5-1989

Effects of Methylene Chloride on Immune Function in Mice and the In Vitro Effect of Methylene Chloride in Immunologic Assays

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EFFECTS OF METHYLENE CHLORIDE ON IMMUNE FUNCTION IN MICE AND

THE IN VITRO EFFECT OF METHYLENE CHLORIDE

IN IMMUNOLOGIC ASSAYS

by

Man-Ping Wang

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1989

ACKNOWLEDGEMENTS

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ABSTRACT

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in Immunologic Assays

by

Man-Ping Wang, Master of Science

Utah State University, 1989

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Department : Biology

A number of toxicities associated with methylene chloride have been found in both human subjects and mice. However, relatively few studies have probed immunotoxicities of methylene chloride. In order to examine possible immunotoxicities or immunomodulating effects of methylene chloride, several tests of cellular immune function were performed using both human in vitro studies and a mouse model.

Body weights and specific organ weights of thymus, spleen, liver, and kidney were normal in CD-1 mice given various concentrations of methylenechloride. However, a significantly reduced mitogenic response to phytohemagglutinin (PHA) and reduced interleukin-2 (IL-2) production was found in these methylene-chloride-treated mice. The findings in the mouse model provide additional evidence that immune suppression may be associated with exposure to methylene chloride.

Splenic mononuclear cells isolated from CD-1 mice were incubated with various concentrations of methylene chloride in vitro and investigated for blastogenic response to mitogen PHA and IL-2 production. The results show no significant difference between methylene-chloride-treated cells and the cells treated with growth media.

Peripheral blood mononuclear cells isolated from healthy donors were incubated with various concentrations of methylene chloride and tested for blastogenic activity, natural killer (NK) cell activity, and IL-2 production. The findings showed that the NK cell activity, the T-cell blastogenesis in response to PHA mitogen, and IL-2 production activity were not affected.

(60 Pages)

INTRODUCTION

Immunotoxicology is a study of the adverse effects on the immune system resulting from the action of xenobiotics (i. e., chemicals, drugs, and biologicals), which have a potential for long-term exposure and are persistent in the environment. Many studies have shown that exposure of rodents to classes of environmental chemicals at doses that are not toxic induce immune alteration and change host resistance to infection agents (e. g., bacteria and viruses) and neoplastic cells (1). Accidental exposure of human subjects to certain xenobiotics results in immunomodulation. For example, exposure of Michigan residents to dairy-farm products that were contaminated with polybrominated biphenyls (PBB) (2) and exposure of Taiwanese and Japanese to rice oil contaminated with polychlorinated biphenyl (PCB) (3) resulted in immune abnormalities.

The interaction of environmental chemicals or drugs with lymphoid tissue may alter immune function and result in six effects, which were reviewed by Dean et. al (1): 1. Immunosuppression: some chemicals, drugs, and metals (4) are agents that can suppress both cell-mediated immunity and humoral immunity. 2. Uncontrolled proliferation: some chemicals or their metabolites can bind to the genetic material (DNA) and lead to genetic alteration that may result in neoplastic development (5). 3. Decreased host surveillance against pathogens and neoplasm: an increased incidence of infectious disease and neoplasia have been frequently associated with immunodeficiency diseases and immunosuppressant therapy (6). Lymphocyte deficiency: deficiencies of the T cells are mainly associated with severe viral infections, intracellular pathogens, and fungi. Deficiencies of B cells are frequently associated with bacterial infections. 4. Allergy: industrial workers are exposed to many environment chemicals capable of inducing asthma, hypersensitivity, and contact dermatitis (7). 5. Autoimmunity: many

drugs and metals may haptenate self-protein and result in autoimmune diseases (8). Several mechanisms that may explain the induction of autoimmune disorders were reviewed by Theofilepoulos (9). Cross-reaction drugs with self-DNA or nuclear antigens and interference with the immunoregulatory function of suppressor T cells (10) are two possible mechanisms for autoimmune diseases. 6. Alteration of drug metabolism: inhibition of hepatic drug metabolism is a common consequence of pharmacological immunostimulating that so far has been reported with most immunomodulation agents (11). As pharmacological drugs are usually biotransformed in the liver to less active and more soluble metabolites by hepatic enzymes (12), any inhibition of hepatic enzymes, e.g., immuno-chemotherapeutic, is likely to cause a relative overdose and result in toxicity.

