on CH50 activity of MCTP added to serum in vitro, fresh rat serum was collected and 250 µl of the serum was dispensed into each of several test tubes. Increasing amounts of MCTP were added to each sample giving serum containing MCTP at each of the following concentrations: 0.088, 0.176, 0.352, 0.7, 0.8, 1.5 and 2.8 mg/ml. Control serum contained an equivalent volume of DMF. These MCTP concentrations correspond to those to be expected by giving a 200 gram rat doses ranging between 1-34 mg/kg, assuming distribution into the plasma compartment. The tubes were incubated for 30 minutes, and then total hemolytic complement activity was measured in each sample.

7. Ability of MCTP to activate serum complement

Rat serum samples were exposed as described above (section L.2.6.) to MCTP or DMF vehicle and then evaluated in the neutrophil aggregation assay. 50 µl samples of MCTP- or DMF-treated serum were added to 0.5 ml of PMN suspension in the aggregometer cuvettes, and the ability of the serum sample to stimulate neutrophil aggregation was recorded. Subsequently, 50 µl of ZAS was added to the cuvette as a positive control.

To determine whether the alternative pathway of complement could be activated in MCTP-treated serum, serum was exposed to varying amounts of MCTP, resulting in samples containing the following concentration of MCTP: 0.75, 3 and 6 mg/ml. Control serum samples contained an equivalent volume of DMF. After a 30-minute incubation period, each serum sample was split into 2 equal volumes. Half of the samples received zymosan (2 mg/ml) in PBS whereas the other half received an equivalent volume of PBS. Each tube was incubated for 30 min at 37°C to activate serum complement. The tubes were centrifuged to remove the zymosan and the supernatants were transferred to clean tubes. 50 µl of each sample was tested for its ability to stimulate neutrophil aggregation.

After the aggregation response was recorded, 50 µl of ZAS was added to the aggregometer cuvette as a positive control.

M. Effect on MCTP Toxicity of Interventions that Alter Production or Metabolism of Toxic Oxygen Metabolites

1. Treatment of rats in vivo

All rats treated with MCTP received a dose of 3.5 mg/kg, i.v. Controls received an equivalent volume of DMF.

2. Effect of desferroxamine mesylate (DF) on MCTP-induced pulmonary injury

A 2x2 factorial design was used to study the effect of DF on MCTP-induced pulmonary injury. Rats in four groups received either DMF and SAL, DMF and DF, MCTP and SAL or MCTP and DF. Treatment with DF (150 mg/kg, i.m.) or SAL was started twenty-four hours prior to treatment with MCTP or DMF vehicle. Thereafter, rats received either DF (150 mg/kg, 2x/day) or SAL, twice daily, until day 7 after MCTP when the rats were killed and lung injury was assessed.

3. Effect of dimethylsulfoxide (DMSO) on MCTP-induced pulmonary injury

A 2x2 factorial design was used to test the effect of DMSO on MCTP-induced pulmonary injury. Before injection, the DMSO was diluted 50% in SAL. Rats in four groups received either DMF and SAL, DMF and DMSO, MCTP and SAL or MCTP and DMSO. DMSO or SAL treatment was started eight hours prior to treatment with MCTP or DMF. Thereafter, rats received DMSO or SAL three times daily. Separate studies were run to test three DMSO doses. DMSO doses tested were 0.67, 1.0 or 1.3 ml/kg, s.c., given three times daily. Rats were killed 7 days after MCTP and lung injury was assessed.

4. Effect of catalase (CAT) on MCTP-induced pulmonary injury

A 2x2 factorial design was used to test the effect of polyethylene glycol (PEG)-coupled CAT (Enzon Inc., South Plainfield, NJ) on MCTP-induced lung injury. Four groups of rats received either DMF and SAL, DMF and PEG-CAT, MCTP and SAL or MCTP and PEG-CAT. The circulating half-life of PEG-CAT was determined as described by Till et al. (1983) and found to be 31 hours. Accordingly, the rats received injections of PEG-CAT (1000 U) or SAL once daily until day 7 after MCTP when they were killed and lung injury assessed.

In a separate study, rats were given an i.v. injection of 7500 U PEG-CAT or an equivalent volume of SAL just before receiving MCTP. Subsequently, rats received 7500 U of PEG-CAT or SAL daily until day 7 when the rats were killed and lung injury assessed.

N. Effect of Diethylcarbamazine (DEC) on MCTP-induced Pulmonary Injury

1. Treatment with MCTP in vivo

Rats were given a single injection of MCTP at a dose of 4 mg/kg, i.v., on day zero of the study.

2. Treatment with DEC

On day 3 after MCTP, rats received DEC (33 mg/kg, 3x/day or 100 mg/kg, 3x/day, s.c.) or saline (1.0 ml/kg, 3x/day, s.c.). Three daily injections of DEC or saline were continued until 7 days after MCTP, when the rats were killed. To determine the effect of DEC on the development of RVH, a second group of rats was treated. On day 3 after MCTP, rats received either DEC (100 mg/kg, 3x/day, s.c.) or saline (1.0 ml/kg, 3x/day, s.c.), until day 14 after MCTP when the rats were killed and indexes of injury were measured.

O. Statistical Analyses

Data are expressed as mean ± S.E.M. In experiments having only two groups, the Student's t-test was used to compare means (Steele and Torrie, 1980). Comparisons in studies involving 3 or more groups were made using a completely random design one-way analysis of variance (ANOVA). Two-way factorial ANOVA was used to evaluate the effect of time and drug treatments on MCTP-pneumotoxicity. The effect of MCTP on serum complement activity was evaluated using mixed design ANOVA. Homogeneity of variance was tested using the F(max) procedure (Steele and Torrie, 1980). When the variance was not homogeneous, logarithmic transformation of the data was performed. The least significant difference test (LSD) was used for individual comparisons (Steele and Torrie, 1980). If the variance remained non-homogeneous after transformation, then pre-planned comparisons were made using the non-parametric Wilcoxon-Mann-Whitney two sample test (rank sum test) (Steele and Torrie, 1980). Correlation between the degree of right ventricular hypertrophy and mean electrical axis of the electrocardiogram was tested using Spearman's coefficient of rank correlation (Steele and Torrie, 1980). In all cases, a 95% confidence level was used as the criterion for significance.

RESULTS

A. Time Course of Injury After a Single Injection of MCTP

The purpose of this study was to characterize the evolution of pulmonary damage, pulmonary hypertension, right ventricular hypertrophy and hematologic changes induced by the administration of a single dose of chemically-synthesized MCTP. Additionally, determination of the mean electrical axis of the electrocardiogram as a non-invasive method to measure the development of right ventricular hypertrophy in rats was evaluated.

Rats were given a single dose of MCTP (5 mg/kg, i.v.) or DMF and were killed at various times after treatment. There was no difference in the rate of weight gain between treated and control rats at 3 or 5 days after treatment (Figure 2). At 7, 10 and 14 days after treatment control rats exhibited gain in weight as expected. However, rats treated with MCTP maintained their body weight at levels similar to those observed at the time of treatment.

Relative lung weight remained constant in control rats throughout the duration of the study (Figure 3). There was no significant change in lung weight at 3 or 5 days after the administration of MCTP. There was, however, a significant increase in lung weight at 7 days, and this increase became larger with time.

The onset of lung injury also was evaluated by monitoring the activity of LDH in the cell-free bronchopulmonary lavage fluid. No change in activity occurred in bronchopulmonary lavage fluid from MCTP-treated rats 3 days after dosing (Figure 4). There was, however, a marked increase in lavage activity at 5,

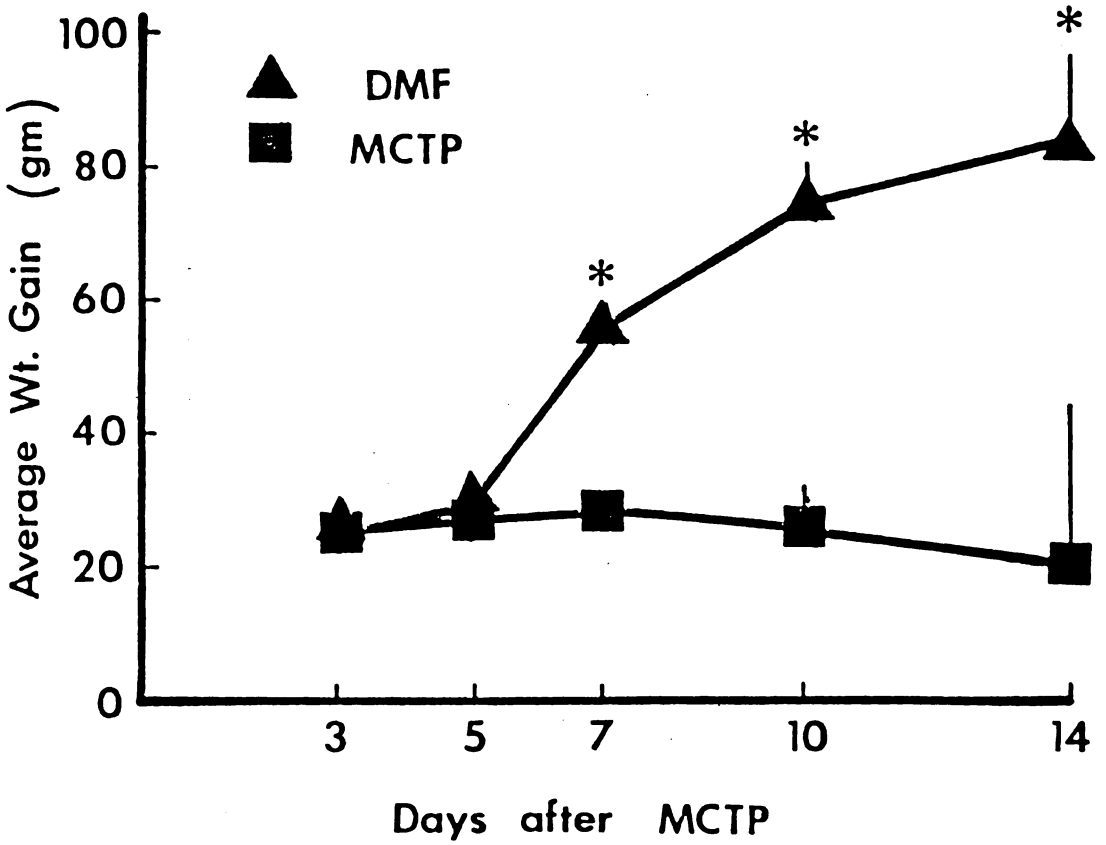


Figure 2. Effect of MCTP on average weight gain. Rats were treated iv either with 5 mg/kg MCTP or with dimethylformamide (DMF) vehicle. Weight gained by rats beween treatment and time of death was determined at 5 times. Symbols represent means ± SEM of 8 rats. Points lacking error bars indicate SEM is less than area covered by symbol. Asterisks indicate significant difference from control ($p < 0.05$).

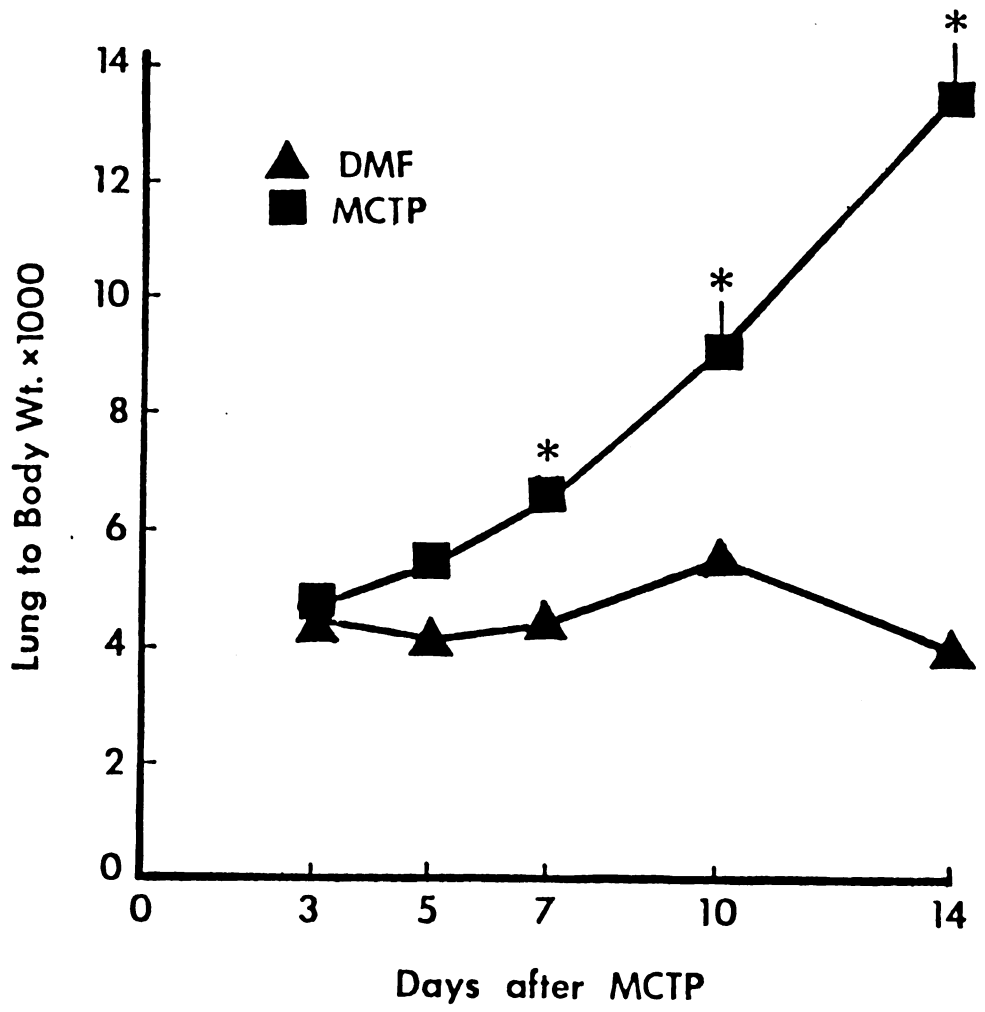


Figure 3. Effect of MCTP on relative lung weight. Lung weight-to-body weight ratio was calculated at 5 different times after treatment iv with either 5 mg/kg MCTP or dimethylformamide vehicle. Symbols represent means ± SEM of 8 rats. Points lacking error bars indicate SEM is less than the area covered by symbol. Asterisks indicate a significant difference from control ($p < 0.05$).

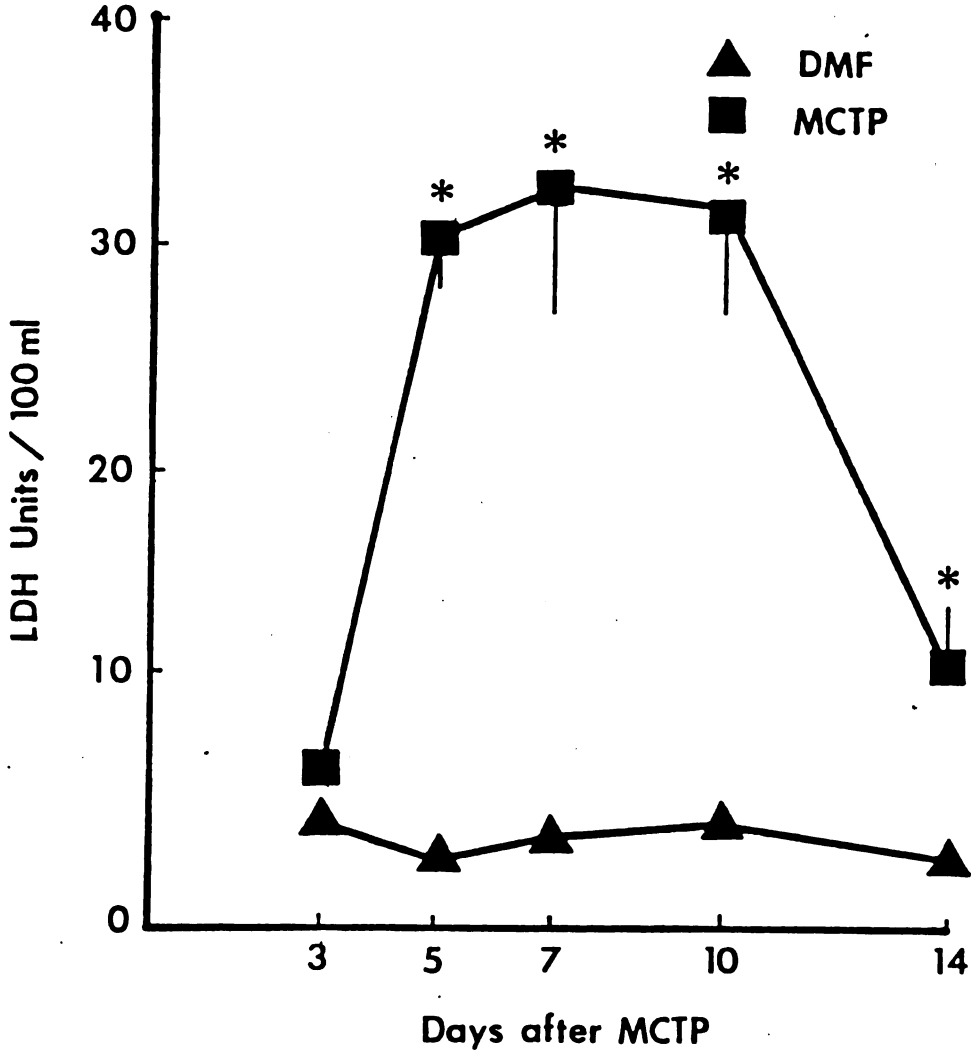


Figure 4. Effect of MCTP on cell-free bronchopulmonary lavage fluid lactate dehydrogenase (LDH) activity. Rats were treated iv either with 5 mg/kg MCTP or with dimethylformamide vehicle; lavage fluid LDH activity was determined as described in MATERIALS AND METHODS at 5 times after treatment. Symbols represent means $\pm$ SEM of 8 rats. Points lacking error bars indicate SEM is less than area covered by symbol. Asterisks indicate a significant different from control ($p < 0.05$).

7, 10, and 14 days after the administration of MCTP. The LDH activity 14 days after treatment, while still significantly greater than controls, was markedly less than at the previous three time points. To define more precisely the early changes in release of LDH into the airway, lavage fluid activity also was examined 4 days after MCTP treatment and was found to be significantly elevated (control 2.0±0.3 U/100 ml, n=3; MCTP treated 17.0±3.3, U/dl, n=3). Thus, the LDH activity began to increase after 3 days and rose through 5 days post-treatment.

The total protein concentration in the bronchopulmonary lavage fluid followed a time course similar to that observed with the LDH activity (Figure 5). However, one difference was that the protein concentration remained markedly elevated at 14 days after dosing. Also, the total protein concentration in lavage fluid was determined 4 days after the administration of MCTP. Similar to the LDH activity, the protein concentration was elevated in treated animals (2.25±0.49 g/dl) compared with controls (0.64±0.07 g/dl).

During the course of MCTP toxicity, various cell types accumulate in the alveoli of the lungs. The cell counts in the bronchopulmonary lavage fluid obtained from control animals remained constant (Figure 6). Cell counts obtained from MCTP-treated animals were significantly elevated over control values at 7 and 10 days after treatment. Cell types present included neutrophils, lymphocytes and pulmonary alveolar macrophages.

Total white blood cell counts (WBC) obtained from control rats remained constant throughout the study (Table 1). There was no change in WBC 3 and 5 days after MCTP administration, but WBC was significantly increased at the rest of the time points examined, and the magnitude of the count increased with time after dosing. Differential WBC showed that the increase in total circulating WBC was not due to one specific type of leukocyte (Table 1). There was no change in

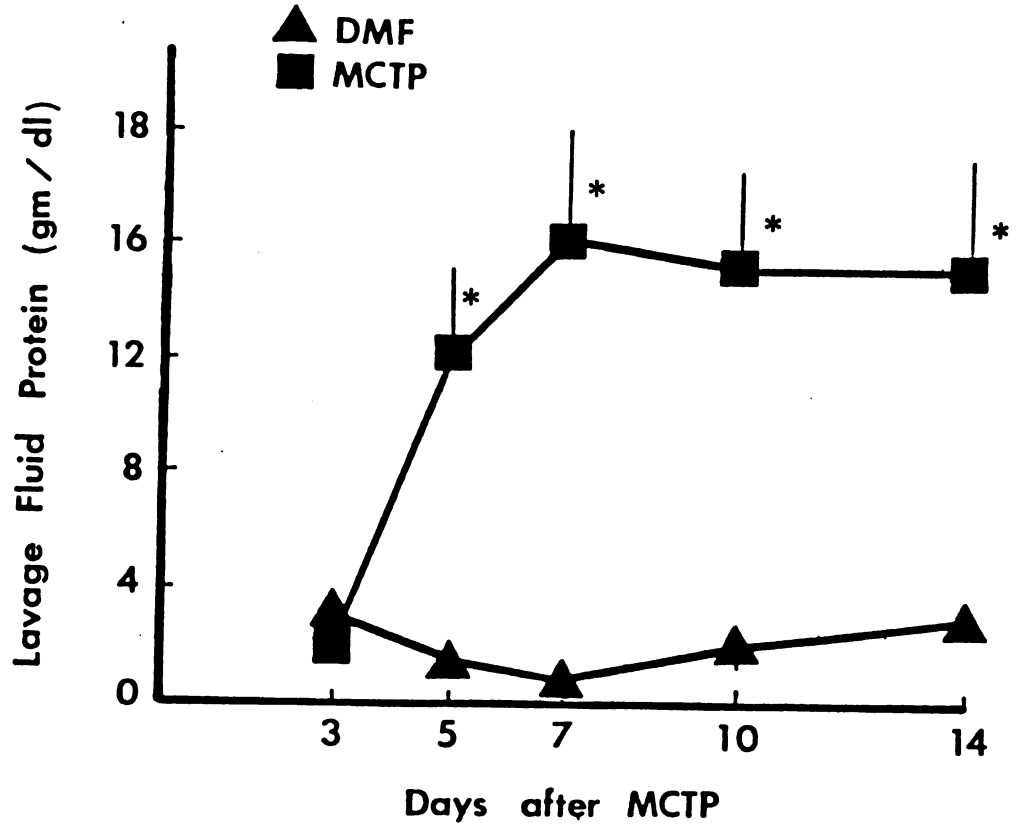


Figure 5. Effect of MCTP on protein concentration in cell-free bronchopulmonary lavage fluid. Rats were treated iv either with 5 mg/kg MCTP or with dimethylformamide vehicle. Total protein concentration was determined as described in MATERIALS AND METHODS. Symbols represent means $\pm$ SEM of 8 rats. Points lacking error bars indicate SEM is less than area covered by symbol. Asterisks indicate a significant different from control ($p < 0.05$).

Figure 6. Effect of MCTP on cell count in bronchopulmonary lavage fluid. Rats were treated iv either with 5 mg/kg MCTP or with dimethylformamide vehicle. At 5 different times, cell count in bronchopulmonary lavage fluid was determined. Symbols represent means $\pm$ SEM of 8 rats. Points lacking error bars indicate SEM is less than area covered by symbol. Asterisks indicate a significant difference from control ($p < 0.05$).

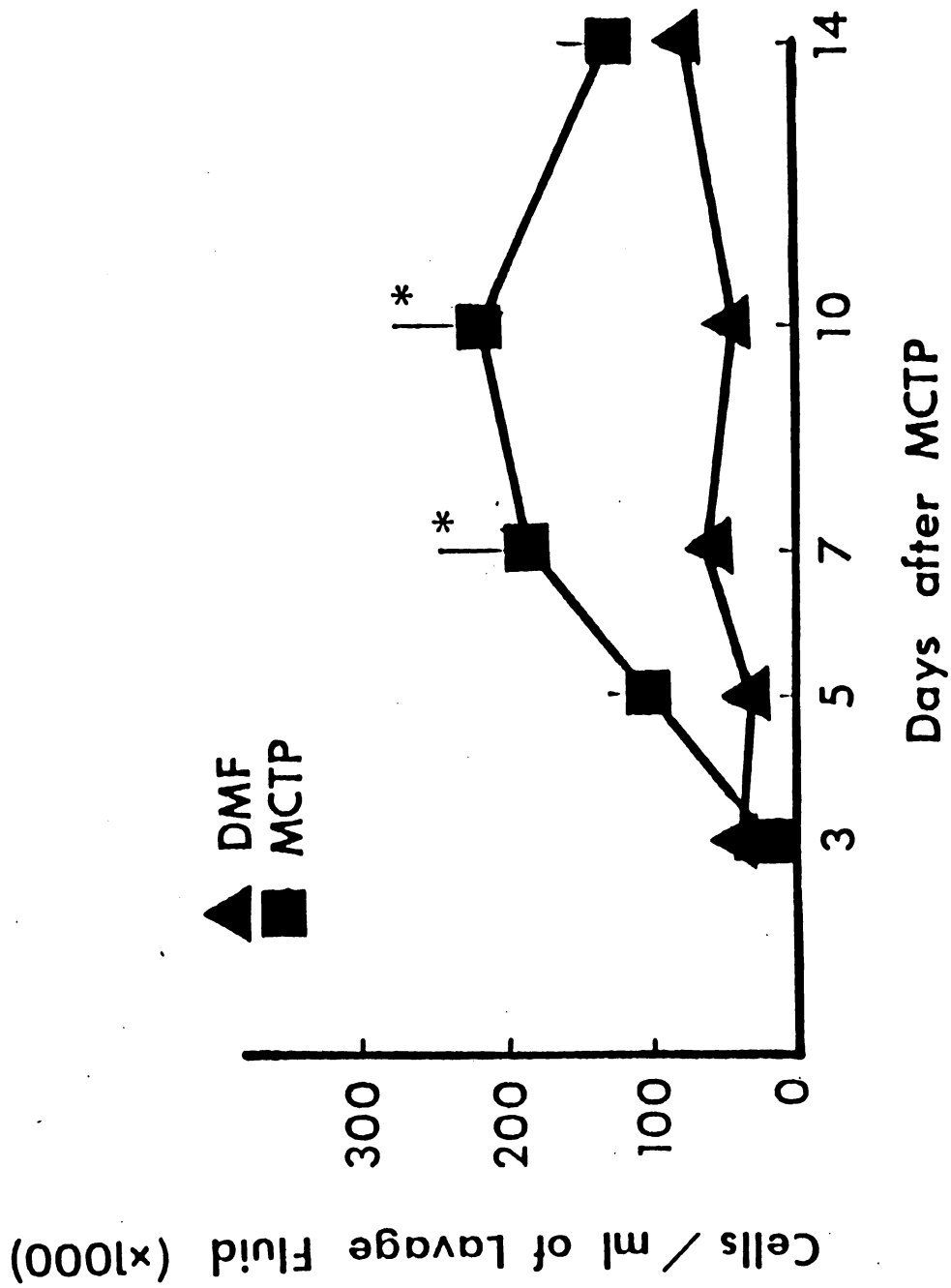

Cells / ml of lavage Fluid (x1000)
300
200
100
0
DMF
MCTP
*
*
3
5
7
10
14
Days after MCTP

Figure 6.

TABLE 1

Effect of MCTP on Hematologic Values

Days After Treatment	Treatment	Hematocrit, %	Platelets, $\times 1{,}000\ mm^{-3}$	White Blood Count, mm^{-3}	Segmented Neutrophils, mm^{-3}	Band Neutrophils, mm^{-3}	Lymphocytes mm^{-3}	Monocytes, mm^{-3}	Eosinophils, mm^{-3}	Basophils, mm^{-3}
3	MCTP	40±1	1,048± 58	8,119±1,486	919± 151	17±17	6,803±1,381	350±128	30±20	0±0
	Control	41±1	1,067± 58	7,040± 499	1,048± 185	69±65	5,229± 483	193± 69	35±66	0±0
5	MCTP	38±1	1,042± 62	7,508±1,190	2,270± 810	18±16	4,803± 442	364±112	52±45	0±0
	Control	38±1	1,070± 58	8,259±1,055	2,208± 245	20±17	5,888± 964	287± 59	39±39	0±0
7	MCTP	41±1	1,071± 68	10,408± 724*	2,178± 701	34±24	8,323± 655	409±241	87±76	0±0
	Control	39±1	980± 47	8,384± 999	2,353± 342	0±0	5,547± 831	414± 76	22±19	0±0
10	MCTP	47±2	1,187± 50	11,269±1,205*	1,883± 469	41±38	8,635±1,266	423±131	100±74	29±10
	Control	41±1	1,244± 61	7,026± 717	1,023± 228	28±26	6,066± 748	80± 32	24±23	0±0
14	MCTP	49±1*	1,023± 78	14,065±1,780*	3,415±1,461	65±45	10,003±1,974	582±264	0±0	0±0
	Control	42±1	1,134±214	8,006±1,267	649± 115	7±3	7,164±1,239	174± 37	12±12	0±0

[a]Rats were given an i.v. injection of either MCTP (5 mg/kg) or DMF vehicle. Determinations were made at 5 times as described in MATERIALS AND METHODS.
*Significantly different from control ($p < 0.05$).

eosinophil or basophil count throughout the study. Although not statistically significant, there was a tendency for segmented neutrophils, band neutrophils, lymphocytes and monocytes to increase in number in treated animals, especially at the later time points.

Platelet count did not change in treated animals at any time point examined (Table 1). Hematocrit values also remained constant until 14 days after treatment when a slight increase occurred in treated animals. An earlier report (Hilliker et al., 1982) indicated that platelet count decreased by 48 hours after subcutaneous administration of MCT. Accordingly, the effect of MCTP administration on platelet count at earlier time points was examined. As indicated in Table 2, platelet count was not affected in MCTP-treated animals 2, 12, 24, and 48 hours after treatment.

There was no increase in mean pulmonary arterial pressure in the rats at 3 or 5 days after MCTP treatment (Figure 7). Those rats killed at 7, 10, or 14 days after MCTP showed an elevation in mean pulmonary arterial pressure.

No right ventricular hypertrophy occurred in rats killed 3, 5, 7, or 10 days after MCTP treatment as measured by the weight ratio of RV/LV+S (Table 3). At 14 days, however, right heart hypertrophy was present in MCTP-treated rats.