Halogenated hydrocarbons are industrially important agents used as solvents, chemicals, intermediates, and consumer products. Many of them are liver and kidney toxins in both animals and humans (13). Several chlorinated hydrocarbons are known to be carcinogenic. Vinyl chloride has been reported to be carcinogenic in man (14). Chloroform, carbon tetrachloride, and other chlorinated hydrocarbons are carcinogens (15). Carbon tetrachloride induces liver and adrenal tumors in mice (16) and neoplastic nodules in the liver of rats (16). Chloroform is known to cause hepatocellular carcinomas in mice and kidney tumors in male rats (16). Recently, many immunotoxicities associated with chlorinated hydrocarbons have been studied in experimental animals. (17). Alterations of various immune parameters have been noted following aliphatic-hydrocarbon exposure in laboratory animals (18, 19).

The present study investigated the effects of methylene chloride on the immune systems of man and of mice. Three approaches were employed to investigate the effects of methylene chloride on immune function. Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors and studied for NK cell activity, T-cell blastogenic activity, and IL-2 production. Splenic mononuclear cells were isolated from CD-1 mice

and studied for T-cell blastogenic activity and IL-2 production activity as in the human *in vitro* study. A murine model to study the effects of methylene chloride on immune function was developed by giving mice short-term exposure to methylene chloride and studying their lymphocytes for IL-2 production and blastogenic activity. The specific objectives of the study were to determine if human PBMC exposed *in vitro* to methylene chloride will have altered T-cell blastogenic responses, NK cell function, or IL-2 production; if murine splenic mononuclear cells exposed *in vitro* to methylene chloride will have altered T-cell blastogenic responses or IL-2 production activity; and if mice exposed *in vivo* to methylene chloride will have altered T-cell blastogenic responses, IL-2 production, or body and major organ (thymus, liver, spleen, and kidney) weights. It is hoped that the results will aid in better understanding the interactions between methylene chloride (a wide-spread environmental pollutant) and the immune system.

CHAPTER II

REVIEW OF THE LITERATURE

Description of Methylene Chloride

Methylene chloride (dichloromethane) is a halogenated aliphatic hydrocarbon that is produced by chlorinating either methane or methyl chloride, or by reduction of chloroform or carbon tetrachloride (20). The current major uses of methylene chloride in the United States are in paint removers, aerosol sprays, solvent degreasers, and in plastics processing (20). Methylene chloride is also used in the textile industry as a solvent for cellulose triacetate in rayon yarn manufacture, in the production of PVC (polyvinyl chloride) fiber, as a low-temperature extractant (e. g., edible fat, butter, and cocoa), in beer flavoring, and as a substitute for trichloroethylene for decaffeinating coffee. The demand of methylene chloride has grown in the past decade in paint removers and in solvent-degreasing operations, manufacturing of aerosol propellants, plastics, and photographic film. It is estimated that about 70,000 U.S. employees are exposed to a working environment containing methylene chloride in one year (20).

Methylene chloride is a cheap, nonflammable, colorless, low-boiling point, organic solvent with a boiling point and properties similar to chloroform and carbon tetrachloride. It can enter the body by inhalation of vapors, absorption of liquid, ingestion, skin and eye contact, or food and water contamination. Due to its high chemical stability, methylene chloride has become distributed throughout the biosphere. Recently, methylene chloride was recognized by the Environmental Protection Agency (EPA) as a priority pollutant.

Toxicities Associated with Methylene Chloride

Renal necrosis. A case of nephrotoxicity was reported to occur after inhalation of methylene chloride and manifested as acute renal failure, myoglobinuria,

hypocomplementemia, and liver-enzyme elevations (21). Serum enzymes were also elevated, including lactate dehydrogenase (LDH) and serum glutamate oxaloacetic transaminase (SGOT). Increased levels of creatine kinase and myoglobin in the urine also occurred after acute renal failure (22). With light microscopy, the cytoplasm of the tubular cells appeared degenerated and contained cellular debris. Both the proximal and distal tubules were affected. Under electron microscopy, the tubular cells were extremely electron lucent and in disarray. In some cells, the plasma membrane was greatly disrupted and devoid of cellular contents. The mitochondria were swollen and the intercristae spaces widely separated (21).

   Pulmonary injury. A case of pulmonary edema and pleural effusion resulting from methylene chloride inhalation was described by Buie et al. (23). Two cases of pulmonary parenchymal damage, one of which was fatal, were reported to have occurred following methylene chloride intoxication (24). Gerritsen and Buschmann (24) showed that phosgen, a combustion product of methylene chloride, damage the lung parenchyma and cause airway and alveolar edema (24). Methylene chloride forms hydrochloric acid in the presence of warmth and moisture (25). Hydrochloric acid may also be the agent responsible for the pulmonary parenchymal reaction (23).

   Central nervous system (CNS) damage. Toxicity of methylene chloride on the CNS was first described in 1936 (26) when Collier reported that workers in the paint industry in England suffered from headache, sedation, and confusion. Effects on the CNS are related to the anaesthetic properties of the solvent. Intermittent exposure of rats to dichloromethane for two weeks causes neurochemical changes, including signs of increased protein breakdown in the cerebrum (27).

   In man, certain brain areas are especially vulnerable to methylene chloride exposure, for example, the anterior parts of the cerebral cortex and the vermis posterior of the cerebellum (28). Briving et al. (29) observed that long-term exposure of Mongolian gerbils *(Meriones ungiculatus)* to dichloromethane leads to decreases

levels of glutamate, gamma-aminobutyric acid and phosphoethanolamine in the frontal cerebral cortex, while glutamine and gamma-aminobutyric acid are elevated in the posterior cerebellar vermis.

Some studies show that traumatic damage to a CNS tissue frequently causes hypertrophy or proliferation of astroglial cells   detected by immunohistochemical methods using specific antibodies to astroglial marker proteins (30). Rosengren et. al (31) found increased concentrations of astroglial marker proteins in the frontal cerebral cortex and the sensory motor cerebral cortex, but DNA concentration was significantly decreased in the hippocampus and in the cerebellar hemispheres after exposure to methylene chloride.

Carbon monoxide toxicity.   Carbon monoxide toxicity is caused by elevated blood levels of carboxyhemoglobin (COHb) (27). The mechanism of carbon monoxide toxicity is associated with carbon monoxide's high affinity for hemoglobin which is two hundred times higher than that of oxygen, and its interference with the release of oxygen from oxyhemoglobin present in the blood. Carbon monoxide poisoning can be a serious threat to the central nervous system (CNS) and myocardium, which are most sensitive to oxygen deprivation (32). Carbon monoxide is a serious hazard to patients with coronary artery disease since only a slight elevation in carboxyhemoglobin concentration necessitates greatly increased coronary blood flow to prevent ischemia (33). Studies found that methylene chloride is metabolized to carbon monoxide by human subjects (34), rats, and mice (35). Exposure of the experimental rat and human subject to $[^{14}C]$ methylene chloride ($^{14}CH_2Cl_2$) leads to the highest tissue uptakes of ^{14}C in the liver, kidney, and adrenal gland (34). After exposure to CH_2Cl_2, the concentration of blood carboxyhemoglobin is increased. Methylene chloride is released slowly from body tissues, resulting in carbon monoxide remaining in circulation for a prolonged period. The half-life of carboxyhemoglobin resulting from methylene chloride is much higher than that caused by carbon monoxide inhalation. Hence, the carboxyhemoglobin

concentration is higher after inhaling methylene chloride. Therefore, cardiovascular compromise and cerebral damage by inducing hypoxia (36) from elevated carboxyhemoglobin may be the result of dichloromethane exposure.