There was little change in the mean electrical axis of the ECG of rats receiving MCTP until 14 days after treatment (Table 3). In those rats killed 14 days after dosing, the average mean electrical axis in MCTP-treated rats was to the right of the normal range, which is 1-105 degrees (Sanbhi and White, 1960). At this time, the deviation of the mean electrical axis was positively and significantly correlated with the extent of right ventricular hypertrophy (Spearman's rank correlation coefficient (= 0.64).

Rats killed 3 and 5 days after MCTP had the same relative sequestration of ^{125}I-BSA as did rats treated with DMF (Figure 8). Between 5 and 7 days after

TABLE 2

Platelet Counts in Rats Treated with MCTP[a]

Hours After MCTP Injection	Platelet Count, x 1,000 mm^{-3}	
	DMF	MCTP
2	919±451	912±150
12	889± 98	971±102
24	981±145	995± 80
48	933± 57	1,066± 54

[a]Rats were treated i.v. with 5 mg/kg MCTP or DMF vehicle. Determinations of circulating platelet count were made at 2, 12, 24, and 48 hr after treatment. There were no significant differences between treated and control animals ($p < 0.05$).

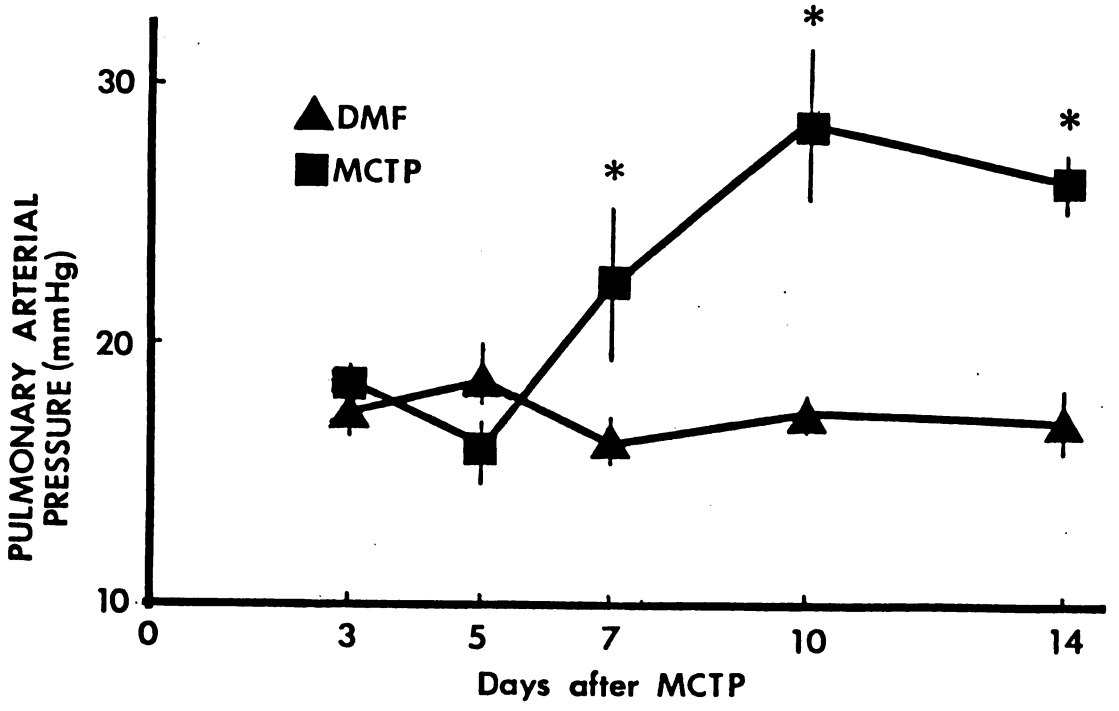


Figure 7. Effect of MCTP on mean pulmonary artery pressure. Rats were treated iv either with 5 mg/kg MCTP or with dimethylformamide vehicle. Mean pulmonary arterial blood pressure was determined at 5 different times as described in MATERIALS AND METHODS. Symbols represent means $\pm$ SEM of 8 rats. Points lacking error bars indicate SEM is less than area covered by symbol. Asterisks indicate a significant differnce from controls ($p < 0.05$).

TABLE 3

Effect of MCTP on Mean Electrical Axis of Electrocardiogram and Relative Right Heart Weight[a]

Days After Treatment	MCTP				Control			
	n	Average MEA	Range of MEA	RV/LV+S Ratio	n	Average MEA	Range of MEA	RV/LV+S Ratio
3	8	63°	30°–120°	0.318±0.005	8	34°	0°–60°	0.335±0.014
5	8	71°	30°–120°	0.322±0.009	8	79°	60°–120°	0.326±0.013
7	8	75°	60°–120°	0.298±0.013	8	60°	30°–90°	0.325±0.011
10	8	90°	0°–(-150°)	0.339±0.015	8	41°	0°–60°	0.315±0.007
14	11	-131°	0°–(-30°)	0.429±0.015[b]	9	62°	30°–90°	0.320±0.015

[a]Rats were treated with a single i.v. injection of MCTP (5 mg/kg) or with DMF vehicle. Determinations were made at 5 times after treatment. Values presented for mean electrical axis (MEA) are mean and range of MEA measured in each group. Values for right ventricle (RV) to left ventricle + septum (LV+S) weight ratios are means ± SEM.

[b]Significantly different from control group ($p < 0.05$).

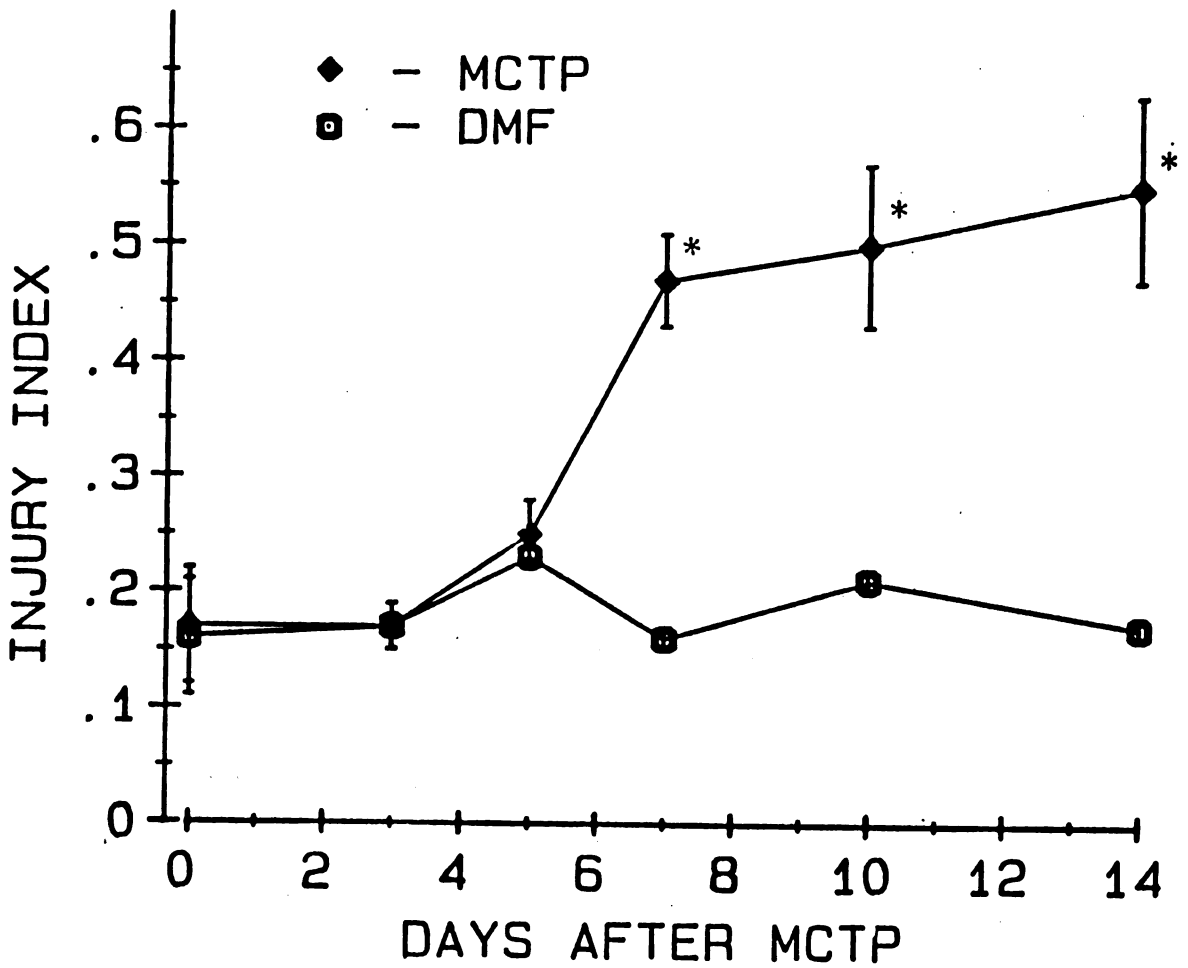


Figure 8. Effect of MCTP on sequestration of ^{125}I-BSA. Rats received MCTP (4 mg/kg, i.v.), or DMF and were killed at the indicated times after treatment. The injury index was determined as described in MATERIALS AND METHODS and represents sequestration of ^{125}I-BSA due to MCTP treatment. Values are mean ± SEM for groups of 3-8 animals. * indicates means significantly different from controls (ANOVA, LSD test, $p < 0.05$).

treatment, there was a marked increase in ^{125}I-BSA pulmonary sequestration in animals that received MCTP, and at 10 and 14 days these values remained elevated compared to DMF-treated controls (Figure 8).

The effect of MCTP on sequestration of ^{125}I-BSA also was evaluated at times shortly after MCTP treatment. Sequestration of the radiolabelled BSA in MCTP-treated rats was not different from rats treated with DMF vehicle (Table 4) when killed 30, 60, 120 and 240 minutes after MCTP.

B. Effect of an Inducer and Inhibitor of Mixed Function Oxidase Activity on MCTP-induced Pulmonary Injury

MCTP is a reactive electrophile that is unstable in aqueous solutions. Even though unstable, some of the MCTP that is produced in the liver apparently survives long enough in the circulation to reach the lung, still in the highly reactive form, and binds covalently with tissue nucleophiles. This bound pyrrole is thought to be responsible for causing the tissue injury. It is also possible that circulating MCTP degradation products could be metabolized by the MFO to other reactive species that are capable of causing lung injury. If so, then co-treatment with drugs that induce or inhibit MFO activity might alter the toxicity of MCT. Accordingly, the effect of phenobarbital and SKF-525A on MCTP-induced lung injury was tested in vivo.

1. Effect of PB on MCT toxicity

Rats were co-treated with MCT and PB to test the efficacy of the PB treatment regimen as an inducer of MFO activity. Rats treated with PB/MCT lost weight over the 7 days of the study whereas the other groups all gained weight. The weight gain in rats treated with PB/MCT and S/MCT was less than in those rats treated with S/SAL or PB/SAL (Table 5).

Treatment of rats with PB/MCT caused an increase in pulmonary lavage LDH activity compared to S/MCT-treated rats (Figure 9A). Lung

TABLE 4

Sequestration of ^{125}I-BSA in Lungs of MCTP-treated Rats

Time After Treatment (min)	Treatment[a]	
	DMF	MCTP
30	0.16±0.05[b]	0.09±0.02
60	0.07±0.01	0.18±0.06
120	0.15±0.01	0.18±0.05
240	0.16±0.01	0.18±0.04

[a]Rats received MCTP (4 mg/kg, i.v.) or DMF and were killed at the times indicated after treatment. Sequestration of ^{125}I-BSA in lungs was determined as described in MATERIALS AND METHODS. There were no differences between any of the groups treated ($p < 0.05$).

[b]Values represent mean ± SEM, n = 2-4/group.

TABLE 5

Effect of Phenobarbital (PB) or SKF-525A on MCT-induced Pneumotoxicity

Experiment	Treatment[a]	Body Wt Change[b]
PB	SAL/SAL	56±2
	PB/SAL	39±2[c]
	SAL/MCT	15±7[c]
	PB/MCT	-2±5[c,d]
SKF-525A	SAL/SAL	66±2
	SKF-525A/SAL	52±5[c]
	SAL/MCT	22±4[c]
	SKF-525A/MCT	42±3[d]

[a]Rats were co-treated with MCT and PB or with MCT and SKF-525A as described in MATERIALS AND METHODS. Controls received equivalent volumes of saline (SAL) vehicle. Rats were killed 7 days after MCT. Values represent mean ± SEM. n = 4-9/group.

[b]Body weight change is the difference between the body weight at treatment and the weight when killed.

[c]Significantly different from SAL/SAL group ($p < 0.05$).

[d]Significantly different from SAL/MCT group ($p < 0.05$).

Figure 9. Effect of phenobarbital (PB) on MCT- and MCTP-induced release of LDH into bronchopulmonary lavage fluid.

9A. Rats received PB (75 mg/kg/day, i.p.) or saline (SAL) four days prior to MCT (105 mg/kg, s.c.) or SAL. Rats were killed 7 days after MCT and airway LDH activity was measured as described in MATERIALS AND METHODS. Values represent mean $\pm$ SEM. a: Significantly different from MCT/SAL-treated animals. b: Significantly different from SAL/PB-treated animals. ($p < 0.05$).

9B. Rats received PB (75 mg/kg/day, i.p.) or SAL four days prior to MCTP (3.5 mg/kg, i.v.) or DMF vehicle. Rats were killed 7 days after MCTP and airway LDH activity was determined. c: Significantly different from DMF/SAL-treated rats. d: Significantly different from DMF/PB-treated rats. There was no difference between MCTP/SAL and MCTP/PB-treated rats ($p < 0.05$).

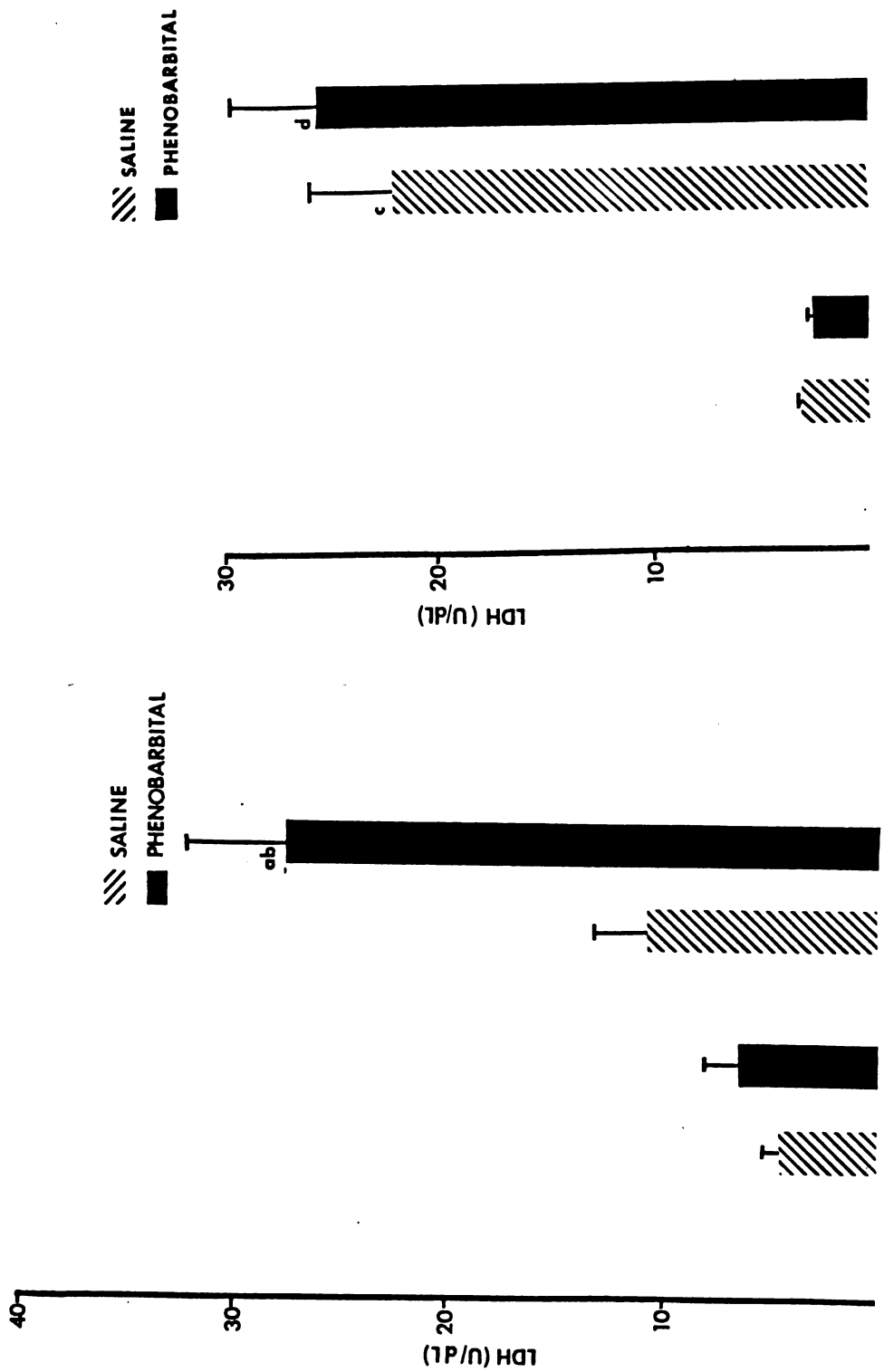


Figure 9A.

Figure 9B.

weight/body weight ratio was increased in rats that received MCTP/PB but not in rats receiving S/MCT (Figure 10A). Sequestration of ^{125}I-BSA in the lungs was not increased in groups treated with MCT (Figure 11A). Two of eight rats treated with PB/MCT died after treatment. No animals in the other groups died.

2. Effect of SKF-525A on MCT toxicity

Rats gained body weight irrespective of treatment (Table 5). However, animals that received SAL/MCT gained significantly less weight than did those in the other groups. The weight gain in the SKF-525A/MCT-treated rats was not different from that of the rats that received SKF-525A/SAL or SAL/SAL (Table 5).

Rats treated with SAL/MCT had increased lavage fluid LDH activity and the lavage fluid LDH activity in SKF-525A/MCT-treated animals was not significantly different from the SAL/SAL or SKF-525A/SAL-treated rats (Figure 12A). Relative lung weight was increased in rats treated with SAL/MCT compared to the other groups (Figure 13A). There was no difference in relative lung weight in SKF-525A/MCT-treated rats compared to those treated with S/S or SKF-525A/S (Figure 13A). Lung injury, as measured by pulmonary sequestration of ^{125}I-BSA, was increased in SAL/MCT-treated animals. There was no difference in sequestration of ^{125}I-BSA in SKF-52A/MCT and SKF-525A/SAL-treated animals (Figure 14A).

3. Effect of PB on MCTP pneumotoxicity

Rats treated with SAL/MCTP or PB/MCTP gained less weight than those rats treated with SAL/DMF or PB/DMF (Table 6). The weight gains in the two groups treated with MCTP were not significantly different from each other (Table 6).

Rats treated with PB/MCTP and SAL/MCTP had increased lavage fluid LDH activity compared to vehicle-treated controls (Figure 9B). Relative lung

Figure 10. Effect of phenobarbital (PB) on MCT- and MCTP-induced changes in relative lung weight.

10A. Rats received PB (75 mg/kg/day, i.p.) or saline (SAL) four days prior to MCT (105 mg/kg, s.c.) or SAL. Rats were killed 7 days after MCT and relative lung weight was measured as described in MATERIALS AND METHODS. Values represent mean ± SEM. a: Significantly different from MCT/SAL-treated animals. b: Significantly different from SAL/PB-treated animals. ($p < 0.05$).

10B. Rats received PB (75 mg/kg/day) or SAL four days prior to MCTP (3.5 mg/kg, i.v.) or DMF vehicle. Rats were killed 7 days after MCTP and relative lung weight was determined. c: Significantly different from DMF/SAL-treated rats. d: Significantly different from DMF/PB-treated rats. There was no difference between MCTP/SAL and MCTP/PB-treated rats ($p < 0.05$).

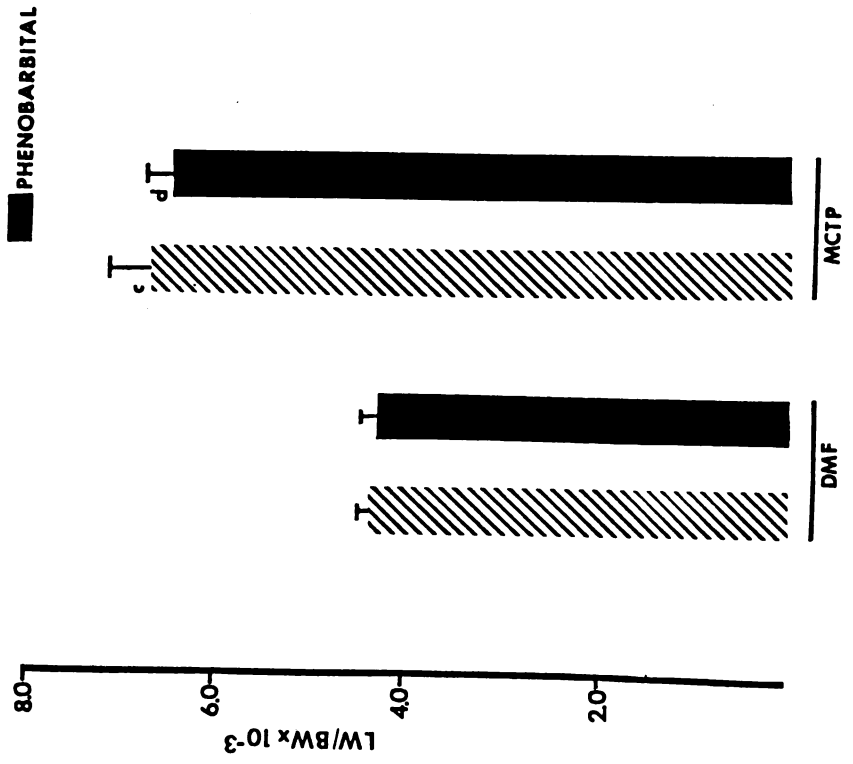


Figure 10B.

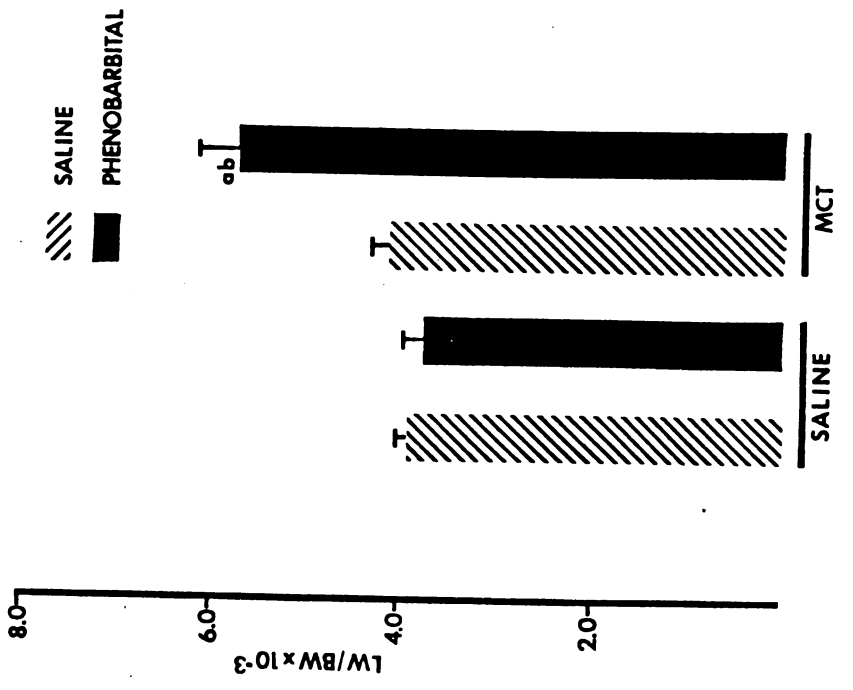


Figure 10A.

Figure 11. Effect of phenobarbital (PB) on MCT- and MCTP-induced pulmonary sequestration of ^{125}I-BSA.

11A. Rats received PB (75 mg/kg/day, i.p.) or saline (SAL) four days prior to MCT (105 mg/kg, s.c.) or SAL. Rats were killed 7 days after MCT and sequestration of ^{125}I-BSA was measured as described in MATERIALS AND METHODS. Values represent mean ± SEM. There were no significant differences among any of the groups treated. ($p < 0.05$).

11B. Rats received PB (75 mg/kg/day) or SAL four days prior to MCTP (3.5 mg/kg, i.v.) or DMF vehicle. Rats were killed 7 days after MCTP and sequestration of ^{125}I-BSA was measured. a: Significantly different from DMF/SAL-treated rats. b: Significantly different from DMF/PB-treated rats. There was no difference between MCTP/SAL and MCTP/PB-treated rats ($p < 0.05$).

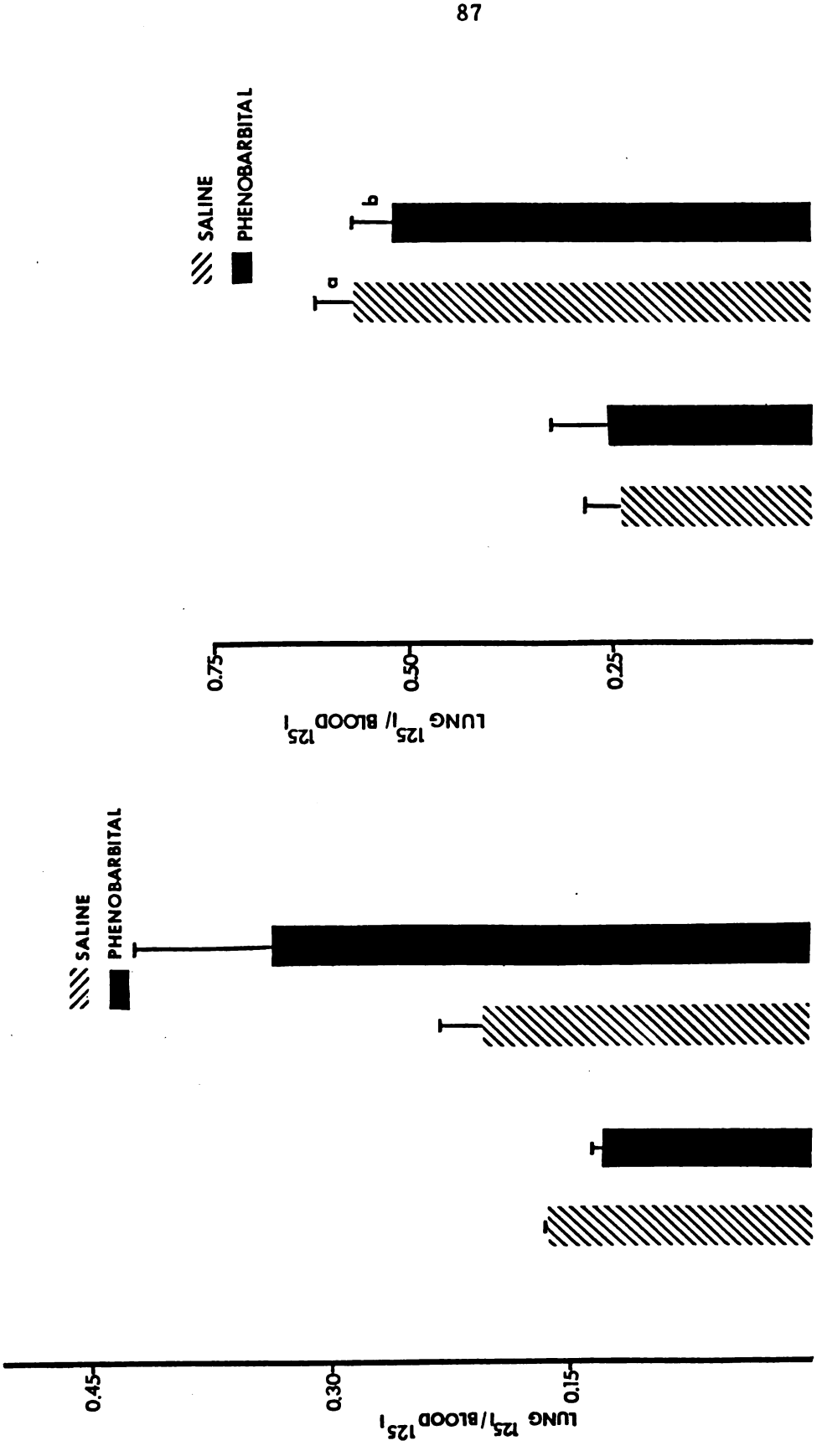


Figure 11A.

Figure 11B.

Figure 12. Effect of SKF-525A on MCT- and MCTP-induced release of LDH into bronchopulmonary lavage fluid.

12A. Rats received SKF-525A (75 mg/kg, i.p.) or saline (SAL) one hour prior to MCT (105 mg/kg, s.c.) or SAL. Rats were killed 7 days after MCT, and airway LDH activity was measured as described in MATERIALS AND METHODS. Values represent mean $\pm$ SEM. a: Significantly different from MCT/SKF-525A-treated animals ($p < 0.05$).

12B. Rats received SKF-525A (75 mg/kg, i.p.) or SAL one hour prior to MCTP (3.5 mg/kg, i.v.) or DMF vehicle. Rats were killed 7 days after MCTP and airway LDH activity was determined. b: Significantly different from DMF/SAL-treated rats. c: Significantly different from DMF/SKF-525A-treated rats. There was no difference between MCTP/SAL and MCTP/SKF-525A-treated rats ($p < 0.05$).