Mutagenicity and carcinogenicity. The potential carcinogenicity of some halogenated aliphatic hydrocarbons is related to their metabolic activation by the hepatic microsomal monooxygenase system (37, 38). Methylene chloride has been reported to be highly mutagenic in some short-term tests using microorganisms, including *Salmonella typhimurium* strains TA 1535 (39) and TA 100 (40). In these assays methylene chloride can cause base-pairing substitutions. Methylene chloride also has been shown to be mutagenic in the *Salmonella typhimurium* TA98 (39, 40), *Saccharomyces cerevisial* (38), and baby hamster kidney (BHK21) cell transformation assays. Thilagar and Kumaroo (41) reported that methylene chloride can induce the chromosome frameshift mutations in chinese hamster ovary cells (CHO). Also, Fishbein (20) has found that methylene chloride can induce tumors when injected into immunosuppressed mice. Burek et al. (42) reported a two-year inhalation study with dichloromethane in rats and hamsters at different levels. In this study, rats of both sexes exhibited an increased number of benign mammary tumors and an increased incidence of salivary gland sarcomas in males. Methylene chloride also has been evaluated for its ability to induce micronucleated polychromatic erythrocytes (MPEs) in the bone marrow of treated mice (43). A recent report from Andrew et al. (44) indicates that methylene chloride is a liver and lung carcinogen in the mouse.

Possible Mechanism of Toxicities Associated with

Methylene Chloride Exposure

Much work has been done to determine the metabolic pathways involved in methylene chloride biotransformation to elucidate possible mechanisms of carbon monoxide production and potentially reactive intermediates that may be involved in the mutagenic effects. Both in vivo and in vitro studies have shown that dihalomethanes are metabolized by two major pathways (45): hepatic microsomal cytochrome P-450 dependent mixed function oxidase system, and glutathione S-transferase dependent pathways in hepatic cytosol. These two pathways have been investigated in detail by Anders et al. (46).

The cytochrome P-450 containing monooxygenase is localized in the smooth endoplasmic reticulum, a complex network of membranes that is continuous with the outer nuclear membrane. When a cell is homogenized, the smooth endoplasmic reticulum disintegrates to form intracellular organelles known as microsomes.

In the oxidative pathway methylene chloride may be metabolized in the hepatic microsomal fraction requiring both molecular oxygen and NADPH (45). Formation of CO from methylene chloride is mediated by the microsomal cytochrome P-450 monooxygenase system (47). The proposed reaction mechanism for the metabolism of CH_2Cl_2 to CO is shown below (7, 47).

$$
\begin{array}{ccc}
\overset{\displaystyle H}{\underset{\displaystyle H}{Cl{-}C{-}Cl}}
& \xrightarrow[\text{Cytochrome P-450}]{\text{NADPH, [O]}}
& \overset{\displaystyle H}{\underset{\displaystyle OH}{Cl{-}C{-}Cl}}
\end{array}
\xrightarrow[\text{-X}^-]{\text{-H}^+}
\underset{\displaystyle O}{Cl{-}C{-}H}
$$

(A):

(1)
$$\xrightarrow[-X^-]{-H^+} \quad C=O$$

(2)
$$\xrightarrow[\text{RNuH}]{\text{Bio. Nuc. (N, O, S)}} \quad RNu-\underset{\underset{O}{\|}}{C}-H \quad \text{or}$$

(B):

$$Cl-\underset{\underset{O}{\|}}{C}-H \xrightarrow{GSH} \underset{\underset{H\ H}{|\ |}}{G\text{-}S\text{-}C}=O \xrightarrow{-H^+} \underset{\underset{H}{|}}{G\text{-}S\text{-}C}=O \xrightarrow{GSH} CO_2$$
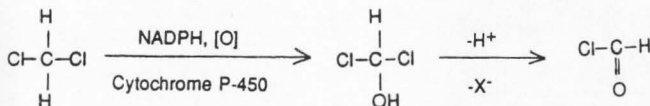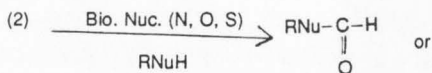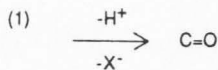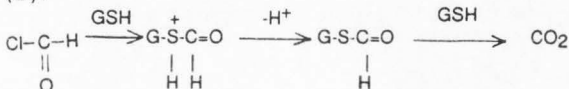
N: Nitrogen

O: Oxygen

S: Sulfur

RNu: Biological Nucleophiles (i. e. DNA, RNA and proteins)

The above reaction shows an initial oxygen insertion reaction to produce a hydroxydichloromethane intermediate ($HOCHCl_2$) which would be rearranged to produce a formyl chloride intermediate. The formyl halide intermediate may be either decomposed spontaneously to carbon monoxide or it can acylate tissue nucleophiles. Due to the reaction, dichloromethane becomes covalently bound to both microsomal proteins and lipids (46, 47).

The glutathione S-transferase (GST) pathway produces CO_2 but no CO (45). The proposed reaction mechanism is shown below:

$$Cl-\underset{\underset{H}{|}}{\overset{\overset{H}{|}}{C}}-H \xrightarrow[\text{GSH} \quad -X-]{\text{Cytosol} \ -H +} GS\text{-}CH_2\text{-}OH \xrightarrow{NAD+} \underset{\underset{H}{|}}{GS\text{-}C}=O \xrightarrow{HCOOH} CO_2$$
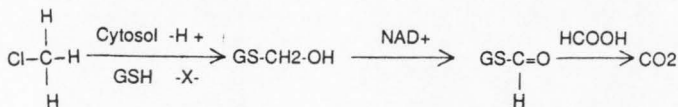
Reactive, potentially toxic intermediates are formed in both pathways (47). Either metabolic pathway can produce carbon dioxide, but only the cyt P-450 pathway yields carbon monoxide.

Review of the Immune System And Its Functions

Recently, immunologists have become concerned about the immune system as a target organ for toxicity (48). One possible reason for this is that toxic chemicals may alter immunologic function and increase disease susceptibility (48). The main role of immune system is to sustain host defense mechanism and to maintain homeostasis. The immune system can be divided into two parts, central lymphoid organs and peripheral lymphoid organs. The central lymphoid tissues include thymus and bone marrow (bursa of fabricius). The peripheral lymphoid tissues consist of spleen, lymph nodes, tonsils, and Peyer's patches. All type of blood cells originate from a pluripotent stem cell found in the yolk sac and fetal liver during fetal development and in the bone marrow, thymus, and spleen of the adult (49, 50). In most mammals, bone marrow is the major source of red cells, platelets, and granulocytes as well as lymphocytes and monocytes. The pluripotent stem cell differentiates into another type of cell called the progenitor cell. One type of progenitor cells passes through the thymus, becomes mature and develops into the thymus-derived T-lymphocyte (T-cell). (51) Mature T lymphocytes are found in the thymic dependent areas of spleen and lymph nodes and comprise about 55-74% of total peripheral blood lymphocytes. The bursa of fabricius (birds) or bone marrow (other species) processes a different type of progenitor cell which mature into the mature B-lymphocyte (B-cell). Mature B lymphocytes are found in the B-cell dependent areas of spleen and lymph nodes and represent approximately 25-40% of peripheral blood lymphocytes (51). Following activation B lymphocytes proliferate and

differentiate into antibody-producing cells and plasma cells. T and B cells can be distinguished by their cell surface markers (52).