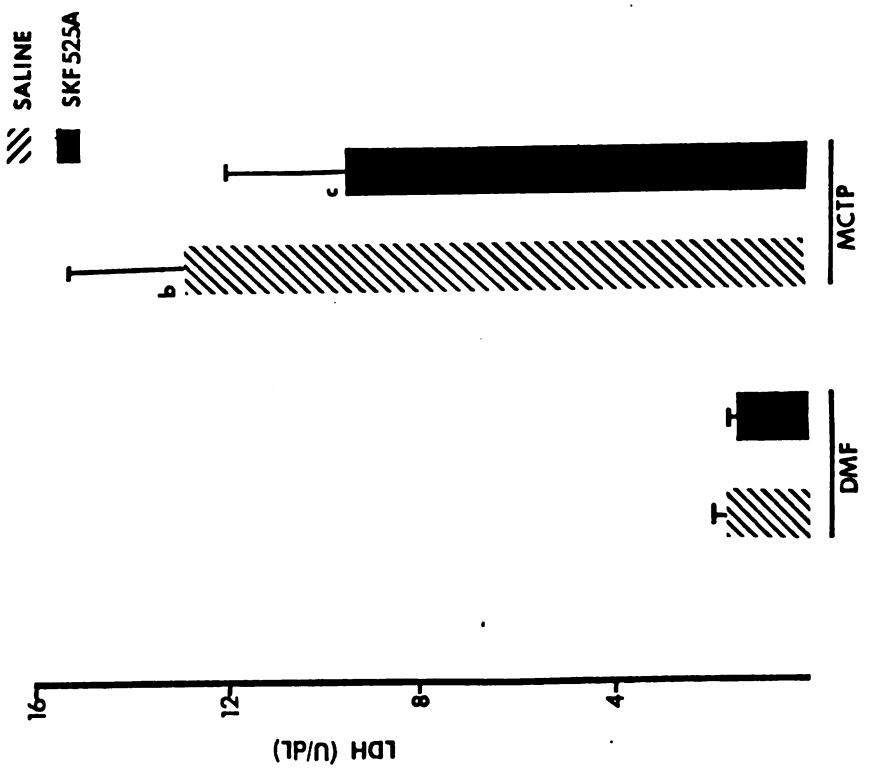


Figure 12B.

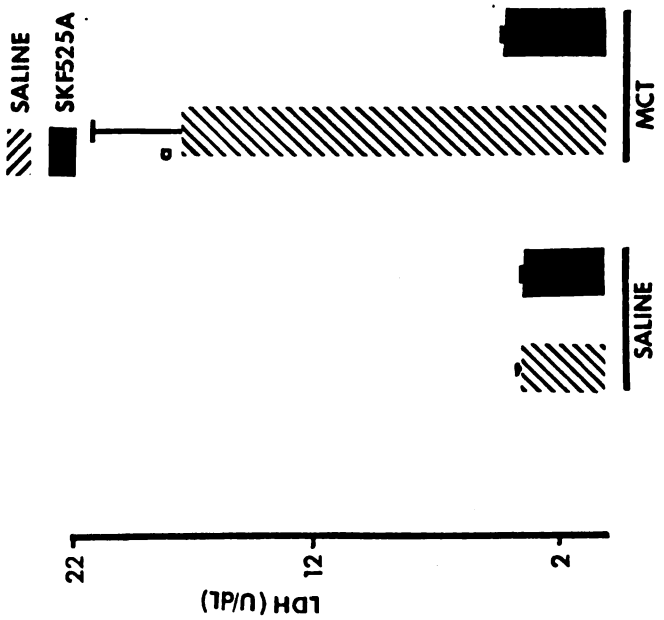


Figure 12A.

Figure 13. Effect of SKF-525A on MCT- and MCTP-induced changes in relative lung weight.

13A. Rats received SKF-525A (75 mg/kg, i.p.) or saline (SAL) one hour prior to MCT (105 mg/kg, s.c.) or SAL. Rats were killed 7 days after MCT and relative lung weight was measured. Values represent mean $\pm$ SEM. a: Significantly different from MCT/SKF-525A-treated animals ($p < 0.05$).

13B. Rats received SKF-525A (75 mg/kg, i.p.) or SAL one hour prior to MCTP (3.5 mg/kg, i.v.) or DMF vehicle. Rats were killed 7 days after MCTP and relative lung weight was determined. b: Significantly different from DMF/SAL-treatd rats. c: Significantly different from DMF/SKF-525A-treated rats. There was no difference between MCTP/SAL and MCTP/SKF-525A-treated rats ($p < 0.05$).

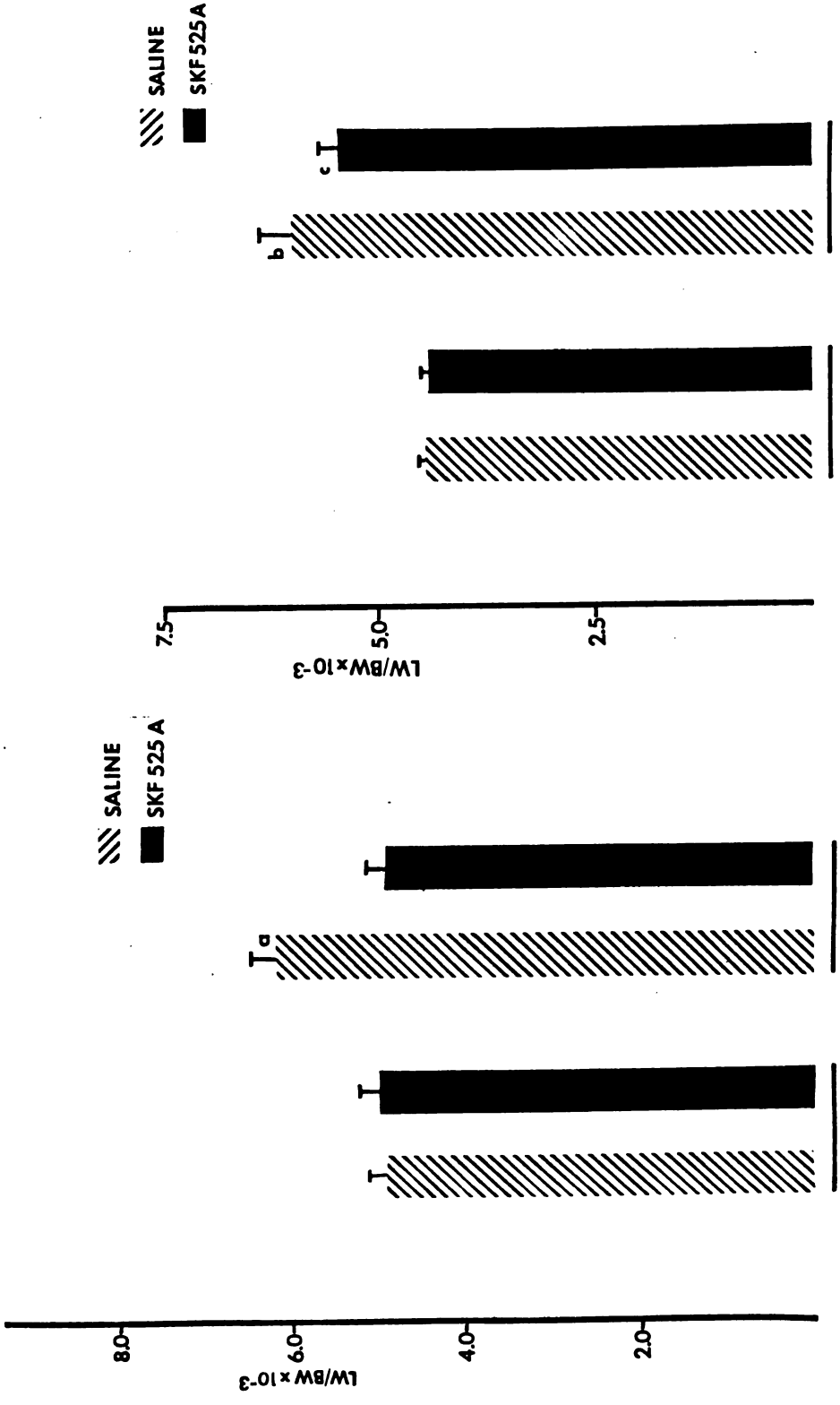


Figure 13A.

Figure 13B.

Figure 14. Effect of SKF-525A on MCT- and MCTP-induced changes in pulmonary sequestration of ^{125}I-BSA.

14A. Rats received SKF-525A (75 mg/kg, i.p.) or saline (SAL) one hour prior to MCT (105 mg/kg, s.c.) or SAL. Rats were killed 7 days after MCT and sequestration of ^{125}I-BSA was measured. Values represent mean ± SEM. a: Significantly different from SAL/SAL- and MCT/SKF-525A-treated animals. There was no difference between MCT/SKF-525A-treated and SAL/SAL-treated animals. ($p < 0.05$).

14B. Rats received SKF-525A (75 mg/kg/day, i.p.) or SAL one hour prior to MCTP (3.5 mg/kg, i.v.) or DMF vehicle. Rats were killed 7 days after MCTP and sequestration of ^{125}I-BSA was determined. b: Significantly different from DMF/SAL-treated rats. c: Significantly different from DMF/SKF-525A-treated rats. There was no difference between MCTP/SAL and MCTP/SKF-525A-treated rats ($p < 0.05$).

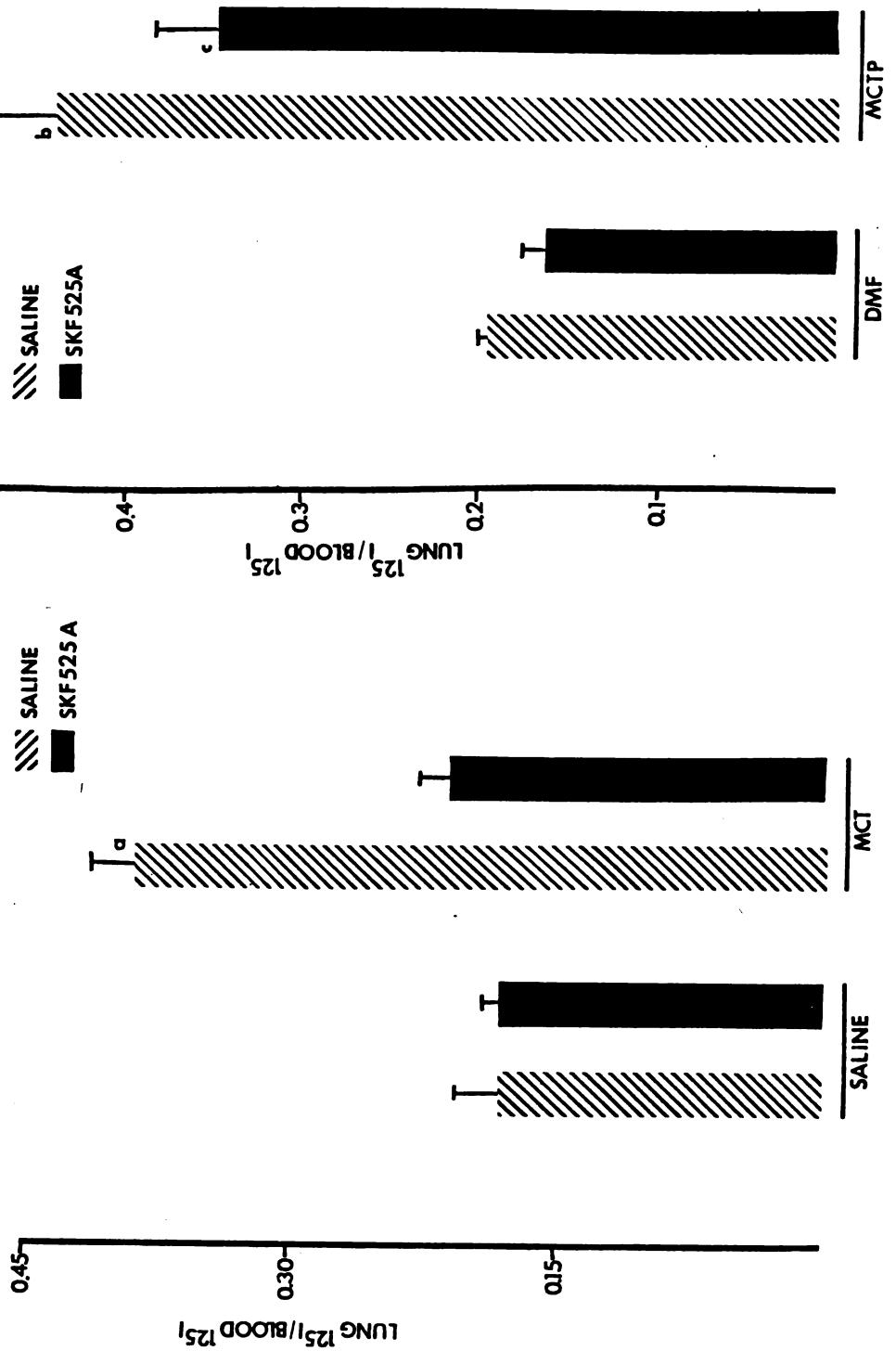


Figure 14A.

Figure 14B.

TABLE 6

Effect of Phenobarbital (PB) or SKF-525A on MCTP-induced Pneumotoxicity

Experiment	Treatment[a]	Body Wt Change[b]
PB	SAL/DMF	42±4
	PB/DMF	40±2
	SAL/MCTP	23±3[c]
	PB/MCTP	28±3[d]
SKF-525A	SAL/DMF	45±6
	SKF-525A/DMF	31±4[c]
	SAL/MCTP	30±6[c]
	SKF-525A/MCTP	21±4[c]

[a]Rats were co-treated with MCTP and PB or with MCTP and SKF-525A as in MATERIALS AND METHODS. Controls received equivalent volumes of DMF or SAL vehicle. Rats were killed 7 days after MCTP. Values represent mean ± SEM, n = 8-15/group.

[b]Body weight change is the difference between the body weight at treatment and the weight when killed.

[c]Significantly different from SAL/DMF-treated group ($p < 0.05$).

[d]Significantly different from PB/DMF-treated group ($p < 0.05$).

weight also was increased in the PB/MCTP and SAL/MCTP-treated groups compared to their respective, vehicle-treated control groups (Figure 10B). Sequestration of ^{125}I-BSA in the lungs was increased in PB/MCTP and in S/MCTP-treated animals compared to those animals treated with S/DMF and with PB/DMF, respectively (Figure 11B). However, there were no differences in any of the indices measured between rats receiving PB/MCTP and those receiving SAL/MCTP (Figures 9B, 10B and 11B).

4. Effect of SKF-525A on MCTP pneumotoxicity

Rats treated with SKF-525A/DMF, SAL/MCTP or SKF-525A/MCTP gained less weight than rats treated with SAL/DMF (Table 6). There was no difference in body weight gain between SKF-525A/MCTP and SAL/MCTP-treated rats (Table 6).

Rats treated with SKF-525A/MCTP or with SAL/MCTP had lavage fluid LDH activity greater than the activity in S/DMF or in SKF-525A/DMF-treated animals, respectively (Figure 12B). However, there was no difference in lavage fluid LDH activity between the SKF-525A/MCTP and SAL/MCTP-treated animals (Figure 12B). Relative lung weight was increased in both SKF-525A/MCTP and SAL/MCTP-treated groups compared to their respective DMF-treated controls, but there was no difference between the two MCTP-treated groups (Figure 13B). Sequestration of ^{125}I-BSA was significantly greater in both the SKF-525A/MCTP-treated and the SAL/MCTP-treated groups compared to rats receiving SKF-525A/DMF and SAL/DMF, respectively (Figure 14B). There was no difference between the SKF-525A/MCTP and SAL/MCTP-treated groups in sequestration of ^{125}I-BSA (Figure 14B).

C. Relative Toxicity of MCT, MCT N-Oxide and MCTP

MCT, MCT N-oxide and MCTP were given to rats in DMF vehicle at a dose of 5 mg/kg. Fourteen days after treatment, only the MCTP-treated animals had lung injury. These rats had increased lavage fluid LDH activity (Figure 15A), increased relative lung weight (Figure 15B) and increased RV/LV+S (Figure 15C), indicating that right ventricular hypertrophy had developed. The lungs from groups receiving MCT or MCT N-oxide were normal in appearance grossly, and indices of lung injury were not different from rats receiving only DMF (Figures 15A, B and C). Rats treated with MCTP gained less weight than did rats treated with MCT, MCT N-oxide or DMF (Figure 15D). Four of the 8 rats treated with MCTP died before the end of the 14-day study. No deaths occurred in the other groups treated.

D. Toxicity of MCTP in Plasma or Saline Vehicle

Recently, Lafranconi and Huxtable (1984) reported that perfusion of isolated livers with MCT resulted in Ehrlich positive (E+) metabolites in the perfusion medium. This medium was found to be toxic to isolated lungs when it was subsequently used to perfuse them. Since several minutes elapsed between collection of perfusion medium from isolated livers and its use in perfusing isolated lungs, the toxic MCT metabolite must be somehwat stable in aqueous solution. Accordingly, it is unlikely that the toxic MCT metabolite in these studies was MCTP, since MCTP rapidly degrades in aqueous media. However, the possibility remains that more stable pyrrolic degradation products of MCTP are also pneumotoxic and may have been responsible for injury when perfused into isolated lungs. Whether soluble breakdown products of MCTP are toxic in vivo is not known. If such products are toxic, then MCTP prepared in aqueous media

Figure 15. Relative ability of MCT, MCT N-oxide and MCTP to produce cardiopulmonary injury. Rats received either MCT, MCT N-oxide or MCTP (5 mg/kg, i.v. in DMF vehicle) or DMF. Fourteen days later, rats were killed and indices of injury were measured as described in MATERIALS AND METHODS. Values represent mean $\pm$ SEM. *Significantly different from DMF-, MCT N-oxide- and MCT-treated groups. ($p < 0.05$).

15A. Lavage fluid LDH activity
15B. Relative lung weight.

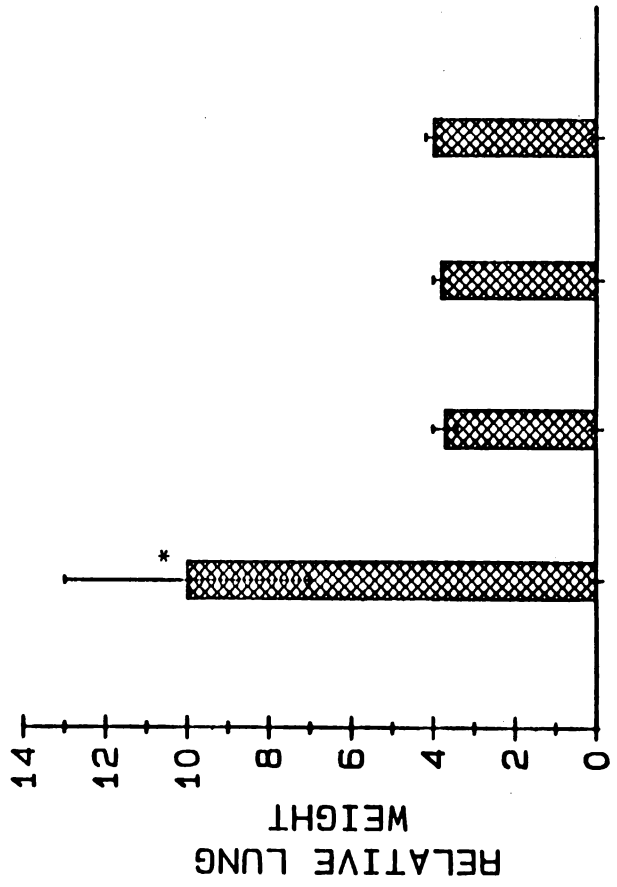


Figure 15B.

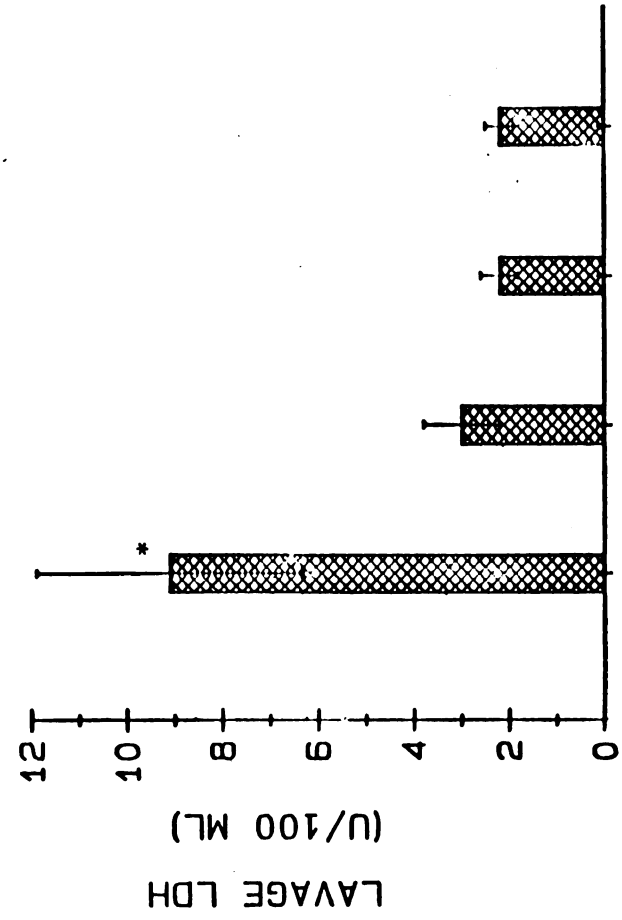


Figure 15A.

Figure 15 (continued)

15C. RV/(LV+S).
15D. Weight gain.

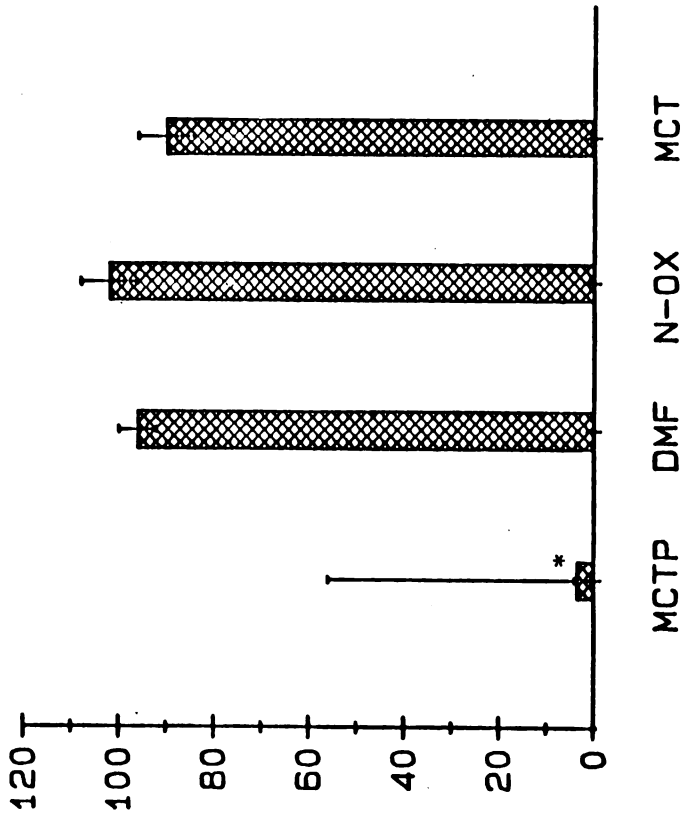


Figure 15D.

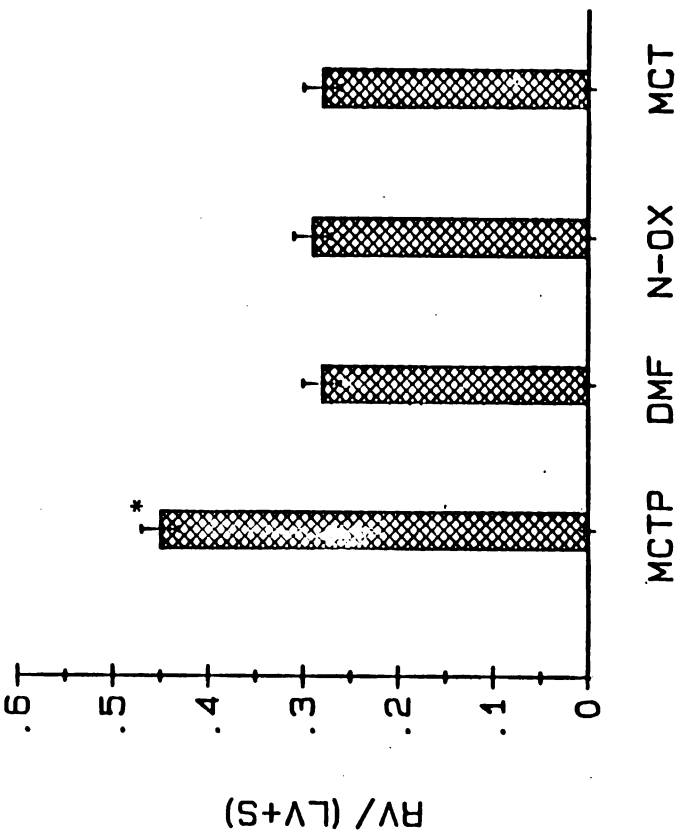


Figure 15C.

should produce lung injury when given to rats. To test whether or not chemically-syntheized MCTP exposed to an aqueous medium is capable of causing lung injury in vivo, MCTP prepared in serum or in saline was given to rats and pneumotoxicity was evaluated.

Rats treated with MCTP in DMF vehicle had increased relative lung weight (Figure 16A) and increased RV/LV+S compared to rats treated with MCTP in serum or saline (Figure 16B). Indeed, values of lung weight and RV/LV+S in the latter two groups were similar to those of rats which had not received MCTP (see Figure 15). There were no differences in body weight gain between any of the groups (Figure 16C). Two of the 10 rats treated with MCTP in DMF died by the end of the 2-week study. There were no deaths in the groups treated with MCTP carried in plasma or in saline. Serum treated with MCTP, when tested in the Ehrlich assay, was strongly positive at 5 and 30 minutes after addition of MCTP.

E. Color Change in Plasma Treated with MCTP

When measured against a serum blank containing no MCTP, a peak in absorbance of light occurred at 477 nm in samples of MCTP dissolved in serum (Figure 17A). MCTP was added to serum and the absorbance change versus time was recorded. Figure 17B shows that the increase in absorbance of light at 477 nm initially is very rapid, but that the absorbance then continues to increase more slowly for more than one minute after the addition of MCTP (Figure 17B).

F. Antilymphocyte Serum (ALS) Efficacy

ALS was used as an immunosuppressant in these studies to test the role of the immune system in the pathogenesis of MCTP pneumotoxicity. The efficacy of ALS as an immunosuppressant was tested by evaluating its ability to prolong the survival of Sprague-Dawley derived skin grafts on F-344 recipients. Xenografts

Figure 16. Effect of aqueous vehicles on MCTP toxicity. MCTP was prepared in DMF, plasma or saline vehicles and was given to rats (5 mg/kg, i.v.) as described in MATERIALS AND METHODS. Rats were killed 14 days after treatment and injury was assessed. *Significantly different from groups treated with MCTP in plasma vehicle and with MCTP in saline vehicle. ($p < 0.05$). MD = MCTP in DMF; MP = MCTP in plasma; MS = MCTP in saline.

16A. Relative lung weight.
16B. RV/(LV+S).

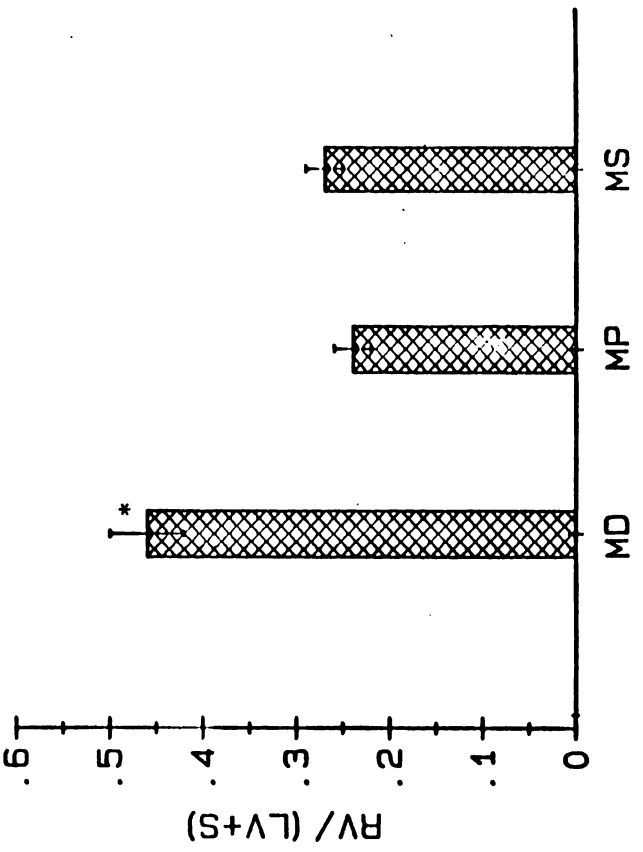


Figure 16B.

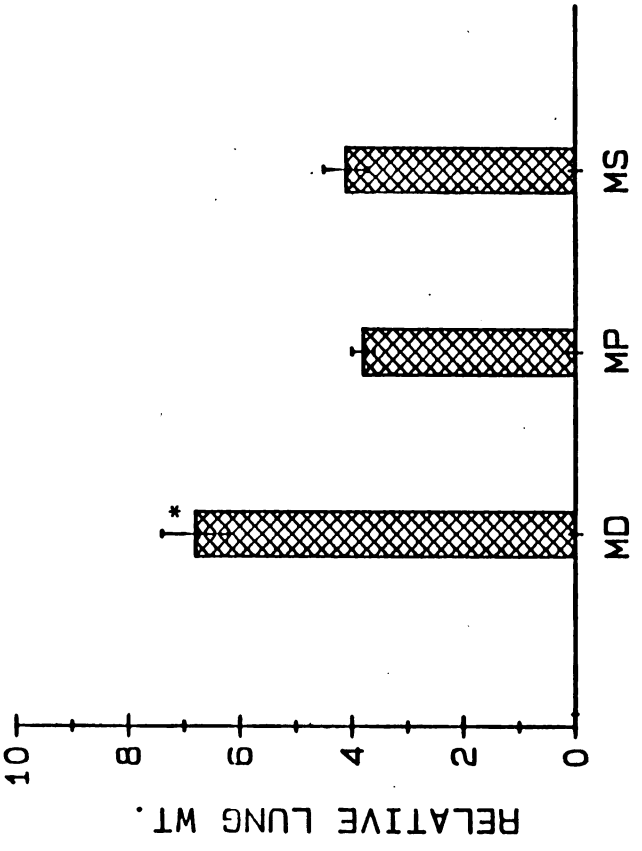


Figure 16A.

Figure 16 (continued)

16C. Body weight gain. There were no differences among the groups treated.

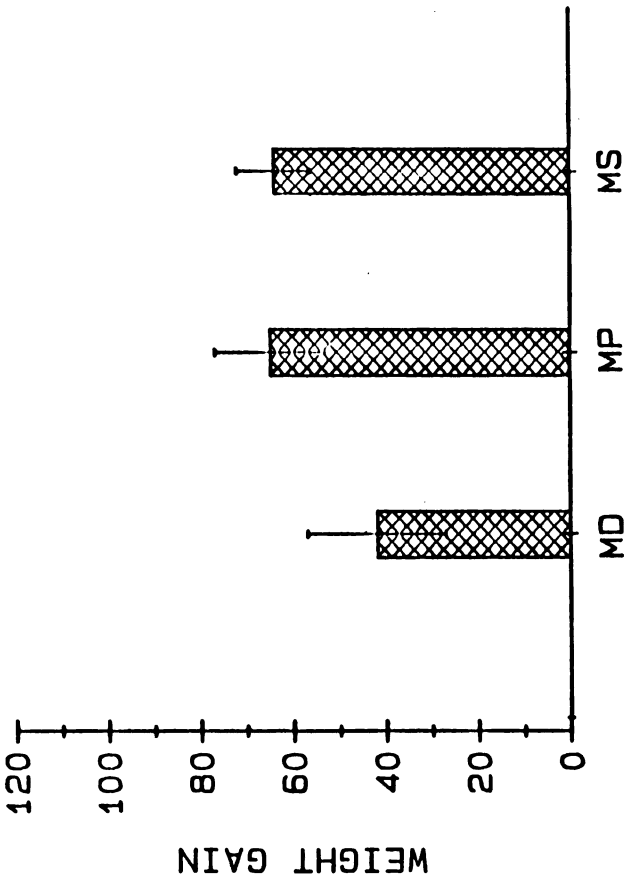


Figure 16C.

Figure 17. Color change in serum after MCTP addition.

17A. MCTP was added to serum, allowed to incubate for 30 min at room temperature, and then measured spectrophotometrically against a serum blank. The wavelength of maximum absorbance was 477 nm.

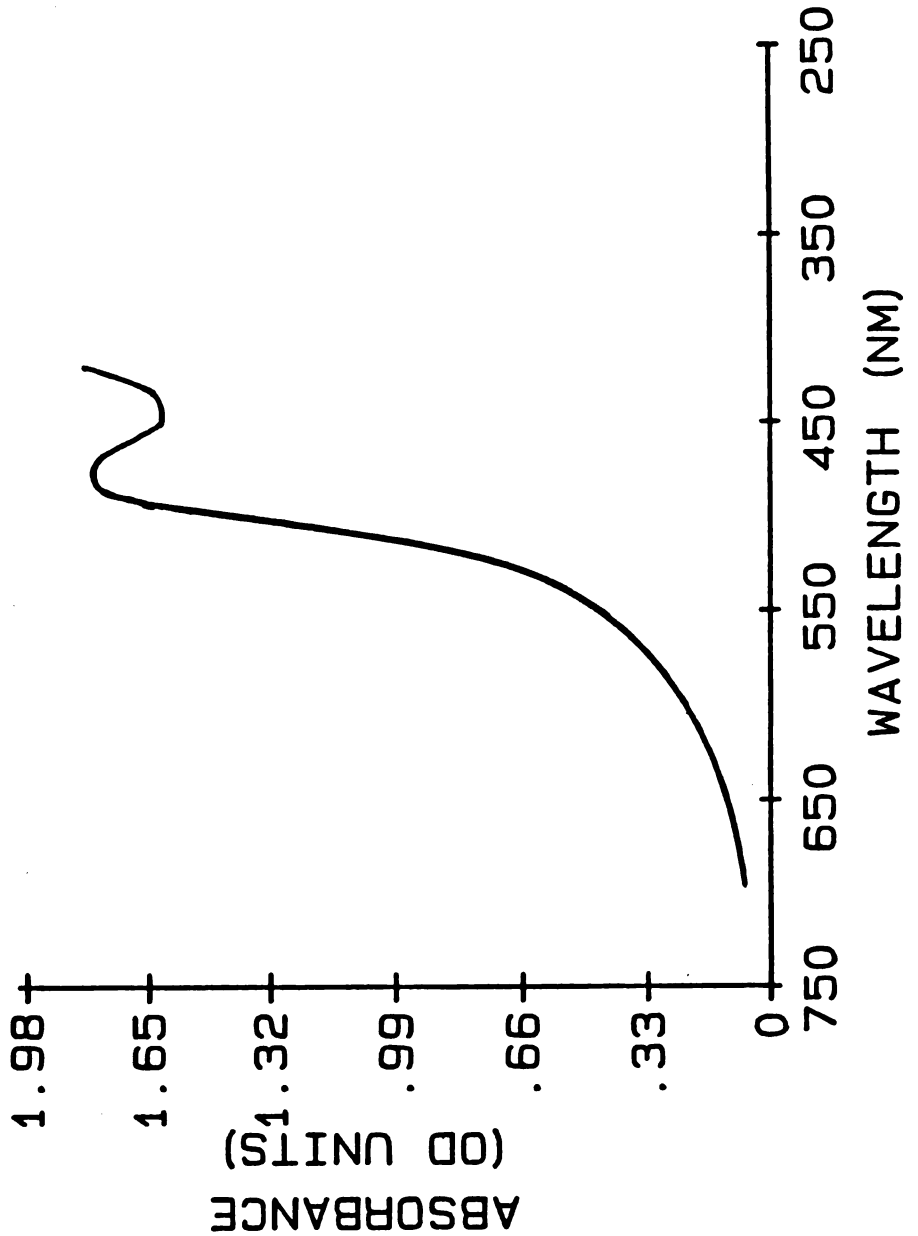

ABSORBANCE
(OD UNITS)
1.98
1.65
1.32
.99
.66
.33
0
750
650
550
450
350
250
WAVELENGTH (NM)

Figure 17A.

Figure 17 (continued)

17B. The change in OD at 477 nm versus time after MCTP was added to fresh rat serum.

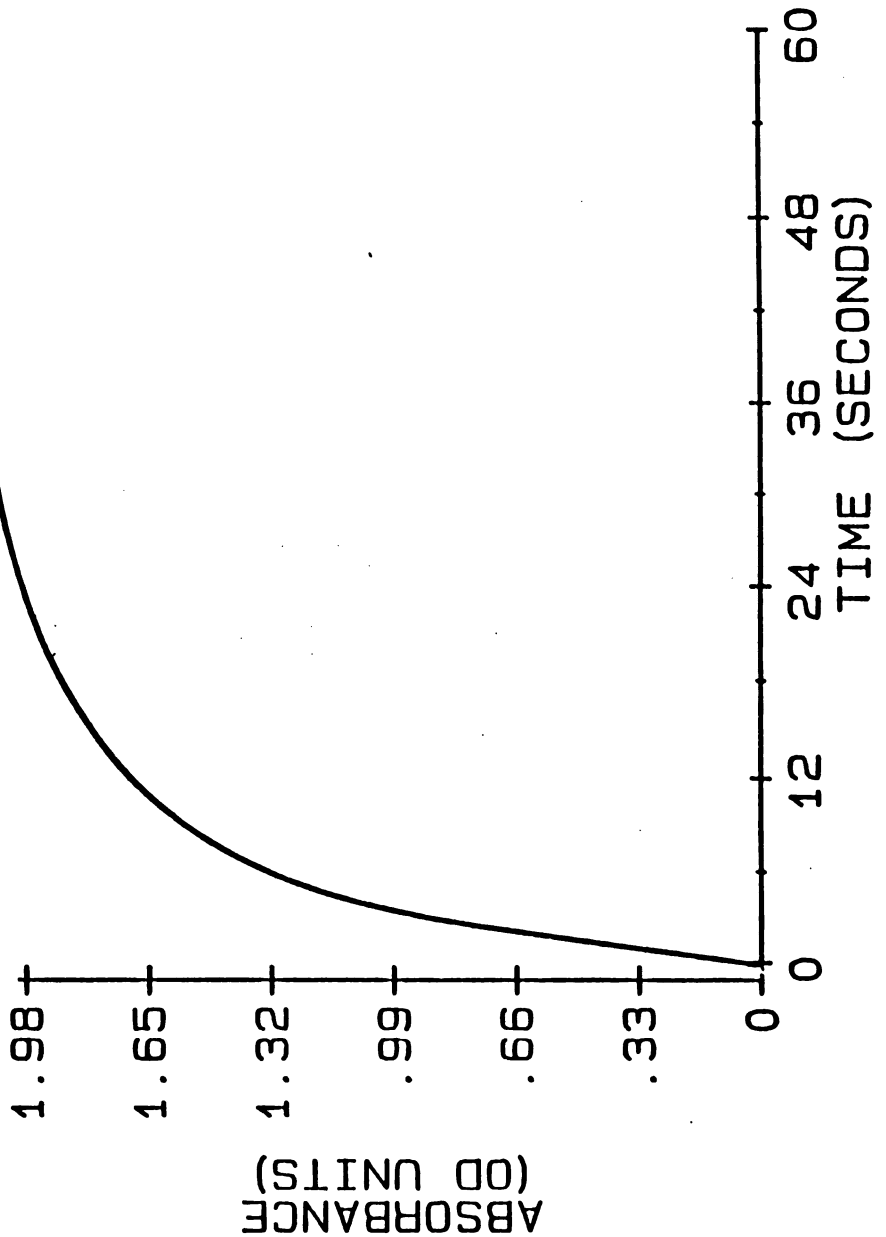

ABSORBANCE
(OD UNITS)
1.98
1.65
1.32
.99
.66
.33
0
0
12
24
36
48
60
TIME (SECONDS)

Figure 17B.

from a Sprague-Dawley rat survived an average of 12 days after placement on F-344 recipients treated with rabbit CS (Table 7). In contrast, xenografts on rats treated with ALS survived an average of 35 days before being rejected (Table 7). Allografts transferred from the tail of each rat to its own lateral thoracic wall survived until the study was terminated at day 45 (Table 7). At no time did the allografts show any sign of rejection. Thus, the loss of the xenografts in the CS-treated rats was not due to non-specific effects such as infection or improper graft placement.

G. Effect of ALS on MCTP-induced Pulmonary Injury

The effect of ALS on MCTP-induced pulmonary injury was tested by co-treating rats with CS/DMF, ALS/DMF, CS/MCTP or ALS/MCTP (Table 8). Rats treated with ALS/MCTP lost more weight than controls treated with ALS/DMF. Relative lung weight was significantly increased in rats treated with CS/MCTP or with ALS/MCTP compared to their respective DMF-treated controls. However, there was no difference in lung weight between rats treated with CS/MCTP and with ALS/MCTP. Similarly, the lavage fluid LDH activity and sequestration of ^{125}I-BSA in the lungs were significantly increased in rats receiving CS/MCTP and ALS/MCTP compared to respective controls. There was no difference in lavage fluid LDH activity between the rats receiving CS/MCTP and ALS/MCTP, but there was greater sequestration of ^{125}I-BSA in lungs of rats receiving ALS/MCTP compared to those treated with CS/MCTP (Table 8).

H. Effect of Cyclosporin A (CYA) on MCTP-induced Pulmonary Injury

The immunosuppressant CyA also was tested for its ability to protect against pulmonary injury due to MCTP. Two doses of CyA were tested in separate studies (Tables 9 and 10). Rats treated with OI/MCTP or with CyA (10

TABLE 7

Ability of Antilymphocyte Serum (ALS) to Prolong Survival of Skin Grafts

Treatment[a]	Days of Graft Survival	
	Graft from Sprague-Dawley	Graft from Self
CS	12±0	>45
ALS	35±2[b]	>45

[a]Rats were treated with ALS or CS as in MATERIALS AND METHODS. Each rat was given 2 skin grafts, one from an unrelated Sprague-Dawley donor and one from its own tail. Grafts were examined daily until rejection occurred. The mean number of days ± SEM the grafts survived is shown for each group. The study was terminated 45 days after grafting. n=3/group.

[b]Significantly different from CS-treated group ($p < 0.05$).

TABLE 8

Effect of ALS on MCTP-induced Pulmonary Injury

Treatment[a]	Body Wt Change (gm)	Relative Lung Wt ($\times 10^{-3}$)	Lavage Fluid LDH (U/100 ml)	^{125}I Lung: Blood
CS/DMF	26±4	4.569±0.056	3.1±0.2	0.13±0.01
ALS/DMF	19±2	4.556±0.118	3.3±0.2	0.11±0.01
CS/MCTP	13±1	5.309±0.362[c]	9.6±3.1[c]	0.19±0.02[c,d]
ALS/MCTP	7±1[b]	5.485±0.152[b]	14.1±1.6[b]	0.23±0.02[b]

[a]Rats were given either CS/DMF, ALS/DMF, CS/MCTP or ALS/MCTP as in MATERIALS AND METHODS. Rats were killed and lung injury was assessed at 7 days after MCTP or DMF. Values represent mean ± SEM.

[b]Significantly different from ALS/DMF group ($p < 0.05$).

[c]Significantly different from CS/DMF group ($p < 0.05$).

[d]Significantly different from ALS/MCTP group ($p < 0.05$).

TABLE 9

Effect of CyA (10 mg/kg) on MCTP-induced Pulmonary Injury

Treatment[a]	Body Wt Change	Relative Lung Wt	Lavage Fluid LDH	RV/LV+S
OI/DMF	81± 4	3.819±0.241	3.3±0.2	0.308±0.004
CyA/DMF	64± 3[b]	4.548±0.185	3.5±0.1	0.291±0.008
OI/MCTP	0±16[b]	14.930±2.776[b]	18.9±4.0[b]	0.428±0.026[b]
CyA/MCTP	9±11[c]	8.340±0.741[c,d]	14.7±4.4[c]	0.337±0.012[c,d]

[a]Rats were treated with either OI/DMF, CyA/DMF, OI/MCTP or CyA/MCTP as in MATERIALS AND METHODS. The daily CyA dose was 10 mg/kg, s.c. Rats were killed 14 days after MCTP or DMF and cardiopulmonary injury was assessed.

[b]Significantly different from OI/DMF group ($p < 0.05$).

[c]Significantly different from CyA/DMF group ($p < 0.05$).

[d]Significantly different from OI/MCTP group ($p < 0.05$).

TABLE 10

Effect of CyA (20 mg/kg) on MCTP-induced Pulmonary Injury

Treatment[a]	Body Wt Change	Relative Lung Wt	Lavage Fluid LDH	RV/LV+S
OI/DMF	81±25	4.254±0.057	2.9±0.9	0.259±0.001
CyA/DMF	43± 8[b]	4.576±0.199	2.9±0.2	0.280±0.005
OI/MCTP	33±16[b]	10.919±1.712[b]	15.0±1.6[b]	0.349±0.016[b]
CyA/MCTP	-22±11[c,d]	9.730±1.238[c]	30.2±4.2[c,d]	0.218±0.013[c]

[a]Rats were treated with either OI/DMF, CyA/DMF, OI/MCTP or CyA/MCTP as in MATERIALS AND METHODS. The daily CyA dose was 20 mg/kg, s.c. Rats were killed 14 days after MCTP or DMF and cardiopulmonary injury was assessed as in MATERIALS AND METHODS.

[b]Significantly different from OI/DMF group ($p < 0.05$).

[c]Significantly different from CyA/DMF group ($p < 0.05$).

[d]Significantly different from OI/MCTP group ($p < 0.05$).

mg/kg/day)/MCTP gained significantly less weight than did controls treated with OI/DMF or CyA/DMF, respectively (Table 9). Rats treated with CyA/DMF also gained less weight than did rats treated with OI/DMF. Rats treated with OI/MCTP and with CyA/MCTP had increased relative lung weight and increased RV/(LV+S) compared to their respective DMF-treated controls. Relative lung weight and RV/(LV+S) in CyA/MCTP-treated rats was significantly less than in rats treated with OI/MCTP (Table 9). Lavage fluid LDH activity was elevated in rats receiving OI/MCTP or CyA/MCTP compared to rats receiving OI/DMF or CyA/DMF, respectively. However, there was no difference in lavage fluid LDH activity in OI/MCTP-treated rats compared to those given CyA/MCTP (Table 9).

Rats treated with a larger dose of CyA (20 mg/kg/day) were not protected from injury due to MCTP (Table 10). CyA/MCTP-treated rats lost weight over the 14 days of the study, whereas all other groups gained weight. Relative lung weight, lavage LDH activity and RV/(LV+S) were increased in both CyA/MCTP- and OI/MCTP-treated groups compared to their respective controls. The only differences in these parameters between the CyA/MCTP-treated rats and the OI/MCTP-treated rats was that lavage fluid LDH activity was greater in rats receiving CyA/MCTP (Table 10).

I. Adoptive Transfer

Adoptive transfer studies were undertaken to determine if lymphocytes transferred from MCTP-treated donors could alter the onset or severity of lesions in MCTP-treated lymphocyte recipients. In series 1, recipients were given lymphocytes taken from rats treated with MCTP seven days earlier. The transferred cells were 85-90 percent lymphocytes and the remainder were granulocytes and macrophages as determined from Wright's stained smears. Adoptively transferred lymphocytes from lung-associated lymph nodes did not

alter MCTP-induced injury in the recipients. Table 11 shows that there was no difference in body weight gain, relative lung weight or lavage fluid LDH activity due to treatment with lymphocytes/MCTP compared to treatment with HBSS/MCTP. Likewise, recipients treated with splenocytes/MCTP did not have lung injury that was different from the rats treated with HBSS/MCTP. Recipients given only lymph node-derived lymphocytes or splenocyte transferred from MCTP-treated donor rats did not develop lung injury (data not shown). Thus, transferred lymphocytes alone were not capable of causing lung injury in recipients.

In series 2, recipients were given PEC or HBSS vehicle seven days prior to receiving a single injection of MCTP. The peritoneal exudate cells were 45-55 percent lymphocytes, 30-35 percent granulocytes and 20-25 percent macrophages. The recipients were killed at 3 and at 5 days after MCTP and lung injury was assessed. At 3 days after MCTP, there were no differences in body weight gain, relative lung weight or lavage fluid LDH activity in PEC/MCTP-treated rats compared to HBSS/MCTP-treated rats (Table 12). There also were no differences between rats treated with PEC/MCTP and those treated with HBSS/MCTP killed 5 days after MCTP (Table 12). Lavage fluid LDH activity and body weight gain were greater in rats killed 5 days after MCTP compared to those killed 3 days after MCTP (Table 12).

The series 3 protocol was similar to the series 2 protocol, except that PEC recipients received a single injection of MCTP 24 hours after receiving PEC or HBSS instead of 7 days after PEC or vehicle. Table 13 shows that the relative lung weight and lavage fluid LDH activity in PEC/MCTP-treated rats were not different from HBSS/MCTP-treated controls killed 3 days after MCTP. Body weight gain was less in HBSS/MCTP-treated rats compared to PEC/MCTP-treated

TABLE 11

Series I: Effect of Adoptive Transfer of Lung-Associated Lymphocytes on MCTP-induced Pulmonary Injury

Treatment[a]	Cell Source	Body Wt Change	Relative Lung Wt ($\times 10^{-3}$)	Lavage LDH (U/100 ml)
HBSS/MCTP	Lymph Node	10±2	4.94±0.19	4.9±0.6
Lymphocytes/ MCTP		9±2	4.97±0.42	5.0±0.7
HBSS/MCTP	Spleen	12±1	5.10±0.19	6.3±0.6
Lymphocytes/ MCTP		9±3	4.48±0.06	5.2±1.0

[a]Lymphocyte donors were treated with MCTP 7 days prior to harvesting lung-associated lymph nodes and spleens. Recipients were given 5.0×10^7 cells, i.p., 24 hours prior to receiving MCTP. Rats were killed 3 days later and lung injury was assessed as in MATERIALS AND METHODS. Values represent mean ± SEM. n = 4-8/group. There were no significant differences between groups receiving lymphocytes/MCTP and those receiving vehicle/MCTP ($p < 0.05$).

TABLE 12

Series 2: Adoptive Transfer of Peritoneal Exudate Cells (PEC) from MCTP-treated Rats

Treatment[a]	Days After MCTP when Injury was Assessed	Body Wt Change (gm)	Relative Lung Wt	Lavage Fuid LDH (U/100 ml)
HBSS/MCTP	3	2±2	4.42±0.09	3.47±0.33
PEC/MCTP	3	5±1	4.39±0.18	3.33±0.39
HBSS/MCTP	5	13±3[b]	4.83±0.22	9.33±1.83[b]
PEC/MCTP	5	11±1[c]	5.06±0.39	8.20±0.77[c]

[a]Mineral oil-elicited PEC were obtained from rats 14 days after MCTP and were transferred into recipients. Seven days after receiving cells, the receipients were given MCTP. The recipients were killed at 3 and 5 days after MCTP and lung injury was assessed. Values represent mean ± SEM. n = 3-10/group.

[b]Significantly different from HBSS/MCTP-treated rats killed 3 days after MCTP ($p < 0.05$).

[c]Significantly different from PEC/MCTP-treated rats killed 3 days after MCTP.

TABLE 13

Series 3: Adoptive Transfer of Peritoneal Exudate Cells from MCTP-treated Rats

Treatment[a]	Days After MCTP when Injury was Assessed	Body Wt Change (gm)	Relative Lung Wt	Lavage Fuid LDH (U/100 ml)
HBSS/MCTP	3	6±1	7.60±0.37	23.5±4.0
PEC/MCTP	3	12±2[b]	6.74±0.46	21.5±5.1
HBSS/MCTP	5	6±2	8.07±0.17	14.7±4.4
PEC/MCTP	5	12±1[b]	7.54±0.45	13.0±6.6

[a]Mineral oil-elicited PEC were obtained from rats 14 days after MCTP and were transferred into recipients. Twenty-four hours after receiving cells, the receipients were given MCTP. The recipients were killed at 3 and 5 days after MCTP and lung injury was assessed. Values represent mean ± SEM. n = 4-5/group.

[b]Significantly different from HBSS/MCTP-treated rats killed 3 days after MCTP ($p < 0.05$).

rats at 3 days after MCTP. Similar results were found in rats killed 5 days after MCTP (Table 13).

In series 4, recipient rats were treated with lung-associated, lymph node-derived lymphocytes taken from donors treated with MCTP 28 days earlier. The donors had well-developed lung lesions and markedly enlarged lymph nodes. However, transfer of these cells into recipients did not alter the time of onset or severity of MCTP-induced lesions compared to rats treated with HBSS/MCTP (Table 14).

J. Role of Complement in MCTP-induced Pulmonary Injury

1. Effect of MCTP on serum complement activity in vivo

In this study, rats were treated with MCTP and sequential blood samples were taken for assessment of serum complement activity. The rats had increased lung weight and increased pulmonary alveolar lavage fluid LDH activity 14 days after MCTP (Tables 15 and 16). However, neither MCTP nor DMF caused significant changes in serum CH50 in rats at 1, 2, 4 or 7 hours after treatment (Figure 18). A second group of rats was evaluated to determine if serum complement activity changes within 2 weeks after MCTP. The mean serum complement acivity in rats treated with MCTP was significantly greater than the pretreatment complement activity at 7, 10 and 14 days after MCTP (Figure 19). In rats receiving DMF, mean serum complement activity was greater than the pretreatment serum complement activity at 1, 3, 7, 10 and 14 days after DMF (Figure 19). However, on a given day after treatment, serum complement activity in DMF-treated rats was not different than in MCTP-treated rats (Figure 19).

Serum samples were obtained from a third group of MCTP-treated rats to determine if MCTP causes complement activation in vivo using the neutrophil aggregation assay. Preliminary studies indicated that when zymosan-activated

TABLE 14

Series 4: Adoptive Transfer of Lung-Associated Lymph Node Lymphocytes from MCTP-treated Rats

Treatment[a]	Days After MCTP	Body Wt Change (gm)	Relative Lung Wt	Lavage Fuid LDH (U/100 ml)
HBSS/MCTP	3	36±4	5.90±1.55	4.5±1.1
Lymphocytes/ MCTP	3	34±4	6.47±1.52	5.4±1.6
HBSS/MCTP	5	8±4[b]	5.24±0.42	13.2±5.0[b]
Lymphocytes/ MCTP	5	9±3[c]	5.03±0.31	8.5±2.5[c]

[a]Lung-associated lymph node lymphocytes were obtained from rats 28 days after MCTP and were transferred into recipients. Twenty-four hours after receiving cells, recipients were given MCTP. The recipients were killed at 3 and 5 days after MCTP and lung injury was assessed. Values represent mean ± SEM.

[b]Significantly different from HBBS/MCTP-treated rats killed 3 days after MCTP ($p < 0.05$).

[c]Significantly different from lymphocyte/MCTP-treated rats killed 3 days after MCTP ($p < 0.05$).

TABLE 15

Lung Injury in MCTP-treated Rats for Serum Hemolytic Complement (CH50) Assessment Within Hours After Treatment

Treatment[a]	Relative Lung Weight	Lavage LDH (U/100 ml)
DMF	3.96±0.14	2.8±0.3
MCTP	7.54±1.13[b]	23.3±2.8[b]

[a]Rats received 3.5 mg/kg MCTP or DMF vehicle i.v., at time zero. Blood samples were taken just prior to treatment and at 1, 2, 4 and 7 hours after treatment. Rats were killed at 14 days after MCTP or DMF and pulmonary injury was assessed as in MATERIALS AND METHODS. Values are mean ± SEM.

[b]Significantly different from DMF-treated controls ($p < 0.05$).

TABLE 16

Lung Injury in MCTP-treated Rats for Serum Hemolytic Complement (CH50) Assessment Over 2 Weeks After Treatment

Treatment[a]	Relative Lung Weight	Lavage LDH (U/100 ml)
DMF	3.82±0.15	2.4±0.3
MCTP	5.64±0.21[b]	15.5±2.8[b]

[a]Rats received 3.5 mg/kg MCTP or DMF vehicle i.v., at time zero. Blood samples were taken just prior to treatment and at 1, 3, 5, 7, 10 and 14 days after treatment. Rats were killed at 14 days after MCTP or DMF and pulmonary injury was assessed as in MATERIALS AND METHODS. Values are mean ± SEM.

[b]Significantly different from DMF-treated controls ($p < 0.05$).

Figure 18. Hemolytic complement activity in serum taken from rats treated with MCTP or DMF. Blood was collected 1, 2, 4 and 7 hours after MCTP or DMF. Values represent mean $\pm$ SEM. n = 4-8/group. There were no significant differences between the groups ($p < 0.05$).

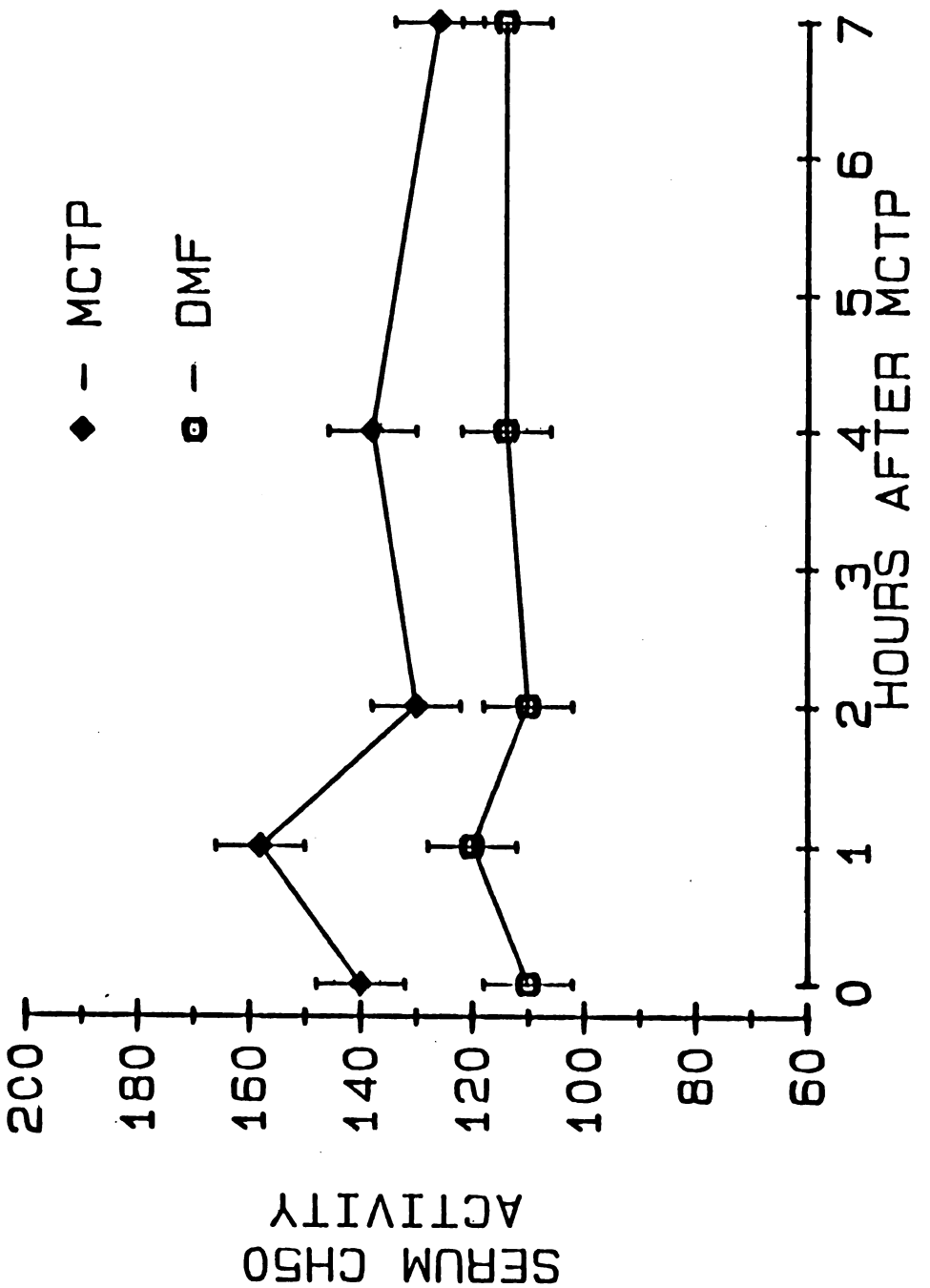
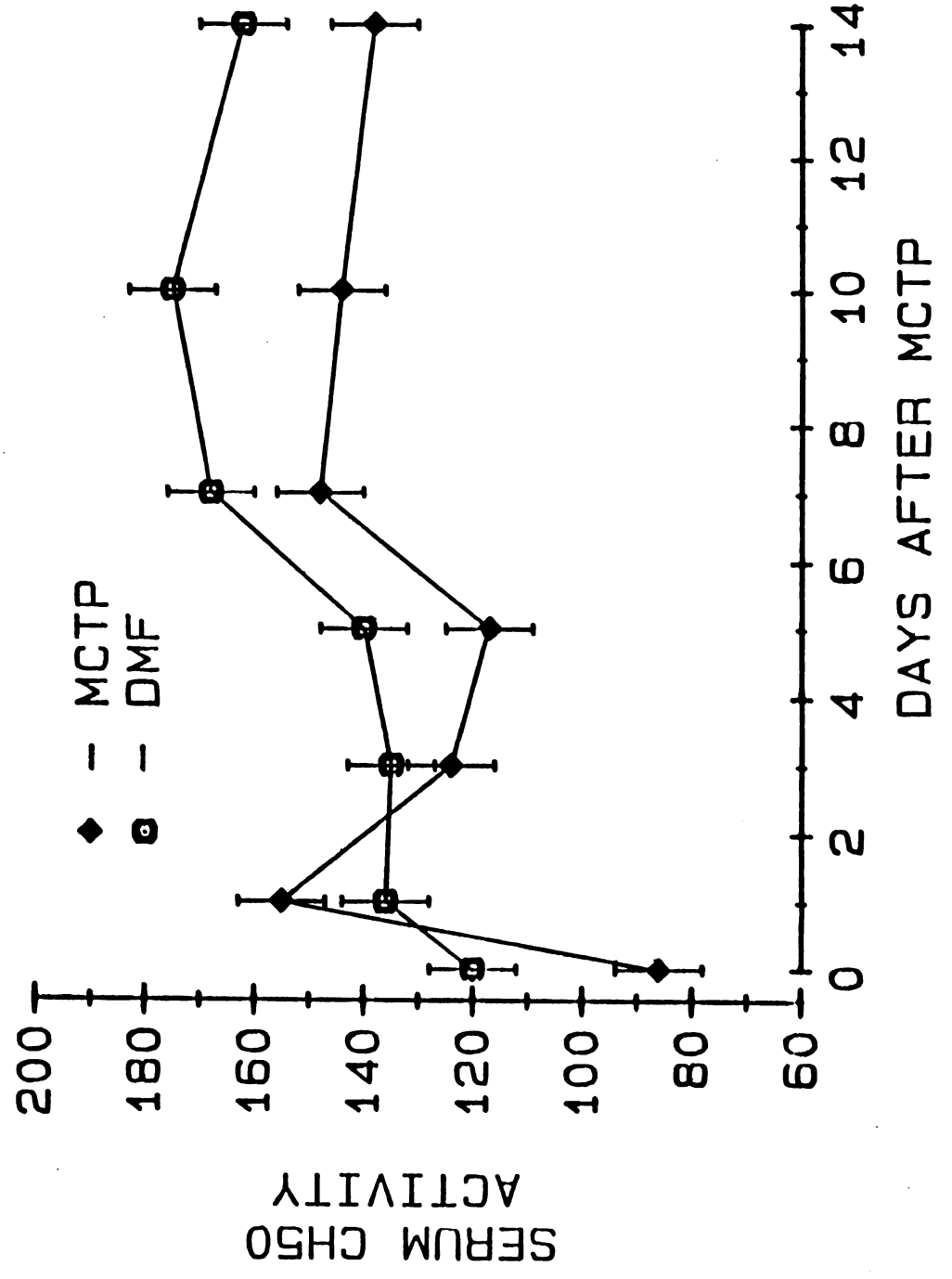

SERUM CH50
ACTIVITY
200
180
160
140
120
100
80
60
0
1
2
3
4
5
6
7
HOURS AFTER MCTP
◆ - MCTP
o - DMF

Figure 18

Figure 19. Hemolytic complement activity in serum taken from rats treated with MCTP or DMF. A blood sample was taken prior to treatment with MCTP or DMF on day zero. Subsequently, blood samples were obtained at 1, 3, 5, 7, 10 and 14 days. There were no significant differences between MCTP- and DMF-treated rats at any time point examined ($p < 0.05$).