When foreign substances (viruses, bacteria, foreign cells, etc.) i. e. antigens, come into the body, they bind to the antigen-presenting cell (macrophage) first. This antigen-presenting cell stimulates the T-lymphocyte to become activated and divide to expand the number of T-helper cells (T_H). These T-helper cells activate antibody responses, delayed-type hypersensitivity (T_{DTH}), suppressor T-cell (T_S), and cytotoxic T-cell. Cytotoxic T cells participate directly as killer cells against virally infected host cells, malignant cells, and foreign tissues.

The mediators of various immune responses are soluble factors, now commonly known as lymphokines, produced by one or another type of cell of the immune system. Following binding of the T-cell to the antigen-presenting cell, an unknown factor from the T-cell is produced. This soluble factor subsequently induces the antigen-presenting cell to produce a small protein with a molecular weight of approximately 15,000 daltons called interleukin-1 also known as lymphocyte activating factor (LAF). Interleukin-1 stimulates the T-cell to release interleukin-2 which drives antigen activated cells into proliferation. In mice, interleukin-2 has a molecular weight of 20,000-30,000 daltons. Interleukin-2 production requires two signals; exposure to mitogen (such as ConA or PHA), or antigen presented on the surface of macrophage and exposure to interleukin-1. Interleukin-2, formerly known as the T-cell growth factor (TCGF) is a strong mitogen for both the T-helper cell and the T-cytotoxic cell, providing these cells have had prior exposure to an antigen. The antigen also can bind to the B-lymphocyte and stimulates one or more specific B cells to clone and produce specific antibody.

In summary, immunity is mediated by B lymphocytes that are responsible for humoral immunity, T lymphocytes that are responsible for cell-mediated immunity, and macrophages that are involved in antigen recognition and phagocytosis. The cytotoxic T-

cell participates directly as a killer cell against viral infected cells, foreign tissues, etc.. Macrophages also play a central role in cell-mediated immunity. They are involved in the initiation of responses as antigen-presenting cells, in regulatory functions, and participate as effector cells in inflammatory and tumor cells.

Assessing T and B Cell Function

Antigen-induced lymphocyte proliferation can be detected in vitro by cultivating lymphoid cells with specific mitogens. Mitogens are defined as lectin proteins derived generally from leguminous plants and bacteria. After binding to the cell surface determinants, mitogens polyclonally stimulate lymphocytes into a lymphoblast through DNA synthesis, blast transformation, and result in division of lymphocytes. Mitogen stimulation of lymphocytes in vitro is believed to reflect the ability of lymphocytes to respond to a foreign antigen in vivo.

T and B cells are activated by different mitogens (53) which can be used as a gross probe for the integrity of T-cell and B-cell function (54). Phytohemagglutinin (PHA), derived from the red kidney bean Phaseolus vulgaris, primarily stimulates both human and mouse T-helper cells and has some effect on T-cytotoxic and T-suppressor cells. Concanavalin A (ConA), a lectin derived from the jack bean, Canavalia ensiformis, stimulates both murine and human T-helper cells, T-suppressor and T-cytotoxic cells. Pokeweed mitogen (PWM), a lectin derived from pokeweed plant, Phytolacca americana, stimulates both human T and B cells. Lipopolysaccharide (LPS), known as endotoxin A, is a protein derived from a variety of gram negative bacteria which stimulates mouse B cells but not human B cells.

T cells carry out functions of immune regulation by participating as amplifying "helper" cells in antibody response, mixed lymphocyte reaction, delayed-type hypersensitivity, and activation of cytotoxic T cells (48). The essence of the immune

response is to activate a small number of precursor cells and expand them into a population of functional effector cells. Lymphocyte proliferation is strongly correlated with DNA replication, protein synthesis, and production of antibodies, lymphokines, and enzymes. Some chemicals can inhibit these macromolecular synthesis. Inhibitors of protein synthesis are also known as immunosuppresants (55).

Since mitosis involves the production of new DNA, the amount of proliferation can be quantitated by measuring the amount of DNA synthesis (56). Measuring DNA synthesis can also determine the ability of lymphocytes to respond to a foreign antigen by cell division and differentiation into blast cells.