SERUM CH50
ACTIVITY
200
180
160
140
120
100
80
60
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2
4
6
8
10
12
14
DAYS AFTER MCTP
MCTP
DMF

Figure 19.

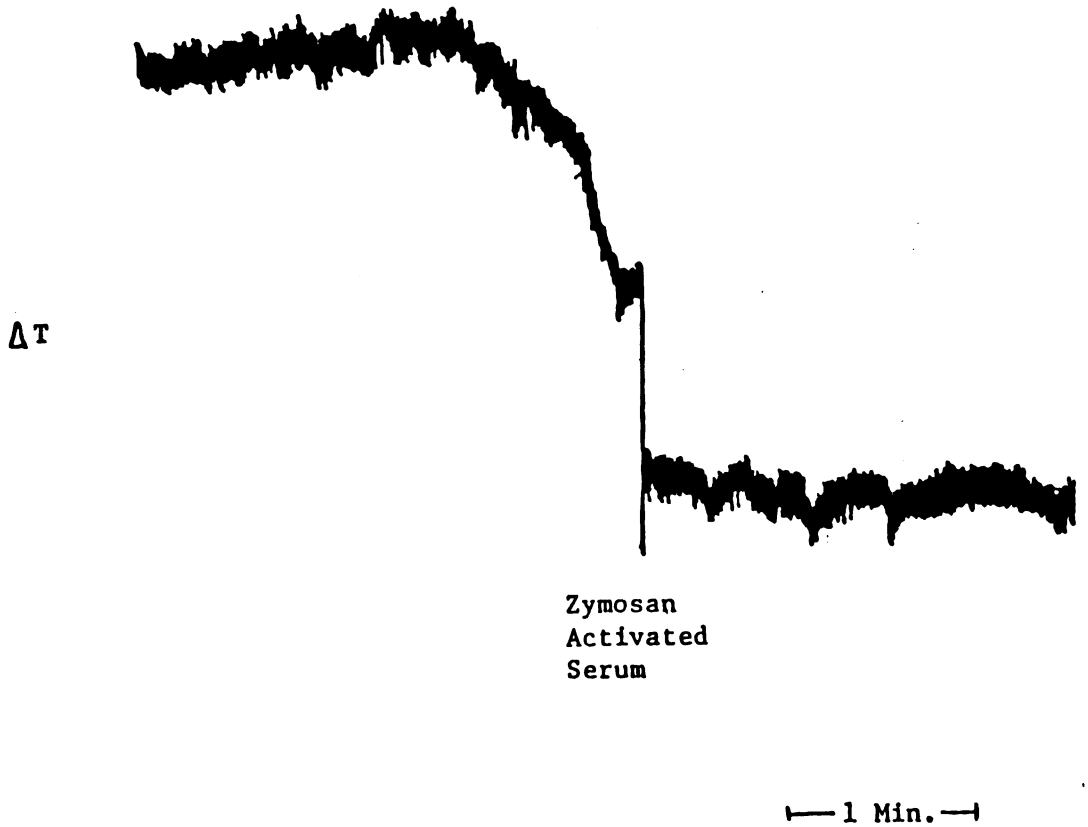

Figure 20. Aggregation of neutrophils in response to zymosan-activated serum. Aggregation was recorded as change in light transmittance (ΔT) induced by addition of 50 µl of zymosan-treated rat serum as outlined in MATERIALS AND METHODS. The rapid increase upon serum addition is dilution artifact. This is followed by a change in transmittance due to neutrophil aggregation.

serum was added to the neutrophil suspension, aggregation occurred as previously reported (Craddock et al., 1977a; Hammerschmidt et al., 1980) (Figure 20). Even though MCTP-treated rats developed lung injury (Table 17), serum samples collected at 1, 3, 5, 7, 10 and 14 days after MCTP did not stimulate neutrophil aggregation (Figures 21A and 21B). When zymosan-activated rat serum was added to the neutrophil-containing aggregometer cuvette subsequent to the addition of serum from either DMF- or MCTP-treated rats, a normal aggregation wave followed (Figures 21A and 21B).

2. Bronchopulmonary lavage fluid and neutrophil aggregation

Lavage fluid was obtained from rats treated with DMF or MCTP to determine if neutrophil aggregating activity is present in the airway. Lavage fluid from rats treated 14 days earlier did not cause neutrophil aggregation (data not shown) even though the activity of LDH in the lavage fluid was increased (Table 17). When zymosan-activated serum was added to the aggregometer cuvette subsequent to bronchopulmonary lavage fluid, a normal aggregation wave followed.

3. Complement depletion in MCT-treated rats

Purified cobra venom factor (CVF) was used to deplete complement from rats in vivo. The efficacy of the CVF as a depletor of complement was evaluated by measuring serum CH50 and elimination of C3 in serum of rats treated with CVF. Using the treatment protocol outlined in MATERIALS AND METHODS, it was possible to deplete serum complement for a period of 5 days.

Serum CH50 in all CVF-treated rats was less than 40 CH50 units compared to controls which had complement activity ranging between 110 and 140 CH50 units (Table 18). C3 was eliminated from the serum of CVF-treated rats compared to saline-treated controls as measured by Ochturlony immunodiffusion. Figure 22A shows an immunodiffusion plate typical of DMF/SAL or MCTP-SAL-

TABLE 17

Lung Injury in Rats Treated with MCTP for Assessment of Complement Activation Using the Neutrophil Aggregation Assay

Treatment[a]	Relative Lung Weight	Lavage LDH (U/100 ml)
DMF	4.04±0.24	4.1±0.4
MCTP	7.11±0.60[b]	21.7±5.5[b]

[a]Rats received 3.5 mg/kg MCTP or DMF vehicle i.v., at time zero. Blood samples were taken just prior to treatment and at 1, 3, 5, 7, 10 and 14 days after treatment. Rats were killed at 14 days after MCTP or DMF and pulmonary injury was assessed as in MATERIALS AND METHODS. Values are mean ± SEM.

[b]Significantly different from DMF-treated controls ($p < 0.05$).

Figure 21. Neutrophil aggregation response to serum from MCTP- or DMF-treated rats.

21A. Representative aggregation of neutrophils exposed first to serum from a DMF-treated rat followed by addition of zymosan-activated serum (ZAS). The rapid increases in transmittance upon addition of serum or ZAS is dilution artifact. Serum from DMF-treated rats did not stimulate aggregation. Subsequent exposure to ZAS resulted in normal aggregation.

21B. Representative aggregation of neutrophils after addition of serum from a MCTP-treated rat followed by ZAS. At no time did serum from a MCTP-treated rat stimulate neutrophil aggregation. Subsequent exposure to ZAS resulted in normal aggregation.

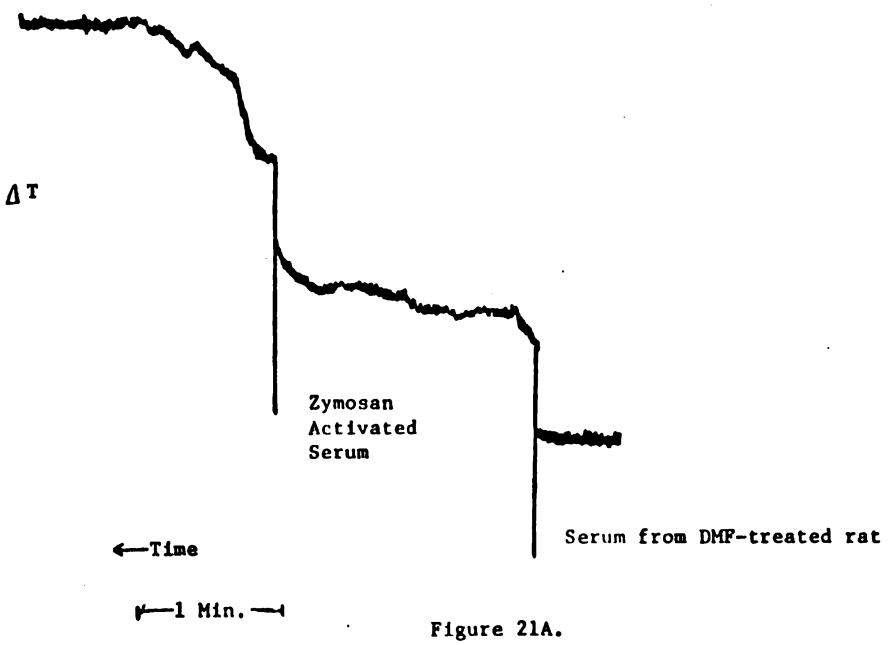

Figure 21A.

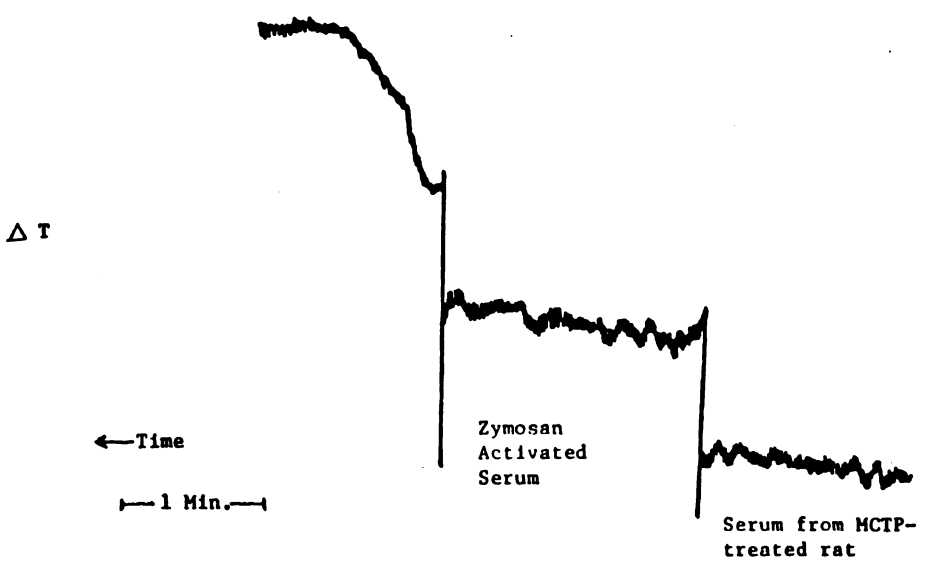

Figure 21B.

TABLE 18

Complement Depletion by Cobra Venom Factor (CVF) in MCTP-treated Rats

Treatment[a]	Mean CH50 Activity on the Indicated Day After CVF[b]					
	2	3	4	5	6	7
DMF/SAL	118±1	127±2	129±1	136±2	138±2	133±2
DMF/CVF	114±2	< 40	< 40	< 40	< 40	< 40
MCTP/SAL	110±2	122±2	144±2	131±2	140±2	140±2
MCTP/CVF	98±2	< 40	< 40	< 40	< 40	< 40

[a]Rats were given MCTP (3.5 mg/kg, i.v.) or DMF vehicle starting on day zero. Starting 2.5 days after MCTP or DMF, rats were given either CVF or saline. Blood samples were taken just prior to the first dose of CVF or SAL and then were taken every 24 hours thereafter. Rats were killed 7 days after MCTP or DMF and lung injury was assessed.

[b]Values represent hemolytic complement activity (in CH50 units) taken from rats at different times after treatment. Values are mean ± SEM, n=8/group.

Figure 22. Confirmation of complement depletion.

22A. The center well of the immunodiffusion plate was charged with anti-rat C3 and the peripheral wells were filled with the serum from a rat treated with MCTP/saline. Well 1 contains serum taken just prior to the start of saline. Wells 2-6 contain serum samples taken at 24, 48, 72, 96 and 120 hours after starting saline, respectively. The white precipitate (arrow) indicates that C3 is present in the serum samples.

22B. The center well is filled with anti-rat C3 serum and the peripheral wells are filled with serum from a rat treated with MCTP/CVF. Well 1 is filled with a serum sample taken prior to CVF. Wells 2-6 contain serum samples taken from rats at 24, 48, 72, 96 and 120 hours after starting CVF, respectively. The white precipitate between well 1 and the center indicates C3 is present in the pretreatment sample (arrow). Absence of a distinct line between the center well and other peripheral wells indicates that CVF effectively depleted C3 from CVF-treated rats, thus rendering the complement system inactive.

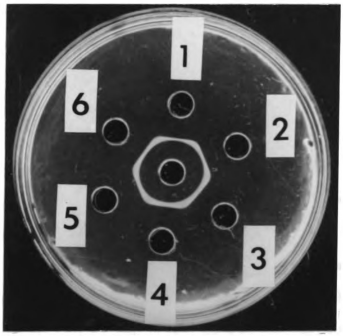

Figure 22A.

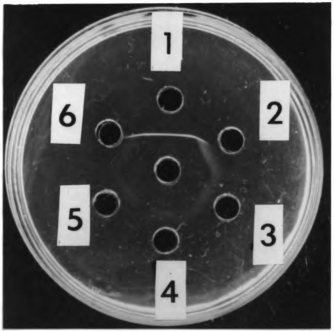

Figure 22B.

treated rats. The center well contains anti-rat C3 and the outer wells contain serum samples taken every 24 hours after SAL. The solid white hexagonal ring surrounding the center well shows C3 was present in serum of SAL-treated rats. Figure 22B shows a band between well 1, which contains a serum sample from a rat prior to CVF treatment, and the center well. There are no distinct bands between the center well and the remaining outer wells of the plate which contain serum samples taken daiy from a rat receiving CVF. This demonstrates that C3 was depleted from the serum, thereby rendering the complement system inactive in DMF/CVF- and MCTP/CVF-treated rats.

There were no differences in body weight gain among groups receiving DMF/SAL, DMF/CVF, MCTP/SAL or MCTP/CVF (Figure 23A). Rats treated with MCTP/SAL or MCTP/CVF had lavage LDH activity greater than the activity in lavage fluid from DMF/SAL-and DMF/CVF-treated animals (Figure 23B). However, there was no difference in lavage fluid LDH activity between MCTP/SAL- and MCTP/CVF-treated animals (Figure 23B). Lavage fluid total protein concentration followed a similar pattern (data not shown). Relative lung weight was increased in both MCTP/SAL- and MCTP/CVF-treated groups compared to rats receiving DMF/SAL and DMF/CVF, but there was no difference between the two MCTP-treated groups (Figure 23C). Sequestration of ^{125}I-BSA was significantly higher in the MCTP/SAL- and MCTP/CVF-treated groups compared to rats receiving DMF/SAL and DMF/CVF, respectively (Figure 23D). There was no difference between the MCTP/SAL- and MCTP/CVF-treated groups in sequestration of ^{125}I-BSA (Figure 23D). At no time was there any difference between lungs from DMF/SAL and those from DMF-CVF-treated rats. Thus, intraperitoneal CVF alone caused no lung injury.

Figure 23. Effect of complement on MCTP-induced pulmonary injury. Rats were treated with DMF/saline (SAL), DMF/CVF, MCTP/SAL or MCTP/CVF as described in MATERIALS AND METHODS. Rats were killed 7 days after MCTP or DMF. All bars represent mean ± SEM. n = 8/group. Bars marked with the same letter represent means significantly different from each other ($p < 0.05$).

23A. Body weight gain.
23B. Bronchopulmonary lavage fluid LDH activity.

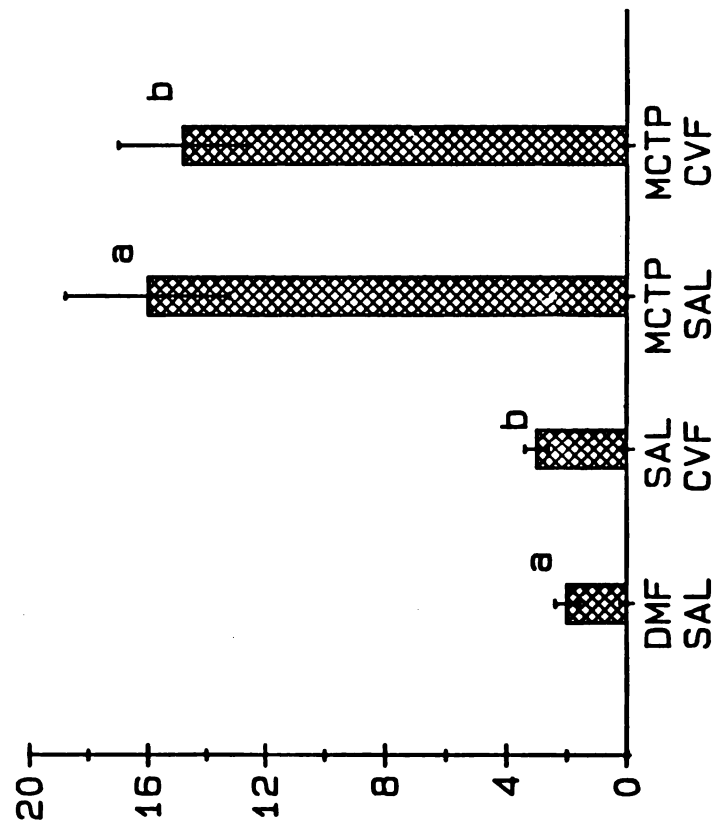

Figure 23B.

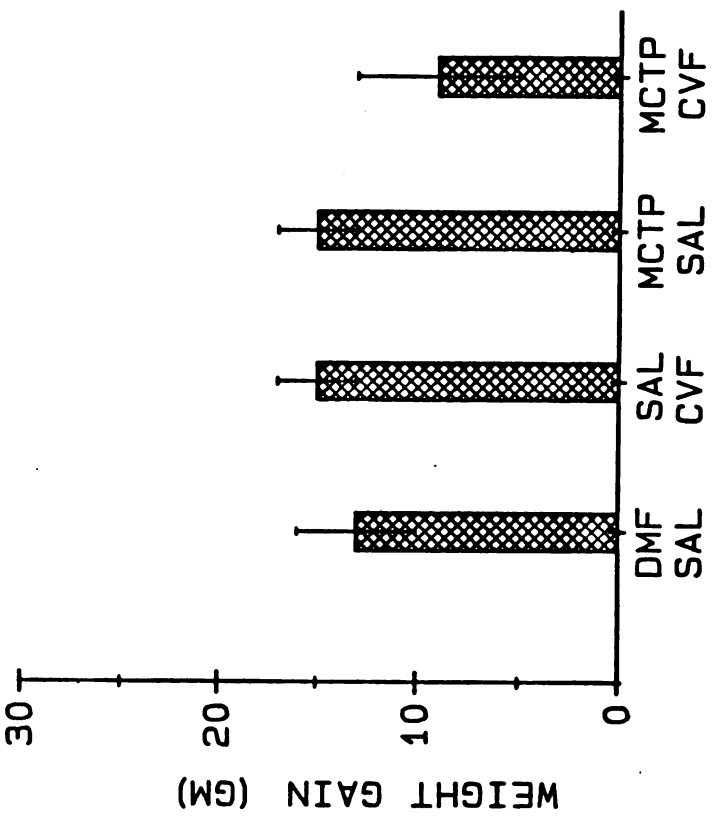

Figure 23A.

Figure 23 (continued)

23C. Relative lung weight.
23D. Lung injury index.

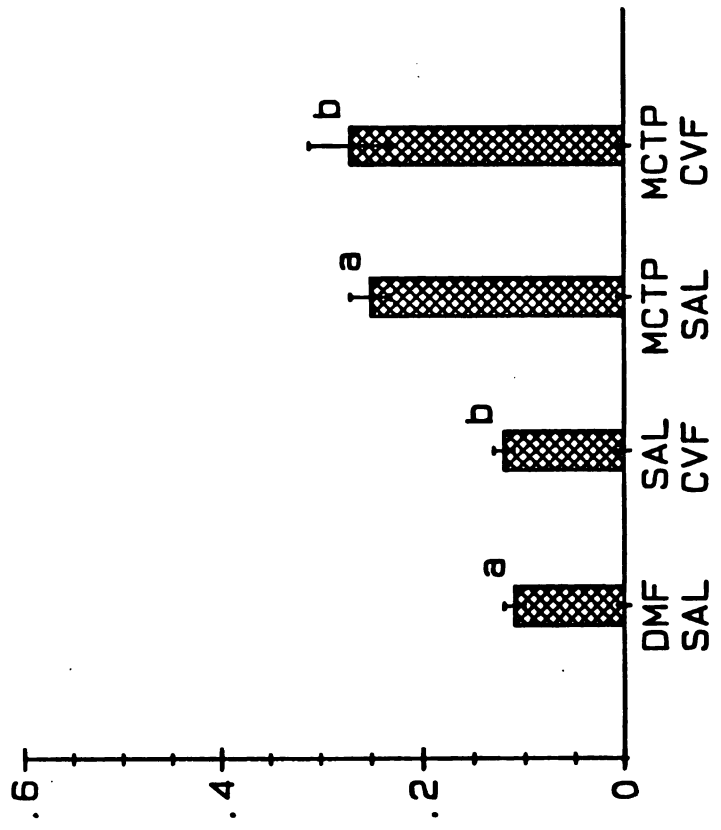

Figure 23D.

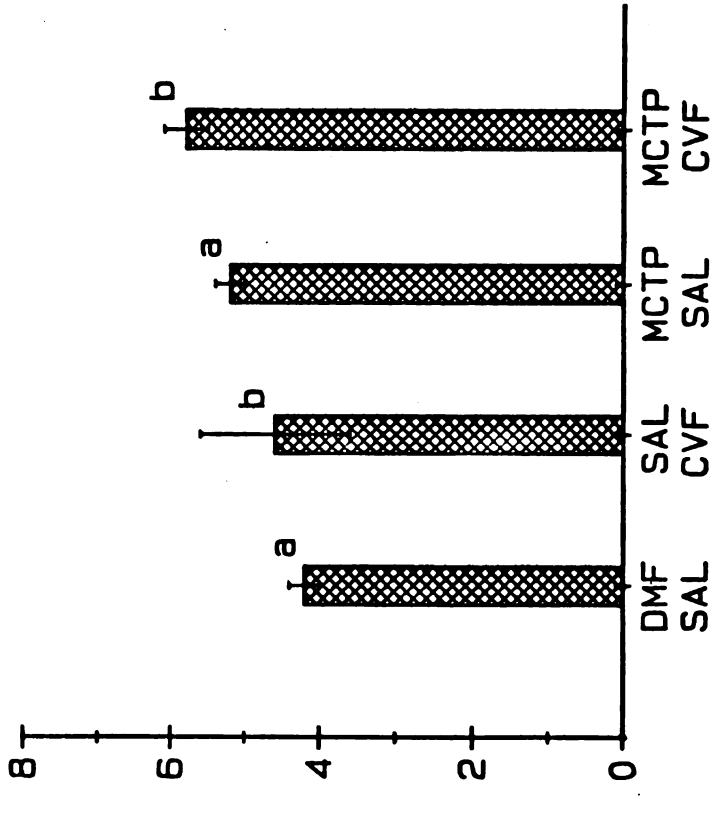

Figure 23C.

4. Effect of MCTP on serum complement activity in vitro

Several doses (see section L.3. of MATERIALS AND METHODS) of MCTP were added to serum in vitro to determine if MCTP directly alters serum complement activity. Serum (250 µl) receiving 12.5 µl DMF had complement activity of 85 CH50 units. The serum CH50 activity declined linearly with dose (Figure 24). MCT and MCT N-oxide, when tested at the same concentrations of MCTP, caused no change in serum complement activity (data not shown).

To determine if the MCTP-induced decrease in CH50 activity was due to complement activation, serum exposed to MCTP or DMF in vitro was tested in the neutrophil aggregation assay. Serum exposed to vehicle or to MCTP in vitro did not cause neutrophil aggregation (Figure 25A and 25B). When zymosan-activated serum was added to the aggregometer cuvette subsequent to vehicle- or MCTP-treated serum, normal aggregation resulted (Figure 25A and 25B).

To determine if serum complement could be activated via the alternative pathway in the presence of DMF or MCTP, serum samples were first exposed to DMF or several doses (see section L.4. of MATERIALS AND METHODS) of MCTP in vitro. After an incubation period, the serum samples were then exposed to zymosan or saline to activate the serum complement. Serum samples exposed to DMF or to increasing doses of MCTP followed by zymosan stimulated neutrophil aggregation, whereas the serum samples exposed DMF or MCTP followed by saline did not (Figure 26A and 26B).

K. Effect of Interventions that Alter Production and Metabolism of Toxic Oxygen Metabolites

1. Effect of desferroxamine mesylate (DF) on MCTP-induced pulmonary injury

Rats were co-treated with MCTP and DF to determine if DF would protect rats from MCTP-induced pulmonary injury. Rats receiving either

Figure 24. Effect of MCTP on complement activity when added to serum in vitro. Increasing amounts of MCTP were added to fresh rat serum. CH50 was measured after a 30-minute incubation period. Values represent the CH50 as a percentage of control corresponding to the amount of MCTP added to the serum. Control complement activity was measured in serum containing an equivalent volume of DMF and was 85 CH50 units. ($Y = 95.7-80.1 \cdot X$, $r^2 = 0.917$).

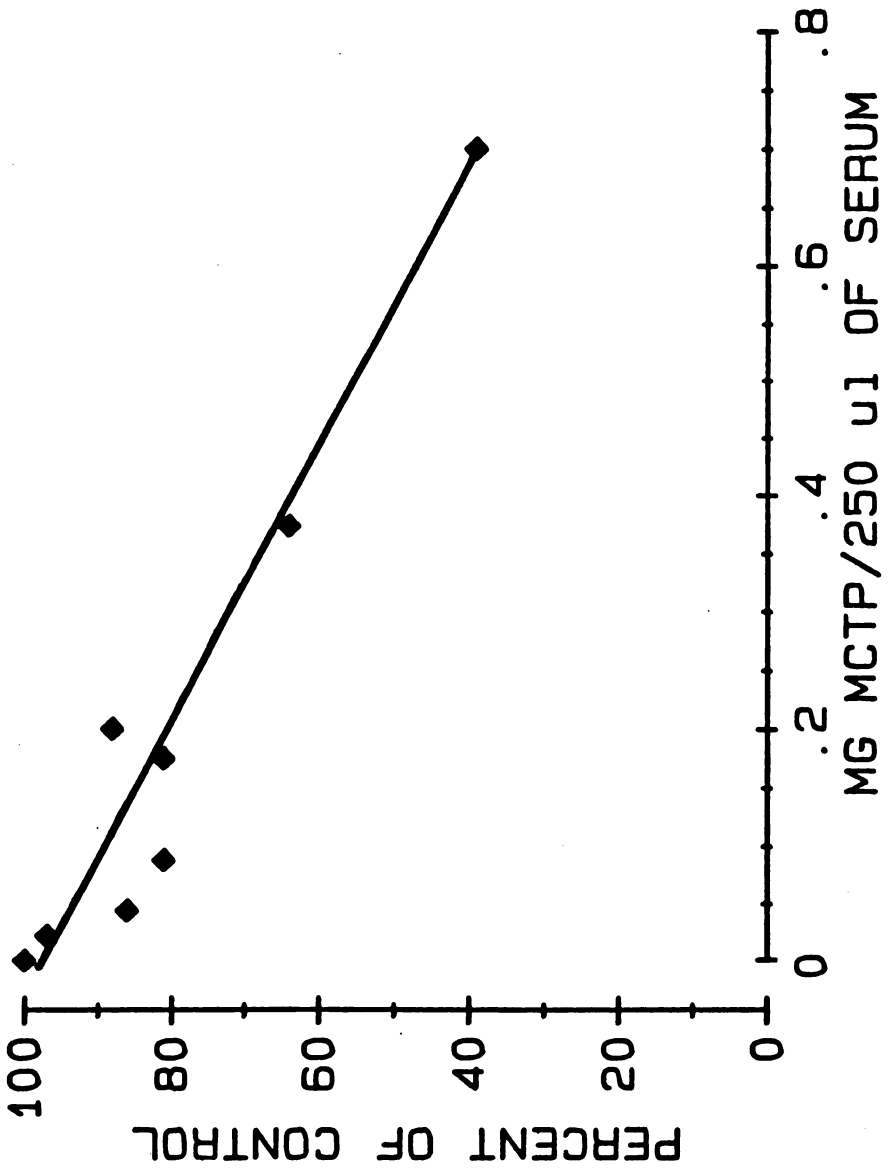
PERCENT OF CONTROL
100
80
60
40
20
0
0
.2
.4
.6
.8
MG MCTP/250 ul OF SERUM

Figure 24.

Figure 25. Ability of serum exposed to DMF or MCTP in vitro to stimulate neutrophil aggregation. Serum was exposed to DMF or MCTP in vitro as in MATERIALS AND METHODS. 50 µl samples were then added to 0.5 ml of a neutrophil suspension in an aggregometer cuvette and the aggregation response was recorded. Subsequently, 50 µl of zymosan-activated serum (ZAS) was added, and the aggregation wave was recorded. The rapid increases in transmittance upon addition of serum or ZAS is dilution artifact. This is followed by a change in transmittance when neutrophil aggregation occurs.

25A. Serum exposed to DMF in vitro did not stimulate aggregation. Subsequent addition of ZAS resulted in aggregation.

25B. Serum exposed to MCTP (6 mg/ml) did not stimulate neutrophil aggregation, whereas, subsequent addition of ZAS stimulated aggregation. Sera exposed to other doses of MCTP in vitro also did not stimulate neutrophil aggregation.

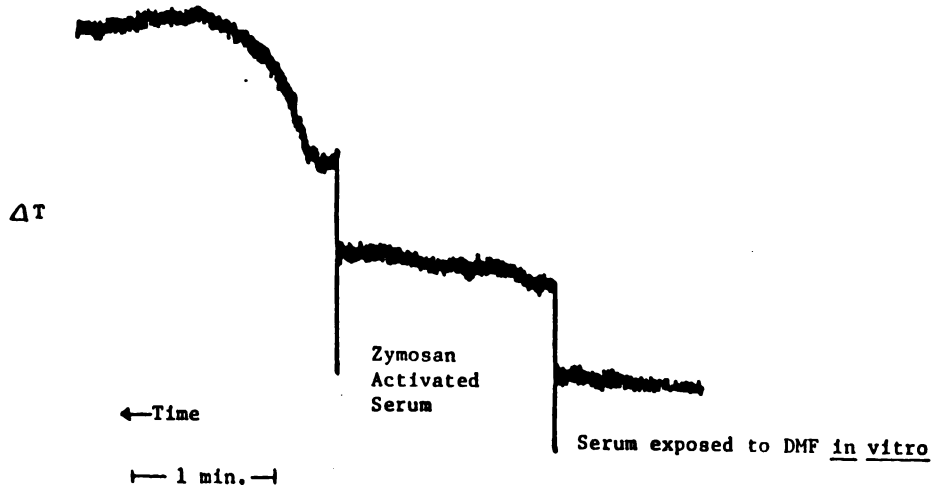

Figure 25A.

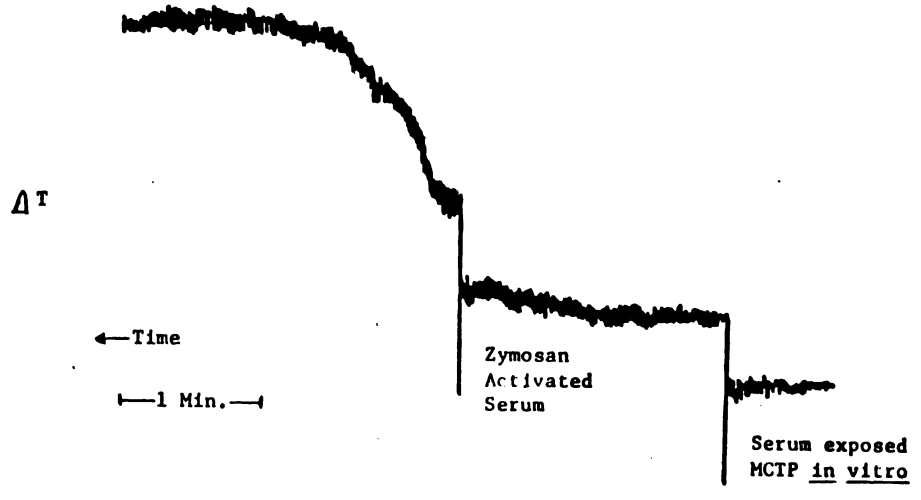

Figure 25B.

Figure 26. Effect of zymosan on MCTP-treated serum.

26A. Serum was first exposed to MCTP and then to saline in vitro as in MATERIALS AND METHODS. 50 µl samples of this serum were then added to 0.5 ml of neutrophil suspension in an aggregometer cuvette. The MCTP/saline-treated serum did not stimulate neutrophil aggregation. Subsequent addition of zymosan-activated serum (ZAS) caused normal aggregation.

26B. Serum was first exposed to MCTP and then to zymosan in vitro as in MATERIALS AND METHODS. 50 µl samples of the serum were then added to 0.5 ml of neutrophil suspension in an aggregometer cuvette. The MCTP/zymosan-treated serum caused neutrophil aggregation. A second wave of aggregation was induced by subsequent addition of ZAS.

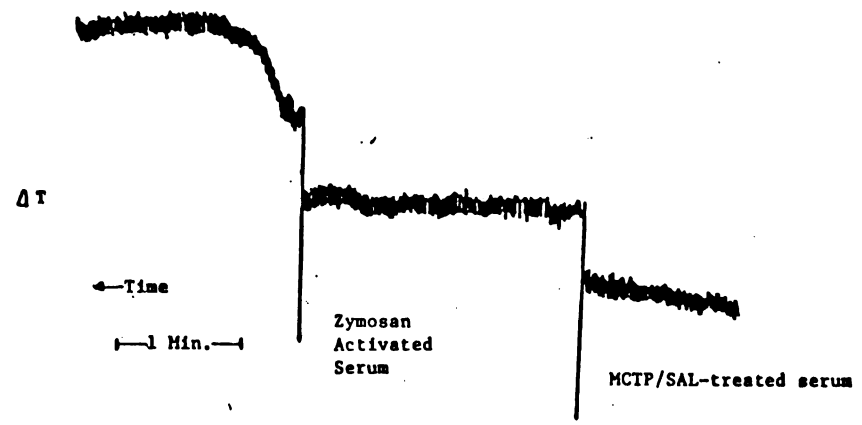

Figure 26A.

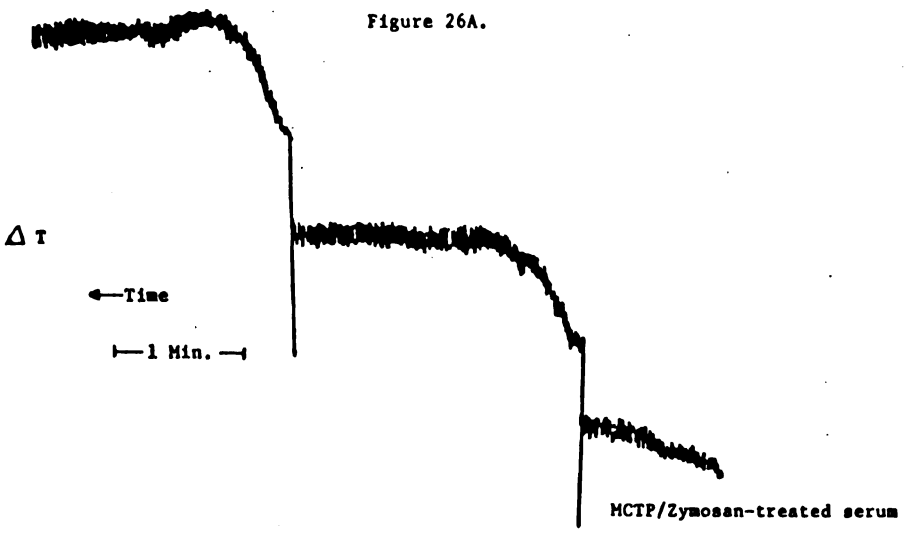

Figure 26B.

Figure 27. Effect of desferroxamine mesylate (DF) on MCTP-induced pulmonary injury. Rats were treated with a single injection of MCTP or DMF and DF (150 mg/kg, 2 times/day) or saline. The effects of treatments on lung injury were assessed in MATERIALS AND METHODS. Bars represent mean ± SEM, n = 8 rats/group. Bars having the same letter are significantly different from each other ($p < 0.05$). Treatment groups: DS = DMF/Saline; DD = DMF/DF; MS = MCTP/Saline; MD = MCTP/DF.

27A. Effect on cell-free bronchopulmonary lavage fluid lactate dehydrogenase activity.
27B. Effect on sequestration of ^{125}I-BSA in the lung.
27C. Effect on body weight gain.
27D. Effect on relative lung weight.

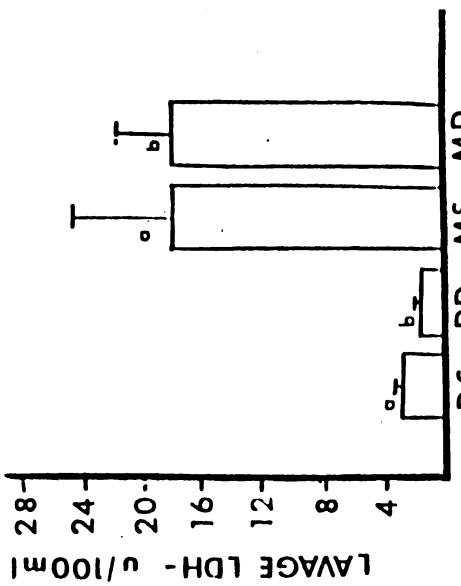

Figure 27A.

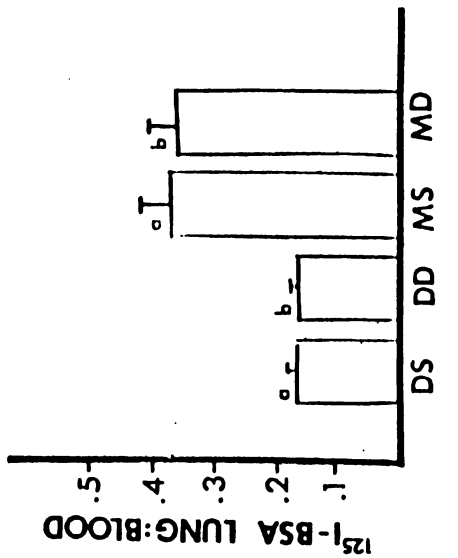

Figure 27B.

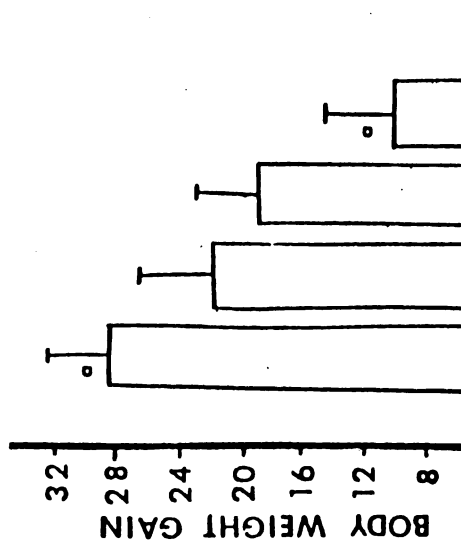

Figure 27C.

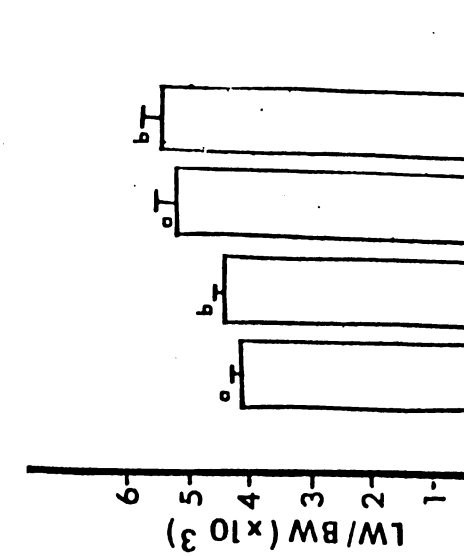

Figure 27D.

MCTP/SAL or MCTP/DF had increased lavage fluid LDH activity (Figure 27A), increased sequestration of ^{125}I-BSA in the lungs (Figure 27B) and increased relative lung weight (Figure 27D) compared to groups treated with DMF/SAL or DMF/DF, respectively. However, there were no differences in the extent of lung injury between the MCTP/SAL- and MCTP/DF-treated groups (Figure 27). There was no difference in body weight gain between groups treated with DMF/SAL and those receiving DMF/DF (Figure 27C).

2. Effect of DMSO on MCTP-induced pulmonary injury

In separate studies, rats were co-treated with MCTP and three different doses of the hydroxyl radical scavenger, DMSO. Rats receiving MCTP and 0.66 ml/kg of DMSO or MCTP and 1.0 ml/kg of DMSO, had increased lavage fluid LDH activity (Figures 28A and 29A), increased sequestration of ^{125}I-BSA (Figures 28B and 29B) and increased relative lung weight (Figures 28D and 29D) compared to their respective DMF/DMSO-treated controls. There were no differences in the extent of lung injury between co-treated with MCTP and 0.66 ml/kg of DMSO compared to rats receiving MCTP/SAL (Figure 28). Similarly, rats receiving MCTP and 1.0 ml/kg of DMSO had lung injury not different from their respective MCTP/SAL-treated controls (Figure 29). Rats treated with DMF and either of the two lowest doses of DMSO employed in this study did not lose weight compared to their respective DMF/S-treated controls (Figures 28C and 29C).

Rats co-treated with MCTP and DMSO at the highest dose tested (1.3 ml/kg) had less sequestration of ^{125}I-BSA in the lungs than did controls treated with MCTP/SAL (Figure 30B). Rats co-treated with 1.3 ml/kg DMSO and DMF lost weight compared to DMF/SAL-treated controls (Figure 30C) and showed other signs of intoxication due to DMSO (ruffled hair coat, listlessness, anorexia). Relative lung weight was increased in MCTP/SAL-treated rats compared to the

Figure 28. Effect of dimethylsulfoxide (DMSO) on MCTP-induced pulmonary injury. Rats were treated with a single injection of MCTP or DMF and DMSO (0.66 ml/kg, 3 times/day) or saline. The effects of treatments on lung injury were assessed as described in MATERIALS AND METHODS. Bars represent mean $\pm$ SEM, n = 8 rats/group. Bars having the same letter are significantly different from each other ($p < 0.05$). Treatment groups: DS = DMF/Saline; DD = DMF/DMSO; MS = MCTP/Saline; MD = MCTP/DMSO.

28A. Effect on cell-free bronchopulmonary lavage fluid lactate dehydrogenase activity.
28B. Effect on sequestration of ^{125}I-BSA in the lung.
28C. Effect on body weight gain.
28D. Effect on relative lung weight.

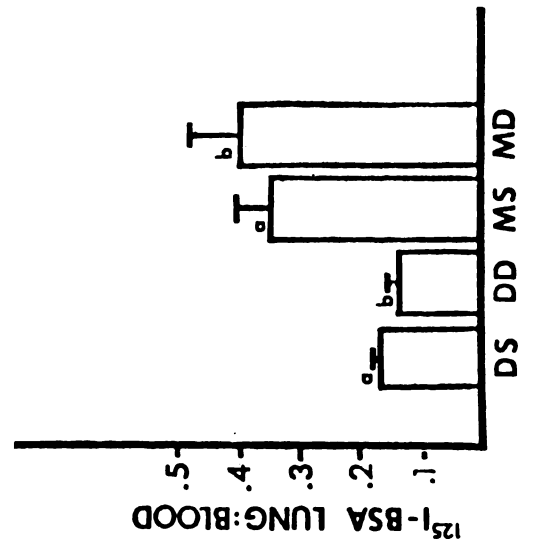

Figure 28B.

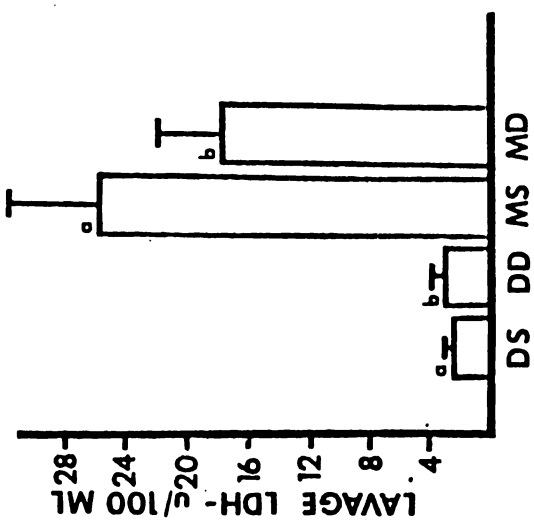

Figure 28A.

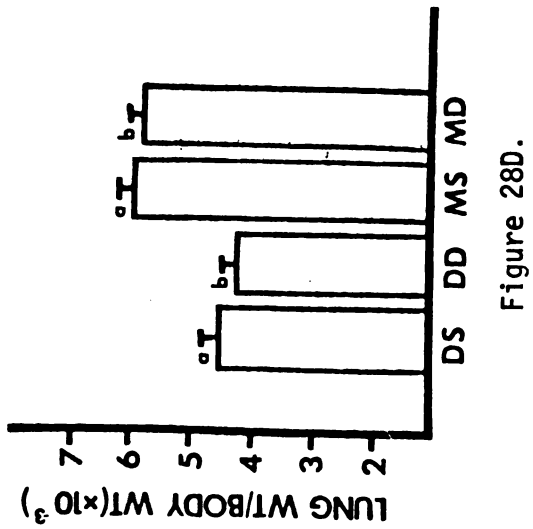

Figure 28D.

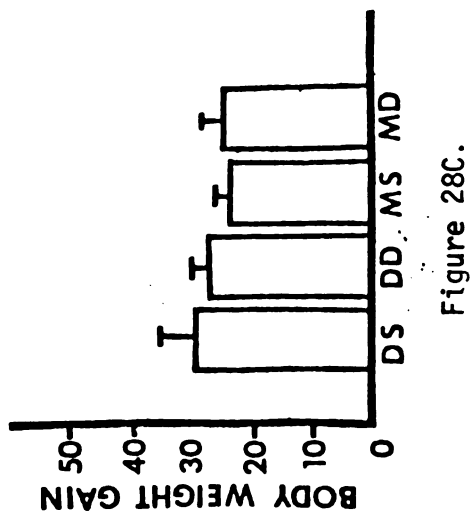

Figure 28C.

Figure 29. Effect of dimethylsulfoxide (DMSO) on MCTP-induced pulmonary injury. Rats were treated with a single injection of MCTP or DMF and DMSO (1.0 ml/kg, 3 times/day) or saline. The effects of treatments on lung injury were assessed as described in MATERIALS AND METHODS. Bars represent mean $\pm$ SEM, n = 8 rats/group. Bars having the same letter are significantly different from each other ($p < 0.05$). Treatment groups: DS = DMF/Saline; DD = DMF/DMSO; MS = MCTP/Saline; MD = MCTP/DMSO.

29A. Effect on cell-free bronchopulmonary lavage fluid lactate dehydrogenase activity.
29B. Effect on sequestration of ^{125}I-BSA in the lung.
29C. Effect on body weight gain.
29D. Effect on relative lung weight.

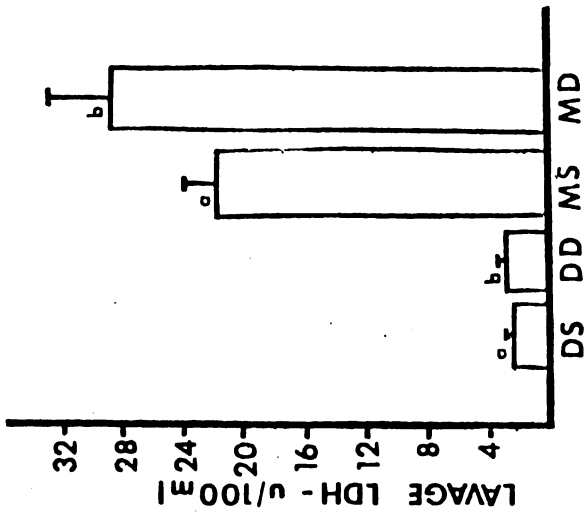

Figure 29A.

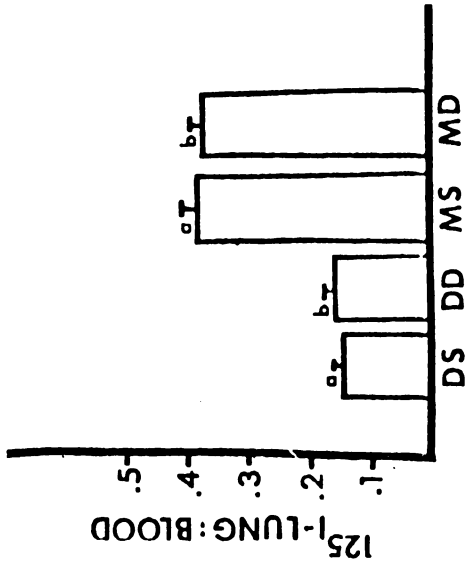

Figure 29B.

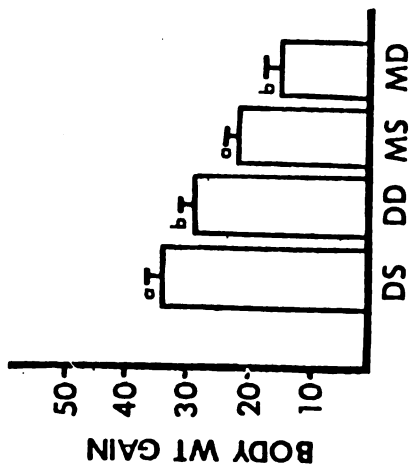

Figure 29C.

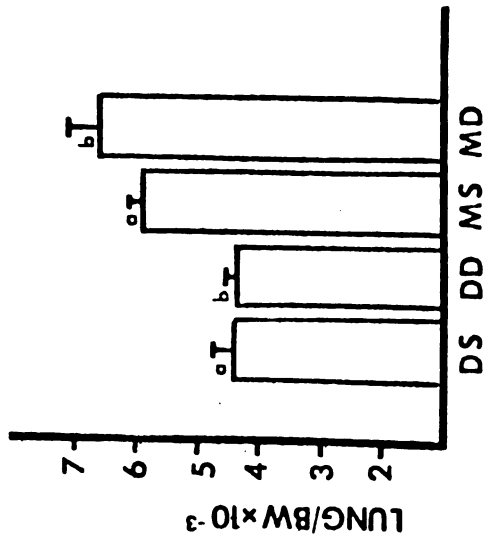

Figure 29D.

Figure 30. Effect of dimethylsulfoxide (DMSO) on MCTP-induced pulmonary injury. Rats were treated with a single injection of MCTP or DMF and DMSO (1.3 ml/kg, 3 times/day) or saline. The effects of treatments on lung injury were assessed as described in MATERIALS AND METHODS. Bars represent mean $\pm$ SEM, n = 8 rats/group. Bars having the same letter are significantly different from each other ($p < 0.05$). Treatment groups: DS = DMF/Saline; DD = DMF/DMSO; MS = MCTP/Saline; MD = MCTP/DMSO.

30A. Effect on cell-free bronchopulmonary lavage fluid lactate dehydrogenase activity.
30B. Effect on sequestration of ^{125}I-BSA in the lung.
30C. Effect on body weight gain.
30D. Effect on relative lung weight.

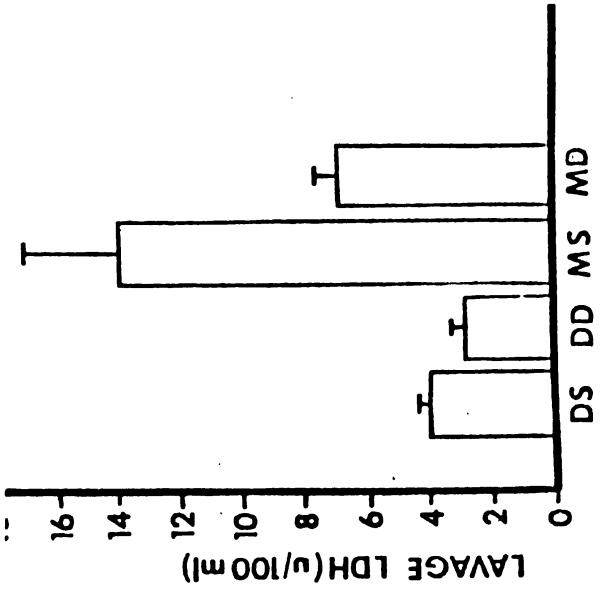

Figure 30A.

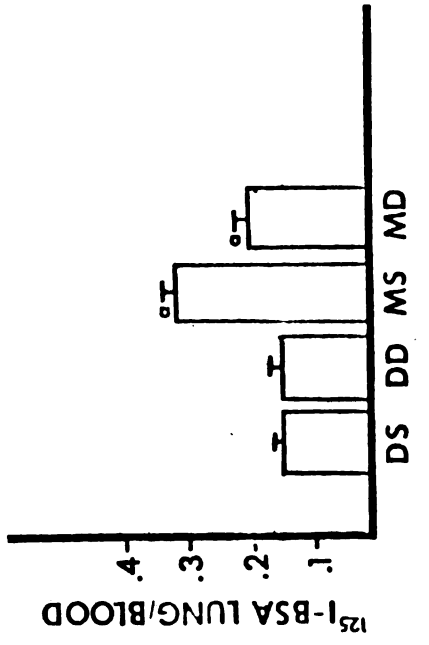

Figure 30B.

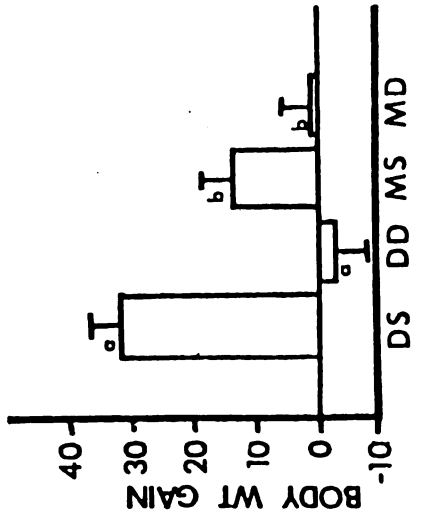

Figure 30C.

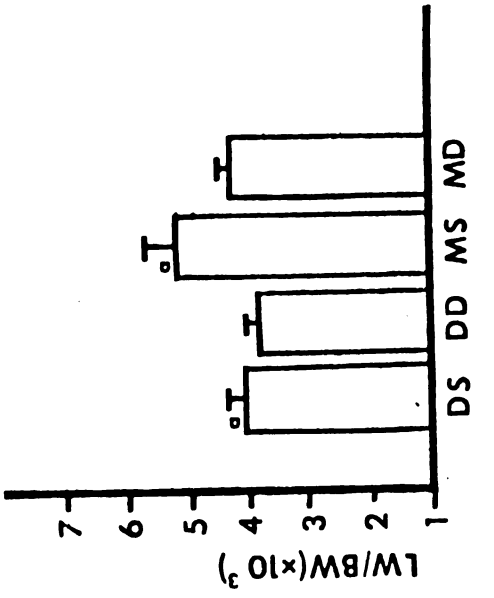

Figure 30D.

DMF/SAL-treated group (Figure 30D). There was no difference in relative lung weight between MCTP/SAL- and MCTP/DMSO-treated rats (Figure 30D). Rats treated with a higher dose of DMSO (4 ml/kg, 3x/day) died within two days due to DMSO intoxication.

3. Effect of catalase on MCTP-induced pulmonary injury

To test whether PEG-CAT would protect against lung injury after MCTP, rats were co-treated with a single dose of MCTP and daily injections of PEG-CAT (1000 U/dose). Rats receiving MCTP/SAL had increased lavage fluid LDH activity, increased sequestration of ^{125}I-BSA and increased relative lung weight compared to DMF/S-treated controls (Figure 31). There were no differences in the extent of lung injury between rats receiving MCTP/PEG-CAT and the group receiving MCTP/SAL (Figure 31).

In a separate study, rats were co-treated with MCTP and a higher daily dose of PEG-CAT (7500 U/injection) or SAL. There were no differences in lavage fluid LDH activity, sequestration of ^{125}I-BSA or relative lung weight in MCTP/PEG-CAT-treated rats compared to those receiving MCTP/SAL (Figure 32).

L. Effect of Diethylcarbamazine (DEC) on MCTP-induced Pulmonary Injury

Since MCTP induces a chronic, fatal, inflammatory injury in the lungs, it was of interest to evaluate the efficacy of anti-inflammatory drugs in preventing lung injury after MCTP. DEC is a drug that has anti-inflammatory properties perhaps due to its abiity to block synthesis of leukotrienes (Mathews and Murphy, 1982; Bach, 1984). Leukotrienes are potential mediators of increased vascular permeability and are potent inflammatory agents. Accordingly, the effect of DEC on MCTP-induced pulmonary injury was evaluated.

Figure 31. Effect of polyethylene glycol-coupled catalase (PEG-CAT) on MCTP-induced pulmonary injury. Rats were treated with a single injection of MCTP or DMF and PEG-CAT (1000 U/day) or saline. The effects of treatments on lung injury were assessed as described in MATERIALS AND METHODS. Bars represent mean $\pm$ SEM, n = 8 rats/group. Bars having the same letter are significantly different from each other ($p < 0.05$). Treatment groups: DS = DMF/Saline; DC = DMF/PEG-CAT; MS = MCTP/Saline; MC = MCTP/PEG-CAT.

31A. Effect on cell-free bronchopulmonary lavage fluid lactate dehydrogenase activity.

31B. Effect on sequestration of ^{125}I-BSA in the lung.

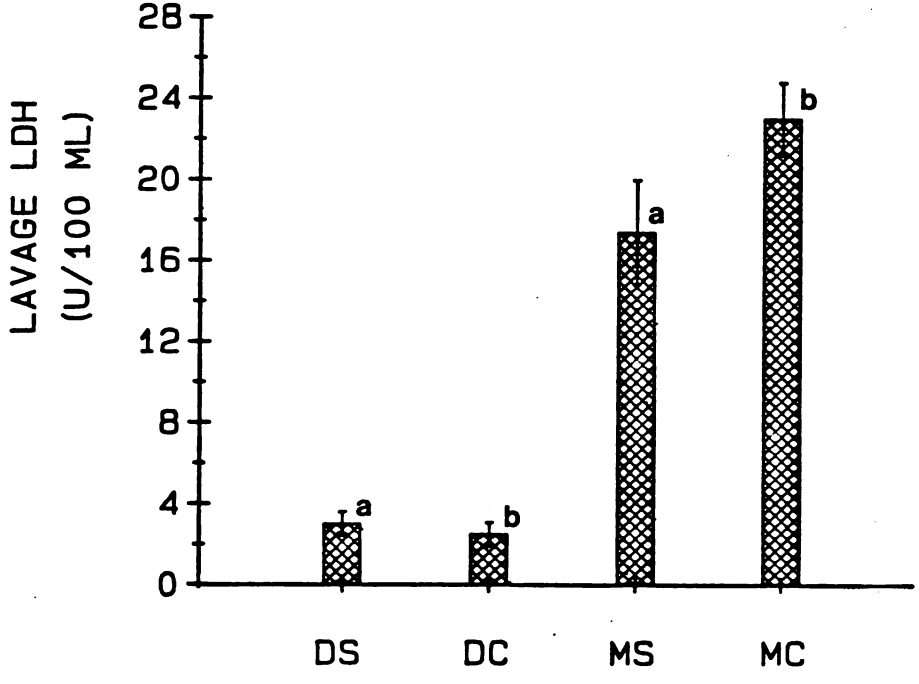

Figure 31A.

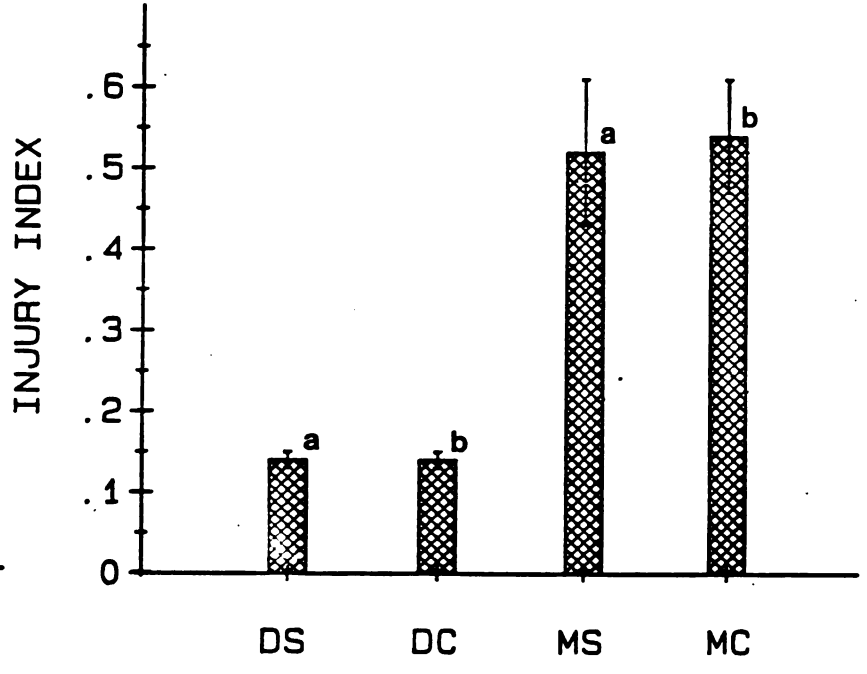

Figure 31B.

Figure 31 (continued)

31C. Effect on body weight gain.
31D. Effect on relative lung weight.

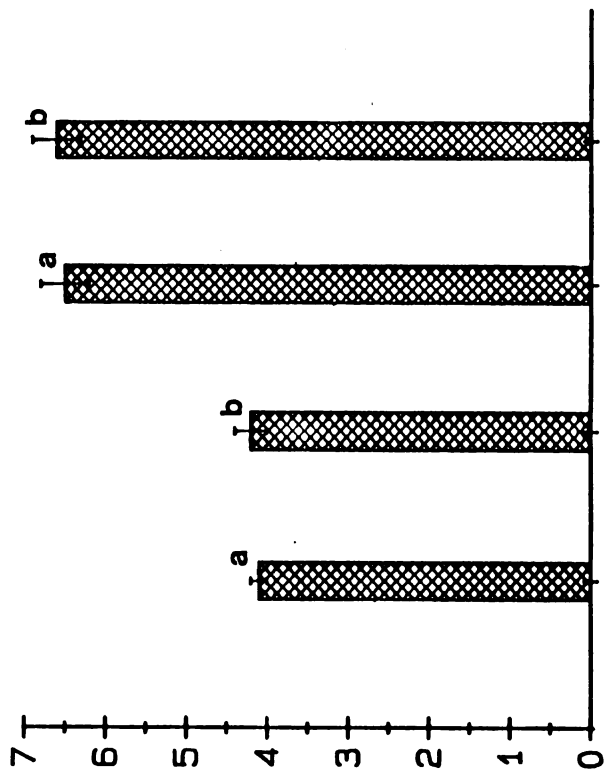

Figure 31D.

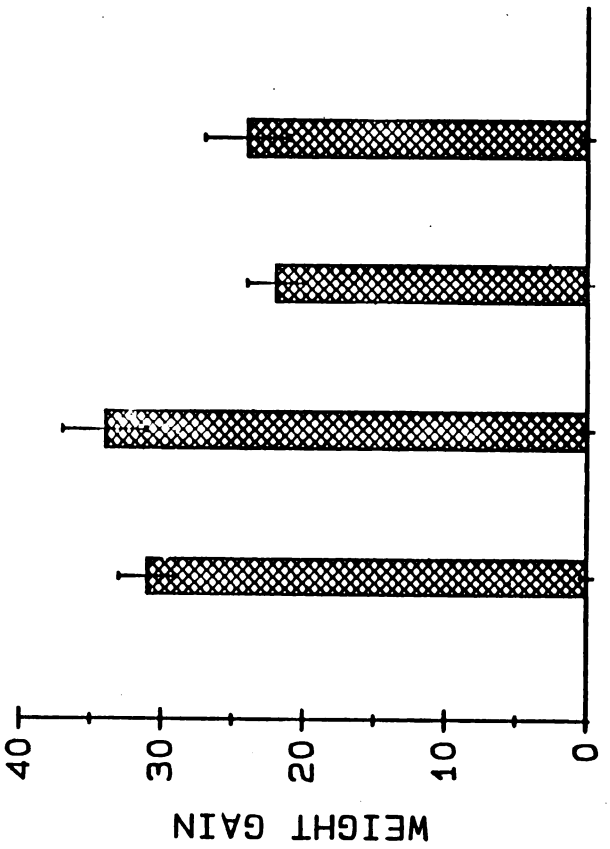

Figure 31C.

Figure 32. Effect of polyethylene glycol-coupled catalase (PEG-CAT) on MCTP-induced pulmonary injury. Rats were treated with a single injection of MCTP or DMF and PEG-CAT (7500 U/day) or saline. The effects of treatments on lung injury were assessed as described in MATERIALS AND METHODS. Bars represent mean $\pm$ SEM, n = 8 rats/group. Bars having the same letter are significantly different from each other ($p < 0.05$). Treatment groups: MS = MCTP/Saline; MC = MCTP/PEG-CAT.

32A. Effect on cell-free bronchopulmonary lavage fluid lactate dehydrogenase activity.

32B. Effect on sequestration of ^{125}I-BSA in the lung.

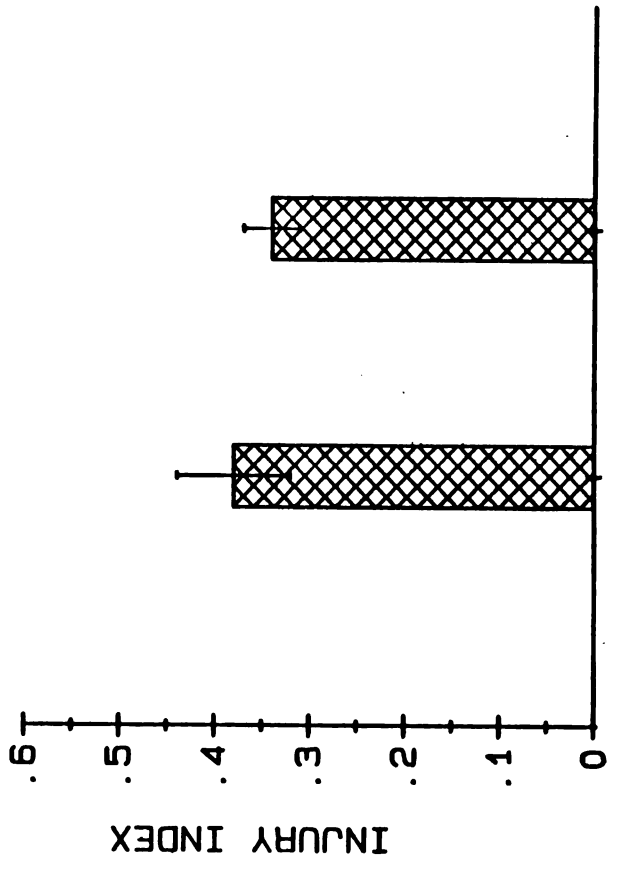

Figure 32B.

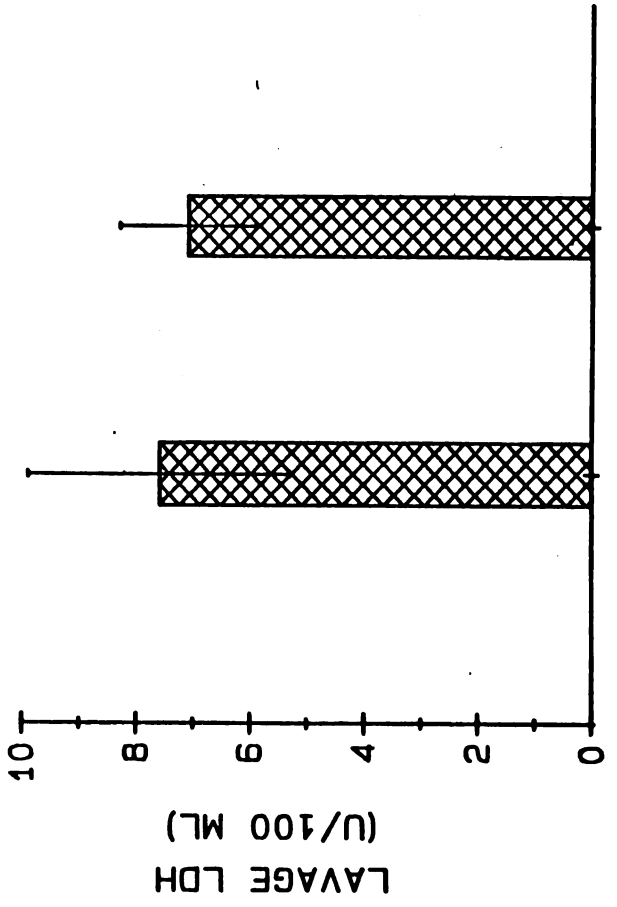

Figure 32A.

Figure 32 (continued)

32C. Effect on body weight gain.
32D. Effect on relative lung weight.

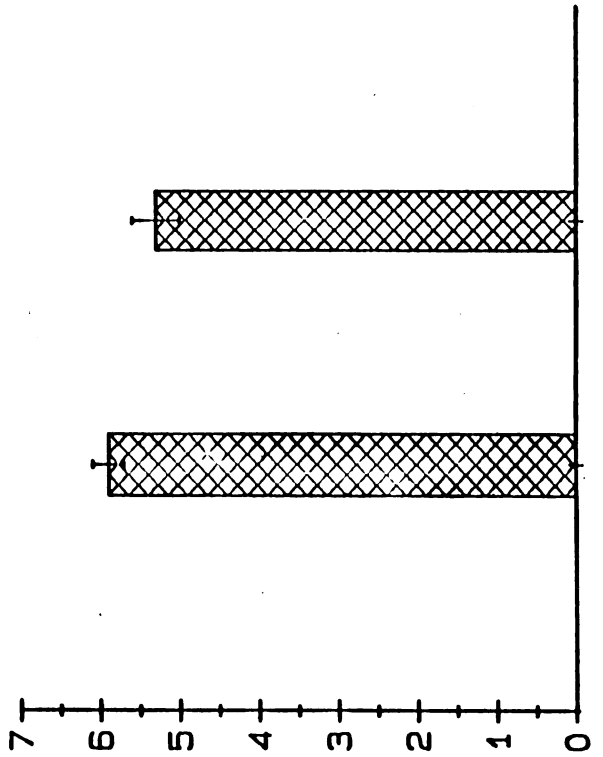

Figure 32D.

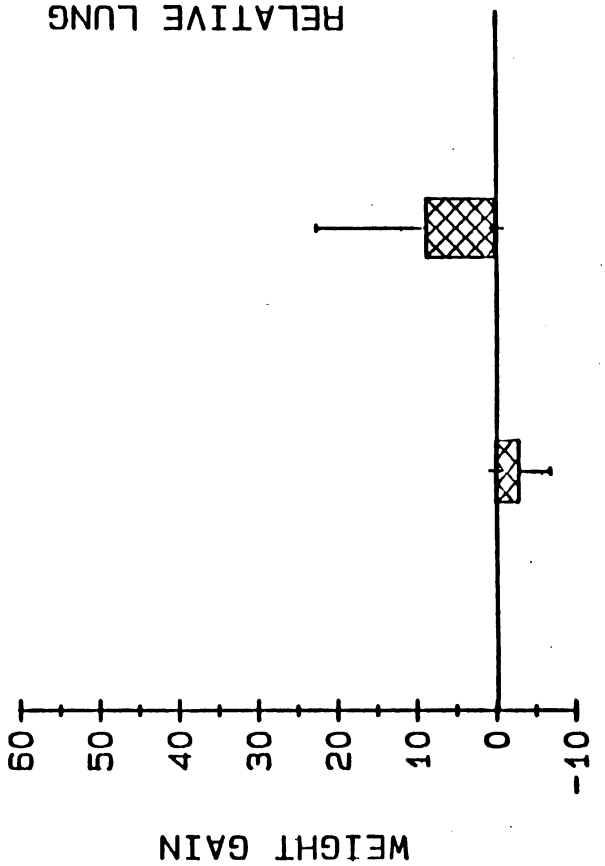

Figure 32C.

TABLE 19

Effect of Diethylcarbamazine (DEC) and MCTP on Body Weight Gain

Days After MCTP	Daily DEC Dose[a]	Body Wt Gain (gm)	n
7	0	12 ± 4	4
7	100	15 ± 7	5
7	300	6 ± 1	6
14	0	10 ± 18	6
14	300	7 ± 20	5

[a]Total daily DEC dose in mg/kg split into equal doses given 3 times/day. Controls receiving no DEC were given saline vehicle. Values represent mean ± SEM. There were no significant differences between any of the groups ($p < 0.05$).

There was no difference in the rate of body weight gain between those rats that received SAL and MCTP and those rats that received DEC and MCTP (Table 19). Thus, treatment with DEC and MCTP did not cause rats to lose more weight than those rats with MCTP alone.

Relative lung weight was in the normal range in rats killed 7 days after MCTP. Thus, there appeared to be no effect on relative lung weight due to MCTP by day 7 (Table 20). In the rats killed 14 days after MCTP, the relative lung weight was markedly increased over values observed in DMF-treated animals (see Figures 4, 9, 12, 23, 28-30 or Tables 9-16). There were no differences in relative lung weight due to treatment with DEC.

Seven days after MCTP, co-treated with DEC resulted in decreased release of LDH into the airway in a dose-dependent manner (Table 21). In contrast, 14 days after MCTP there was no difference in lavage LDH activity between the MCTP/SAL- and MCTP/DEC-treated animals (Table 21).

Lavage fluid protein concentration followed a pattern similar to that observed for LDH activity. At 7 days after MCTP, elevation in lavage fluid protein concentration was ameliorated by DEC in a dose-dependent manner (Table 22). At 14 days after MCTP, there was no difference in protein concentration between the two groups, and the magnitude of protein concentration was much greater at 14 days than at 7 days after MCTP (Table 22).

At 14 days after MCTP, there was no difference in the relative sequestration of ^{125}I-BSA in the lungs of rats treated with MCTP/SAL and MCTP/DEC (Figure 33). Rats treated only with DMF vehicle have ^{125}I lung/^{125}I blood ratios of 0.18±0.06 (see Figure 8). Thus, MCTP-induced sequestration of ^{125}I-BSA by lung was not attenuated by DEC treatment.

TABLE 20

Effect of Diethylcarbamazine (DEC) and MCTP on Relative Lung Weight

Days After MCTP	Daily DEC Dose[a]	Lung Wt/Body Wt ($\times 10^{-3}$)	n
7	0	4.39±0.15	4
7	100	4.32±0.48	5
7	300	3.76±0.12	6
14	0	11.38±1.50	6
14	300	12.39±3.37	5

[a]Total daily DEC dose in mg/kg split into equal doses given 3 times/day. Controls receiving no DEC were given saline vehicle. Values represent mean ± SEM. There were no significant differences between any of the groups ($p < 0.05$).

TABLE 21

Effect of Diethylcarbamazine (DEC) and MCTP on Bronchopulmonary Lavage Fluid LDH Activity

Days After MCTP	DEC Dose[a]	LDH Activity (U/100 ml)[b]	n
7	0	21.9±4.47	4
7	100	11.5±1.13[b]	5
7	300	6.9±1.28[b]	6
14	0	26.9±4.7	6
14	300	29.1±6.3	5

[a]Total daily DEC dose in mg/kg split into equal doses given 3 times/day. Controls receiving no DEC were given saline vehicle. Values represent mean ± SEM.

[b]Significantly different from saline-treated controls ($p < 0.05$).

TABLE 22

Effect of Diethylcarbamazine (DEC) and MCTP on Bronchopulmonary Lavage Fluid Protein Concentration

Days After MCTP	DEC Dose[a]	Lavage Fluid Protein (gm/DL)	n
7	0	58± 8	4
7	100	48± 12	5
7	300	27± 4[b]	6
14	0	420± 86	5
14	300	469±138	5

[a]Total daily DEC dose in mg/kg split into equal doses given 3 times/day. Controls receiving no DEC were given saline vehicle. Values represent mean ± SEM.

[b]Significantly different from saline-treated controls ($p < 0.05$).

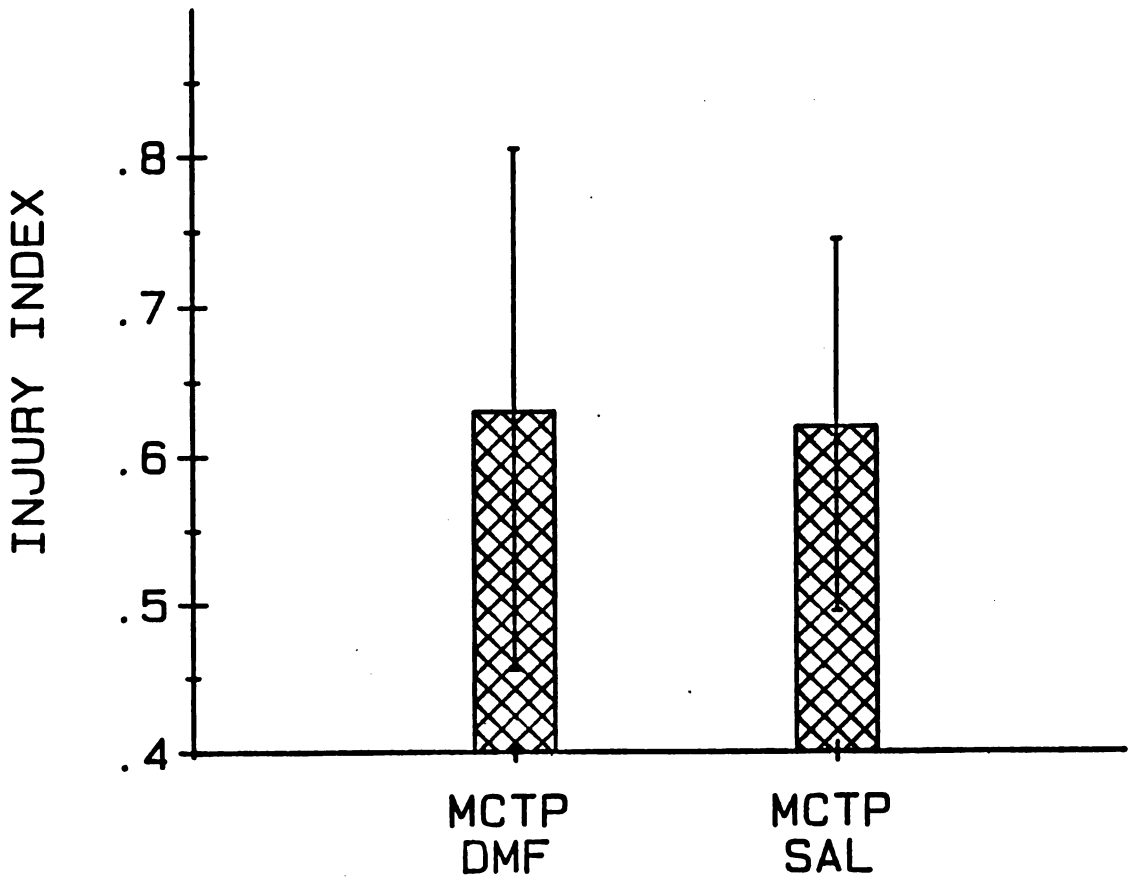

Figure 33. Effect of DEC on the sequestration of ^{125}I-BSA in the lungs of rats treated with MCTP. Rats were treated with MCTP and with DEC or saline as described in MATERIALS AND METHODS. An index of injury was determined by calculating the ratio of ^{125}I-BSA retained in the lung to that in the blood. There was no difference in the index due to treatment with DEC ($p < 0.05$).

RVH is evident 14 days after treatment with MCTP (Table 3). In this study, RV/(LV+S) was determined in rats 14 days after MCTP. The values obtained for RV/(LV+S) are markedly increased compared to values obtained from control rats (Figure 34), indicating that RVH was present in both groups of rats killed 14 days after MCTP. There was no difference in RV/(LV+S) between rats treated with MCTP/DEC and those treated with MCTP/saline (Figure 34).

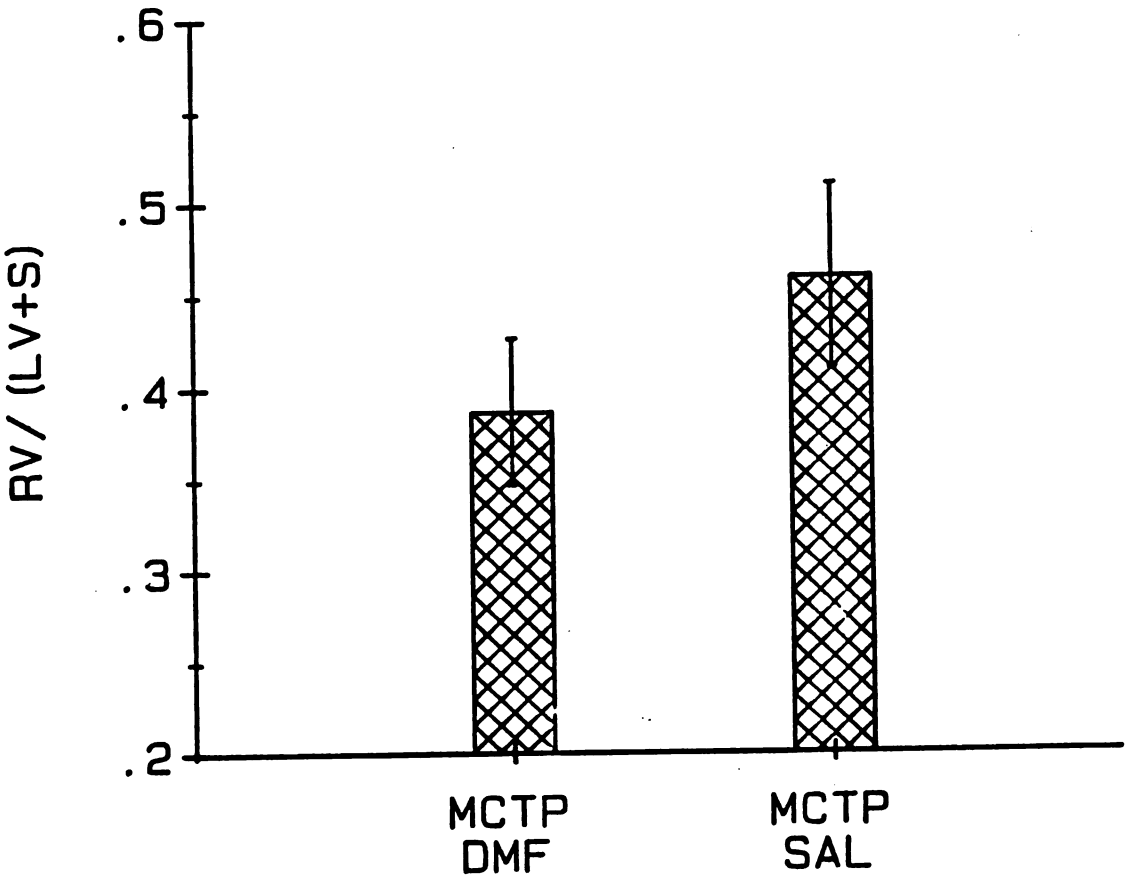

Figure 34. Effect of DEC on the development of right ventricular hypertrophy (RVH). RVH was measured as increases in the ratio of the weight of the right ventricle (RV) to the weight of the left ventricle plus septum (LV+S). There was no difference in the RV/LV+S due to treatment with DEC.

DISCUSSION

A. Time-Course of Injury After a Single Injection of MCTP

The purpose of this study was to evaluate the time-course of the development of cardiopulmonary injury after a single injection of MCTP. Rats were given a single dose of MCTP and controls received a single injection of DMF vehicle. Control rats gained weight, whereas MCTP-treated rats did not. This finding is similar to that which occurs in rats dosed with MCT given by injection (Ghodzi and Will, 1981) or by ingestion (Gillis et al., 1978). The probable cause for the lack of weight gain is that the treated rats consumed less food. Although food intake was not recorded in this study, Gillis et al. (1978) observed a decrease in food consumption in animals receiving MCT in their drinking water.

The presence of pulmonary damage was assessed by measurement of relative lung weight, by determination of LDH activity in cell-free bronchpoulmonary lavage fluid and sequestration of ^{125}I-labelled BSA in the lungs. The lung weight increases observed are consistent with reports of accumulation of edema fluid in the alveolar and interstitial areas of lungs of animals treated with MCTP (Butler et al., 1970) and also with cellular infiltration (Butler, 1970), and endothelial, smooth muscle, and alveolar epithelial cell hypertrophy (Lafranconi et al., 1984) that occur in MCT toxicity. Measurement of bronchopulmonary lavage fluid LDH activity has been used as a sensitive albeit non-specific index of injury caused by pneumotoxicants (Henderson et al., 1978) including MCT (Roth, 1981). MCTP produces lesions similar to those seen in MCT toxicity and, like MCT (Roth, 1981), leads to elevated LDH activity in the lavage fluid. The LDH activity increased

rapidly between 3 and 5 days following MCTP. After remaining markedly elevated from 5 through 10 days after treatment, the LDH activity at 14 days decreased approximately one-third that seen at the previous time points. The reason for this decrease in activity is unknown but may have been due to decreased release of the enzyme as the result of repair processes occurring in the airway. Another possible explanation for the decreasing activity is that the damage is still severe at 14 days, but no more LDH is released from lung cells. Also, many pulmonary alveolar macrophages accumulate during the time-course of MCTP toxicity (Chesney et al., 1974). These cells have phagocytic activity (Sugita et al., 1983) and may remove LDH protein from the airway.

Estimation of radiolabelled protein marker sequestration in the lungs was done to evaluate the procedure as a method for quantification of lung injury after MCTP. There was no accumulation of the radioactive marker in lungs of treated animals in the first few days after MCTP. Even as early as 30-240 minutes after dosing, there were no differences between treated and control animals. Thus, ^{125}I-BSA sequestration corresponds with the onset of other aspects of MCTP-induced lung injury such as increased pulmonary arterial pressure and increased lung weight (Bruner et al., 1983).

The sequestration of the ^{125}I-BSA may be due to changes in pulmonary vascular permeability leading to movement into the interstitium of the larger BSA molecule which is normally restricted to the vascular compartment. There is much endothelial injury after MCTP, and this may allow leak of the BSA into the swollen interstitial spaces and alveoli. This BSA would not have been washed out with the 10 ml saline flush and would thereby result in the increased injury index. Another possible cause for increased sequestration of ^{125}I-BSA is that considerable vascular remodelling due to MCTP (Chesney et al., 1974; Lalich et al., 1977) may result in less thorough flushing of the vasculature, leading to an increased

injury index. Whether the cause of the increased sequestration of ^{125}I-BSA is due to permeability changes or to other vascular alterations, the sequestration of ^{125}I-BSA in the lungs progressively increases after MCTP treatment and is reliably associated with injury during the later stages of MCTP-induced pulmonary pathogenesis.

Changes in several hematologic parameters were evaluated in this study. The increase in hematocrit observed 14 days after treatment with MCTP might be due to a specific effect of MCTP on erythrocytes as was observed by Chesney et al. (1974). However, at 14 days after treatment, the animals were sickly in appearance, anorectic, and possibly dehydrated. They appeared similar to MCT-treated rats, which are known to consume less water (Gillis et al., 1978). It is likely, therefore, that slight dehydration may have caused the increase in hematocrit.

Changes in WBC count and differential count often reflect the nature and severity of a disease process and the systemic response of an individual to a tissue injury. In the present study, the magnitude of the changes among any of the individual cell types is small, making interpretation of such changes difficult. The picture seen is consistent with a generalized inflammatory insult. Whether the increase in WBC represents a response to damage caused by the MCTP or indicates that the damage in the lungs is caused indirectly by leukocytes (Fantone and Ward, 1982) is not known.

Platelet count decreases after a single injection of MCT (Hilliker et al., 1982). By contrast, administration of MCTP did not alter platelet count. This dissimilarity may be due to differences in toxicodynamics of these substances. MCT must be metabolized in the liver to exert toxicity. Hepatic activation of MCT may produce a degree of liver tissue injury that results in sequestration of platelets by liver and a transient thrombocytopenia. MCTP, on the other hand,

does not require activation by liver and probably does not produce liver injury that may lead to platelet sequestration. Another possible explanation for this difference is that a MCT metabolite other than MCTP may be responsible for the thrombocytopenia observed and that MCTP itself may have no direct effect on the platelets.

The temporal relationship between the development of pulmonary hypertension and right ventricular hypertrophy after treatment with MCT is not completely clear. Meyrick et al. (1980) observed pulmonary hypertension 14 days after feeding rats ground C. spectabilis seeds, but right ventricular hypertorphy was not observed until 2 weeks later. Ghodzi and Will (1981) gave rats a single, 60 mg/kg injection of MCT and made evaluations at weekly intervals. Pulmonary hypertension and right ventricular hypertrophy were both observed for the first time 14 days after treatment. In the present study, a detailed examination of the temporal relationship between pulmonary arterial pressure elevation and development of right ventricular hypertrophy was made in rats treated with MCTP. A single intravenous dose of MCT caused a pulmonary arterial pressure change that was similar in magnitude and onset to that seen by Ghodzi and Will (1981) using MCT. This increase in pressure after MCTP treatment was followed by right ventricular hypertrophy 7 days later. It is, therefore, apparent that the onset of right ventricular hypertrophy does not correspond with the onset of increased pulmonary arterial blood pressure, but rather is an adaptive response to increased afterload.

The ECG is a measure of the electrical potential generated within the body due to the sequential depolarization of the cardiac tissue during each heart beat. One of the characteristic electrocardiographic changes observed in right ventricular hypertrophy is a deviation of the mean electrical axis (MEA) of the QRS complex to the right (Roman et al., 1961). An ECG was measured in each rat, and

the MEA of the QRS complex to the nearest 30 degrees was determined to evaluate whether or not this procedure could be used as a non-invasive method for measuring right ventricular hypertrophy in vivo. These data show that, in rats with significant right ventricular hypertrophy, changes in MEA do occur and that these changes are significantly and positively correlated. Therefore, in long-term experiments in which direct measurement of right ventricular hypertrophy is desired, but not feasible without killing animals, the ECG provides a useful tool to monitor repeatedly changes in the right heart.

B. Effect of an Inducer and an Inhibitor of Mixed Function Oxidase Activity on MCTP-induced Pulmonary Injury

PB or SKF-525A pretreatment of rats increased or decreased MCT toxicity, respectively. Thus, the treatments used to induce and inhibit P450-mediated metabolism were effective in these studies. Rats were then co-treated with PB or SKF-525A and MCTP to determine if alterations in hepatic MFO metabolism change the toxicity of MCTP. There were no differences in MCTP toxicity due to co-treatment with PB or SKF-525A, suggesting that there is no further metabolism of MCTP or its aqueous degradation products to metabolites that cause pulmonary injury by forms of cytochrome P450 that are affected by these two agents.

These results also suggest that drugs which inhibit or induce hepatic MFO's will not interfere with MCTP-induced pulmonary injury. Many investigators have used drugs with specific pharmacologic actions to study the mechanism of action of MCT. Results of such studies are difficult to interpret because effects of a co-administered drug on MCT pneumotoxicity may be due to changes in MCT metabolism that cannot be differentiated from a specific and direct pharmacologic effect of the drug being tested. Use of MCTP in such studies eliminates this complication; therefore, use of MCTP rather than MCT in such experimental

studies may be advantageous whenever bioactivation of MCT may be altered by being co-treated with drugs.

C. Relative Toxicity of MCT, MCT N-oxide and MCTP

It is possible that MCTP may be converted to MCT, MCT N-oxide or other toxic products that are responsible for injury after MCTP. It is also possible that small amounts of MCT or MCT N-oxide may be minor contaminants in the MCTP that contribute to the lung injury. Accordingly, rats were treated with i.v. MCT or MCT N-oxide to determine if injury occurred which was similar to that produced by an equivalent dose of MCTP. Rats treated with MCT or MCT N-oxide had no lung injury 14 days after treatment, whereas MCTP-treated rats had significant lung damage, right ventricular hypertrophy and mortality. These results confirm that MCTP is more toxic than MCT or MCT N-oxide. The results also indicate that the toxic effects of the chemically synthesized MCTP are not due to MCT or to MCT N-oxide contaminants. Mass spectral analysis support this contention, since the MCTP was not contaminated with major amounts of MCT or N-oxide.

D. Toxicity of MCTP in Plasma or Saline Vehicle

A recent report indicated that perfusion medium from isolated rat livers exposed to MCT was capable of causing injury to isolated, perfused rat lungs (Lafranconi and Huxtable, 1984). These results suggested the formation of a pneumotoxic, Ehrlich positive, hepatic metabolite of MCT that is stable in aqueous solution. To test whether chemically synthesized MCTP is capable of causing lung injury after exposure to aqueous solutions, MCTP was prepared in serum or saline vehicle and given to rats. Rats receiving MCTP prepared in these vehicles did not develop lung injury as did those rats that received the same dose

of MCTP prepared in DMF, a vehicle in which it is stable. Also, when MCTP was added to the reservoir of medium perfusing isolated lungs, uptake of 5HT was not decreased nor was other lung injury produced (Hilliker and Roth, 1985). However, MCTP dissolved in DMF and introduced directly into the pulmonary artery did result in injury to isolated lung. These results indicate that MCTP, when allowed to exist for several minutes in perfusion medium, serum or saline, undergoes a change that renders it incapable of producing pulmonary injury in vivo or in vitro. Ehrlich positive activity is maintained in these aqueous media, however. These results support the contention that MCTP must be in its reactive, unbound form which is capable of binding directly with tissue nucleophiles in the lung to cause injury (Mattocks, 1968).

The results of the present study contrast with the finding of Lafranconi and Huxtable (1984) that MCT metabolites present in protein-containing perfusion medium from isolated liver caused an acute lung injury in isolated lungs. Since pure MCTP does not cause lung injury in vitro or in vivo after prolonged exposure to aqueous vehicle, it is unlikely that the agent causing lung injury in the experiment of Lafranconi and Huxtable (1984) was derived from MCTP. It is possible, however, that the acute lung injury caused by the liver perfusate was due to a MCT metabolite other than MCTP.

E. Color Change in Plasma Treated with MCTP

The question has been raised as to whether MCTP produced by the liver in vivo can survive long enough in the blood to be able to alkylate nucleophiles in extrahepatic tissues (Lafranconi and Huxtable, 1984). A red-orange color change is observed when MCTP is added to serum and likely represents the change occurring as MCTP binds covalently to serum proteins or degrades to a red-colored polymer (Mattocks, 1969). This color change occurs over a time greater

than 60 seconds after MCTP is added to serum (Figure 17). While the color change may not directly represent MCTP alkylation of proteins, it suggests that some undegraded MCTP is present for at least 60 seconds after being added to plasma. Since the circulation time of the rat is only a few seconds (Cotton et al., 1971; Hanwell and Linzell, 1972), it is not unreasonable to expect that MCTP produced in the liver may survive the trip from the liver to the lungs and may be capable of alkylating tissue proteins there. Other evidence that MCTP survives passage from the liver to the lungs is that covalently bound pyrroles are found in lungs of rats after treatment with MCT even though lung tissue is incapable of synthesizing MCTP from MCT (Mattocks, 1968; Hilliker et al., 1983).

F. The Role of Cell-mediated Immunity in MCTP-induced Pulmonary Injury

The delayed onset of major pulmonary injury after low doses of MCTP suggests that an indirect mechanism(s) mediates MCTP-induced lung damage, and the character of pulmonary lesions suggest that immune mechanisms may be involved in the pathogenesis of the injury. Accordingly, the role of immune mechanisms in the development of lung injury due to MCTP was evaluated.

Altered immune responses against tissue antigens occur after exposure to chemicals other than MCTP. For example, hepatic necrosis in humans is associated with repeated halothane anesthesia. It has been speculated that the hepatic damage may be due to development of sensitization against liver cells that are antigenically altered by halothane or its metabolites (Vergani et al., 1978). Mice exposed to sublethal doses of CCl_4 develop specific lymphocyte sensitivity to liver antigen preparations (Smith et al., 1980). Although an intact immune system is not required for the development of CCl_4-induced liver injury (Smith et al., 1980), these results suggest that chemically-induced damage can

give rise to antigens that are recognized by the immune system, thereby leading to sensitization.

1. Effect of immunosuppression with ALS and CyA

The immunosuppressive agents ALS and CyA were used to evaluate the role of immune mechanisms in injury due to MCTP. The dose of ALS used was effective in suppressing rejection of xenografts placed on F-344 rats. Thus, the ALS was effective in suppressing immune system function in the present study. However, treatment of rats with ALS did not prevent injury due to MCTP.

CyA also was used as an immunosuppressive agent in this study. The doses of CyA used were effective in suppressing rejection of transplanted organs in rats (Kawahara et al., 1980; Fritz et al., 1983; Morris et al., 1983; Hall et al., 1984; Kirkman et al., 1984) and against injury in rat models of autoimmune disease (Nussenblatt et al., 1981; Thompson, 1983). Co-treatment with 10 mg/kg/day of CyA partially protected rats from MCTP-induced increases in relative lung weight and also against the development of right ventricular hypertrophy. However, rats treated with CyA/DMF lost weight compared to OI/DMF-treated controls. This may be important in interpreting the data since MCTP-treated rats maintained on a restricted diet that results in depressed weight gain also are partially protected against development of lung injury due to MCTP (Ganey et al., 1985). Thus, it cannot be ruled out that the protection afforded by 10 mg/kg/day CyA may have been due to non-specific effects of the CyA.

Rats co-treated with 20 mg/kg/day CyA/MCTP were not protected against lung injury. Rather, 20 mg/kg/day of CyA with MCTP resulted in increased lavage fluid LDH activity and marked weight loss compared to rats receiving OI/MCTP. Thus, these results indicate that CyA does not protect against MCTP-induced lung injury.

2. Adoptive transfer

Scheiffarth et al. (1967) reported that mice treated with lymphocytes transferred from CCl_4-treated mice were capable of causing liver lesions in the recipients starting 2-3 days after transfer. Accordingly, adoptive transfer of lymphocytes from MCTP-treated donors was done to determine if transfer of lymphocytes could shorten the onset or alter severity of lesions in MCTP-treated recipients. Transfer of lymphocytes from MCTP-treated rats alone did not cause lung injury in recipients. In the series 1 adoptive transfer studies, recipients were given lung-associated lymph node-derived lymphocytes from rats that had been treated with MCTP seven days earlier. Lymphocytes were obtained from donors at this time because the number of sensitized lymphocytes in the lung-associated lymph nodes peak at 7 days after administration of antigen into F-344 rat lungs (Bice et al., 1982). In the series 4 experiments, lung-associated lymph node-derived lymphocytes were transferred from rats treated with MCTP 28 days earlier. This second time-point was chosen so that lymphocytes would be transferred from rats that had extensive pulmonary damage due to MCTP. Lung injury in the series 4 donors was extensive, and lung-associated lymph nodes were markedly enlarged compared to lymph nodes from normal, non-treated rats. Adoptive transfer of lymphocytes from MCTP-treated donors did not alter the onset of lung injury in MCTP-treated recipients. Thus, transfer of lymphocytes from MCTP-treated donors did not alter the onset of injury after MCTP in the recipients.

In series 2 and 3, mineral oil-elicited PEC harvested from MCTP-treated donors were transferred into recipients and tested for their ability to alter the onset of MCTP-induced lung injury. Oil-elicited PEC were tested because these cells transfer immunity against mammary tumors in F-344 rats (Boyer et al., 1981). However, in the present study, PEC transferred from MCTP-

treated rats did not alter the onset or severity of injury in MCTP-treated recipients. These results suggest that sensitization of lymphocytes is not important in MCTP-induced pulmonary injury.

G. Role of Complement in MCTP-induced Pulmonary Injury

1. Effect of MCTP on serum complement in vivo

The purpose of this study was to examine the role of the complement system in the pulmonary injury caused by MCTP. To determine if complement activation occurs in vivo after MCTP, serum CH50 was measured in rats treated with MCTP or DMF. There was no change in hemolytic complement activity in serum samples taken either within hours after MCTP or at the later time points evaluated. Since complement activation can occur in vivo without causing detectable changes in hemolytic complement activity (Hammerschmidt et al., 1980), nephelometric measurement of neutrophil aggregation was used to determine if complement activation occurred in vivo after MCTP. Craddock et al. (1977) demonstrated that C5a, a complement activation product, causes neutrophil aggregation in vitro. Using this method, C5a generation and, thus, complement activation can be detected in serum when changes in CH50, C3, C3 conversion products or chemotactic activity cannot be measured (Hammerschmidt et al., 1980). Neutrophil aggregating activity also has been found in the serum taken from patients with diseases known to be associated with complement activation in vivo such as systemic lupus erythematosis, rheumatoid vasculitis, acute drug allergy, transfusion reactions and acute migraine using this method (Hammerschmidt et al., 1980). Thus, neutrophil aggregometry is a very sensitive and useful method for detection of complement activation in vivo (Hammerschmidt et al. 1980).

Serum taken from rats at various times after MCTP does not stimulate neutrophil aggregation. To assure there were no factors in serum from MCTP-treated rats that might inhibit neutrophil aggregation in vitro, ZAS was added to the aggregometer cuvettes subsequent to each test sample. The addition of ZAS stimulated neutrophil aggregation in cuvettes containing serum from DMF- or MCTP-treated rats equally, suggesting that there were no inhibitory factors present in serum from MCTP-treated rats. Thus, the results of these experiments indicate that circulating products of complement activation are not detectable in vivo after MCTP.

2. Complement depletion in MCTP-treated rats

Complement depletion with CVF was used to evaluate the role of complement in MCTP-induced pneumotoxicity. In vivo, CVF results in generation of CVF-Bb and CVF-Bb-C3b. These complexes have the ability to convert C3 to C3a and C3b and are resistant to the regulatory inactivator proteins H and I (Maller-Eberhard and Schreiber, 1980; Alper and Balavitch, 1976). CVF thereby causes a sustained depletion of C3 which renders the complement system inactive.

In this study, complement depletion was confirmed by measuring hemolytic complement activity and C3 levels in serum from rats treated with CVF or saline. Rats treated with CVF had less than 40 CH50 units of complement activity and serum C3 levels were undetectable during the 5 days after starting CVF. Major injury due to MCTP occurs starting at 4 days after treatment (Bruner et al., 1983). Since the rats in this study were complement depleted starting at 2.5 days after MCTP, the complement system was inactive in MCTP/CVF-treated animals during the time when major injury occurs. Depletion of complement starting 2.5 days after MCTP did not protect against lung injury due to MCTP, suggesting that the complement system does not play an important role in the development of the pathologic lesions that occur 4-7 days after MCTP.

3. Effect of MCTP on serum complement activity in vitro

Direct addition of MCTP to serum in vitro caused a dose-dependent decrease in hemolytic complement activity. When MCTP-treated serum samples were tested in the neutrophil aggregation assay, the serum did not stimulate neutrophil aggregation, whereas subsequent addition of ZAS resulted in neutrophil aggregation. These results indicate that MCTP added to serum does not inhibit neutrophil aggregation in vitro, and suggest that MCTP does not activate complement in vitro. Thus, MCTP is an inhibitor of hemolytic complement activity in vitro.

Since addition of MCTP to serum in vitro decreases complement activity via the classical pathway (i.e., decreased ability to lyse sensitized SRBC), it was of interest to determine if complement in MCTP-treated serum can be activated via the alternative pathway. Serum containing various amounts of MCTP was exposed to zymosan and then tested for neutrophil activation in the neutrophil aggregation assay. The results of this study indicate that zymosan can activate complement in MCTP-treated serum leading to the generation of C5a via the alternative pathway.

There are a variety of compounds that are capable of inhibiting the classical complement pathway in vitro. These compounds include polypeptides, synthetic polyanions, polynucleotides, pyridinium sulfonylfluorides, benzamidines, guanidines, levopimaric acid derivatives, phenothiazines, phenylindamidines and other compounds (Asghar, 1984). Like MCTP, many are toxic and some are effective only in vitro (Asghar, 1984). These compounds act by a variety of mechanisms on the various constituents of the complement system. The mechanism by which MCTP causes the inhibitory effect is not known and will require more study.

H. Effect of Interventions that Alter Production or Metabolism of Toxic Oxygen Metabolites

There are increased numbers of alveolar macrophages and neutrophils in the lungs of rats treated with MCTP. When activated, these cells are capable of generating oxygen metabolites that can damage tissue. Whether reactive oxygen metabolites from these cells is important in the pathogenesis of lung injury due to MCTP is not known. Accordingly, rats were co-treated with the pneumotoxicant MCTP and with DF, DMSO or PEG-CAT.

1. Effect of DF on MCTP-induced pulmonary injury

At a dose of 20 mg/kg, DF decreased lung injury by 80% after systemic activation of complement by CVF (Ward et al., 1983). Similarly, at a dose of 40 mg/kg, DF protected against the increased vascular permeability in intradermal immune complex-mediated vascular injury (Fligiel et al., 1984). In the present study, DF was given at a dose of 150 mg/kg, twice daily. This dose was used because urinary iron excretion was markedly increased by this regimen without producing signs of intoxication in the rats. Doses of DF greater than 150 mg/kg, 2 times/day, did not proportionately increase urinary iron clearance, and the higher doses caused marked weight loss and other signs of intoxication. Rats receiving DF were not protected from injury due to MCTP. Thus, these results suggest that tissue iron available for chelation by DF does not play an important role in the pathogenesis of MCTP-induced pulmonary injury.

2. Effect of catalase on MCTP-induced pulmonary injury

The effect of PEG-coupled CAT was evaluated in rats treated with MCTP. When CAT is coupled with PEG, the circulating half-life of the CAT is markedly increased. Till et al. (1983) demonstrated that the half-life of uncoupled-CAT is only several minutes, whereas, the PEG-coupled CAT has a markedly increased circulating half-life on the order of several hours. In the

present study, the circulating half-life of the PEG-CAT was approximately 31 hours.

CAT protects against lung injury in several models of oxygen radical-dependent lung injury (Tate et al., 1982; Till et al., 1983, 1985; Ward et al., 1983, 1985). At a dose of 300 U/rat, PEG-CAT protected against the lung injury that occurs after systemic activation of complement (Ward et al., 1985) and from lung damage secondary to thermal injury (Till et al., 1985). In the present study, rats were treated with a single dose of MCTP and with daily injections of PEG-CAT (1000 U) to determine if PEG-CAT would decrease lung injury. The PEG-CAT did not decrease lung injury due to MCTP. In a separate study, rats were given a single injection of MCTP and a higher daily dose of PEG-CAT (7500 U per dose). The increased dose of PEG-CAT also did not decrease MCTP-induced lung damage. Thus, these data indicate that PEG-CAT does not protect against injury due to MCTP and suggest that intravascular generation of hydrogen peroxide does not play an important role in the lung injury due to MCTP.

3. Effect of DMSO on MCTP-induced pulmonary injury

DMSO protects against damage in several models of acute tissue injury that are oxygen radical-dependent (Tate et al., 1982; Ward et al., 1983, 1985; Fligiel et al., 1984; Till et al., 1985). DMSO, at a dose of 1.5 ml/kg, protected against lung injury that occurs after systemic activation of complement (Ward et al., 1985) and against the secondary lung damage that occurs after thermal injury (Till et al., 1985). In immune complex-mediated dermal vascular injury, DMSO treatment also protected against changes in vascular permeability, although higher DMSO doses are required (Fligiel et al., 1984). In the present study, rats were given a single dose of MCTP and daily injections of DMSO. DMSO did not protect from injury due to MCTP when rats received either 0.67 or 1.0 ml/kg DMSO three times per day. When a single injection of MCTP and a larger dose of

DMSO was given (1.3 ml/kg, 3x/day), rats had less sequestration of ^{125}I-BSA in the lungs than did controls, but the DMSO did not protect rats from MCTP-induced increases in lung weight. 1.3 ml/kg of DMSO also was toxic, since the DMF/DMSO-treated rats lost weight and showed other signs of intoxication. This may be important in interpretation of the results since MCT- and MCTP-treated rats maintained on a restricted diet that results in decreased weight gain also are partially protected against development of lung injury (Hayashi et al., 1979; Ganey et al., 1985). Thus, while there may be some uncertainty related to the interpretation of the effects of 1.3 ml/kg, 3x/day of DMSO on MCTP-induced pulmonary injury, the weight of the evidence is against the hypothesis of oxygen metabolite involvement. Thus, the lung injury due to MCTP seems to differ in mechanism from the damage occurring after i.v. CVF (Till et al., 1982; Ward et al., 1985), thermal injury (Till et al., 1983, 1985), intratracheal administration of phorbol myristate acetate (Johnson and Ward, 1982) or immune complex-mediated dermal injury (Fliegiel et al,. 1984), wherein treatment with DF, DMSO or PEG-CAT are effective in decreasing damage.

I. Effect of Diethylcarbamazine (DEC) on MCTP-induced Pulmonary Injury

DEC has antiinflammatory properties mediated perhaps through its ability to block synthesis of leukotrienes (LT) (Hawkins, 1979). DEC inhibits the production of LT from isolated mastocytoma cells in vitro (Mathews and Murphy, 1982), from guinea-pig isolated, perfused lung (Engineer et al., 1978), from guinea-pig chopped lung (Piper and Temple, 1981), from isolated, perfused rabbit lungs (Greenburg et al., 1984), and from isolated, perfused, hypoxic rat lungs (Morganroth et al., 1984). DEC is also effective in decreasing the inflammatory response in pulmonary hypertension and RVH in chronically hypoxic rats (Morganroth et al., 1984). DEC is thought to block LT synthesis by decreasing the activity

of LTA4 synthetase and perhaps other steps in the LT synthesis pathway (Mathews and Murphy, 1982; Bach, 1984).

Many of the changes that occur after MCTP might arise form the physiologic effects of LT. For example, LTB4 is a potent chemotaxin for neutrophils and may in part be responsible for increasing neutrophil number and activity (Samuelson, 1983) in the lungs. LTC4 and LTD4 increase vascular permeability, and LTD4 is a pulmonary vasoconstrictor (Yokochi et al., 1982). Neutrophils and other inflammatory cells are known to release LT (Orange et al., 1980; Ford-Hutchinson et al., 1980). Many inflammatory cells are present in the lungs of rats due to treatment with MCTP and may be a source of LT in lungs of rats exposed to MCTP. Indeed, LT concentrations are increased in bronchopulmonary lavage fluid and in lung homogenates of rats treated with MCT (Stenmark et al., 1985).

Pulmonary injury due to MCTP was assessed in this study by measuring release of LDH and protein into the pulmonary airway, the changes in relative lung weight, sequestration of ^{125}I-BSA in the lung and RVH. At 7 days after MCTP, DEC apparently protected against the development of injury since rats treated with MCTP/DEC had lower alveolar lavage fluid LDH activity and protein concentration compared to those rats receiving MCTP/saline. However, in rats allowed to survive until 14 days after treatment, there was no evidence that DEC protected against lung injury or the development of RVH. These results suggest that DEC delays the onset of pulmonary injury after MCTP, but that it does not prevent pulmonary damage or RVH.

These results with MCTP contrast with those reported by Stenmark et al. (1985) in which MCT-treated rats were protected by DEC from the pulmonary inflammatory response, RVH, and pulmonary hypertension due to MCT. This difference may be due to the fact that MCT requires metabolic activation by the mixed function oxidase (MFO) enzymes of the liver (Mattocks, 1968). Since DEC

is rapidly metabolized by the MFO system (Hawkins, 1979; Hlavaha, 1982), it is possible that the protection from MCT-induced injury was due to decreased hepatic metabolic activation of MCT to MCTP because of competition between MCT and DEC for MFO.

In the present study, the chemically synthesized active metabolite, MCTP, was given 3 days prior to the start of DEC. This method of administration allowed for exposure of rats to MCTP without any other drugs present to interfere with its initial effects. The DEC treatment was started at a point in the time-course of MCTP-induced pneumotoxicity before the onset of measurable lung injury. Thus, if DEC were an effective antiinflammatory agent in this model, the lung injury and RVH should have been lessened. These results, therefore, suggest that DEC interferes with an event, perhaps LT synthesis, early in the pathogenesis of MCTP-induced lung injury but does not afford complete protection from MCTP.

SUMMARY AND CONCLUSIONS

The first studies undertaken in this project were designed to characterize the cardiopulmonary injury induced by the pneumotoxicant, MCTP. After rats are given a single dose of MCTP, pulmonary vascular injury, pulmonary hypertension and right ventricular hypertrophy develops. These changes do not occur immediately after MCTP, but rather, take several days to develop. These results suggest that MCTP acts indirectly to produce injury by initiating a series of secondary events that led to pulmonary vascular injury and pulmonary hypertension.

The hypothesis that a cell-mediated immune response is involved in the cardiopulmonary effects of MCTP was evaluated by testing the effects of the immunosuppressants ALS and CyA on MCTP-induced pulmonary injury. Co-treatment of rats with MCTP and these agents did not reduce the lung injury. The role of cell-mediated immunity also was evaluated by examining the ability of lymphocytes adoptively transferred from MCTP-treated animals to produce lung injury or to alter the time course and/or severity of MCTP-induced damage. Lymphocytes from MCTP-treated donors did not cause pulmonary injury in recipients, nor did transferred cells alter the time-course or severity of lung injury in MCTP-treated donors. Thus, these results indicate that cell-mediated immune mechanisms are not involved in lung injury due to MCTP.

Studies were undertaken to evaluate the role of the complement system in MCTP-induced pulmonary injury. Examination of the complement system was of interest because complement is an integral part of a humoral immune response and its activation products are important inflammatory mediators. The possibility

that MCTP causes complement activation in vivo was tested by measuring complement activity in serum taken from MCTP-treated rats. Since the hemolytic complement assay may not detect complement activity in vivo, the neutrophil aggregation assay also was employed to determine if circulating complement activation products could be detected in serum of MCTP-treated rats. The role of complement also was evaluated by depleting rats of complement with purified CVF. MCTP treatment does not cause detectable complement activation in vivo and complement depletion does not protect rats from lung injury. Thus, these results indicate that complement does not play an important role in the development of major lung injury that occurs several days after treatment of rats with MCTP. MCTP also decreases serum hemolytic complement activity in vitro but does not interfere with zymosan-induced activation of complement. The mechanism by which MCTP depresses complement activity in vitro is unknown.

A series of experiments was undertaken to evaluate the effect of DF, DMSO and CAT on MCTP-induced pulmonary injury. These interventions all interfere with the generation of damaging oxygen metabolites, and each has been shown to protect against injury in other models of oxygen metabolite-dependent pulmonary damage. Lungs of rats treated with MCTP have increased numbers of inflammatory cells that may produce damaging oxygen metabolites capable of causing at least part of the injury due to MCTP. However, the results indicate that DF, DMSO and CAT do not decrease the injury caused by MCTP and suggest that toxic oxygen metabolites do not play an important role in the pathogenesis of MCTP-induced pneumotoxicity.

Diethylcarbamazine also was tested for its ability to decrease injury due to MCTP. Co-treatment of rats with MCTP and DEC resulted in a delay in the onset of MCTP-induced pulmonary injury but did not prevent the development of

pulmonary and cardiac changes. These results suggest that DEC interferes with an event, perhaps LT synthesis, early in the pathogenesis of MCTP-induced lung injury but does not afford complete protection from MCTP.

In conclusion, the role of immune effectors in MCTP-induced pulmonary injury were evaluated in this project. The results indicate that cell-mediated immune mechanisms, the complement system and the intravascular generation of toxic oxygen metabolites are not involved in the pathogenesis of the lung injury.

Even though these studies indicate that immune mechanisms are not involved in the injury, further study of this model should be pursued. The MCTP-treated rat is an excellent model for the study of pulmonary hypertension and also for the study of chronic pulmonary vascular injury. The mechanisms leading to pulmonary hypertension in man are still unknown, and treatment of those having the disease is largely unsuccessful.

Other pulmonary diseases, such as adult respiratory distress syndrome results in pulmonary vascular remodelling and pulmonary hypertension which is not preventable or reversible using currently available therapeutic techniques. Accordingly, if successful treatments are to be developed, therapy will have to be directed toward facilitating endothelial cell repair and interrupting vascular changes that occur after endothelial cell injury. Developing such treatment regimens will require greater understanding of the mechanisms that control vascular homeostasis and growth. The MCTP-treated rat is an excellent model for such research. Future MCTP research projects might involve the study of interactions between injured pulmonary endothelial cells and pulmonary blood vessels. In vitro studies using endothelial cell culture systems may help to identify how MCTP causes endothelial cell injury. Additionally, endothelial cell-smooth muscle culture systems could be employed to evaluate how MCTP-injured endothelial cells may alter smooth muscle cell growth and function. Such

systems, for example, could be employed to determine if chemically-induced endothelial cell injury leads to release of factors that stimulate smooth muscle cell growth. Such research might lead to a better understanding of these basic mechanisms and result in improved treatment of humans suffering from pulmonary hypertension and pulmonary vascular disease.

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