Natural Killer (NK) Cells

Some lymphocytes having neither B nor T cell surface markers are called "null cells". These cells, active in immune cytotoxic functions, are the NK cells, and killer cells, the later mediating the antibody-dependent-cell-mediated-cytotoxicity (ADCC) (57). The mechanisms of natural killing are yet unknown. However, it has been suggested that there are three distinct phases directly binding to the target cells, a Ca++ dependent phase, involving vesicular secretion that modifies the target cells so that it is 'programmed" for lysis (58), and a late phase during which the "programmed" cell undergoes lysis.

The NK cells are cytotoxic against a broad spectrum of target cells in vitro and exercise this function without prior immunization (59). The spontaneous natural cytotoxicity of the NK cells is an important mechanism in resistance to tumor and viral diseases. NK cells appear to be one of the first-line defenses against viral or chemical transformed cells. NK cells are active in tumor surveillance (60) and can control viral infections (61). The NK cell constitutes about 5% of the peripheral blood lymphocytes and splenic lymphocytes in man and other animals. Generally, NK cells can lyse both

allogenic and syngeneic tumor cells (62, 63) and have no requirement for MHC restriction. The NK activity is greatly enhanced by interferon or by anything that can induce interferon production such as chemical, and virus infection.

In Vitro Assessment of NK Cell Activity

NK activity can be assayed based on the diffusion of radioactive chromate $(^{51}Cr_2O_4{}^{-2})$ ion through the cell membrane (64). The susceptible tumor cells are first labeled with radioactive chromium and then incubated with NK cells. Thus, ^{51}Cr is released from a labeled target cell into the supernatant fluid because the cell membrane is damaged to allow the efflux of intracellular molecules. Killing activity is measured by the amount of ^{51}Cr released from lysed cells. The ^{51}Cr release assay represents a simple, rapid, and quantitative test for cell-mediated cytolysis.

Possible Mechanisms of Immune Toxicity

Due to the complexity of the immune system, only a limited number of studies have investigated interaction between chemicals and immune functions. In many cases, the active sites of immunotoxicity are not well understood because the chemical interactions are difficult to investigate (4). Exposure to a chemical may cause a variety of reactions by enhancing or suppressing host immune response or it may have no effect on the immune system at doses that induce toxicity in other organs (4). So it is important in any toxicological study to consider the immune system as a whole (65). The immune system of mice is understood very well and is often used in immunology and toxicology. The use of an animal model provides useful information about the role of chemicals in altering immune function. The animal model can also be compared with results of human data and allow better understanding of the basic immunotoxicity in alteration of the immune functions by methylene chloride.

It is now well known that toxicity of chemicals is due to the formation of reactive intermediates during the metabolism (7). These intermediates might cause some serious toxicities. The roles of toxins on various types of toxicity are (53) alteration of biological factors (such as ions and cofactors), enzymes, hormones, and pharmacological receptors that will cause the acute or chronic toxicity symptoms, such as hypersensitivity, neurotoxicity (66), and blood disorders (67); the reactive intermediates with body macromolecules might cause necrosis followed by cell death (68); chemicals or their metabolites with genetic material might lead to genetic alterations such as mutagenesis and carcinogenesis (14, 15) and many carcinogens are also immunosuppressive (69).

CHAPTER III

MATERIALS AND METHODS

Experimental Design

The present study investigated the effects of methylene chloride on the function of immune cells, in particular cell-mediated immunity. The experiments were performed on spleen cells of mice (CD-1 strain) following both in vivo and in vitro treatment with methylene chloride. Also studied were the peripheral blood mononuclear cells from healthy male volunteers following in vitro exposure to methylene chloride. The immune parameters analyzed were T cell blastogenic responses to mitogen (PHA), IL-2 production, and NK cell activity against the tumor cell line K562.

Procedure of Assay Methods

Experimental animals. Young adult male mice of inbred strain CD-1 (Charles River Laboratory, CA) were obtained at 4-6 weeks old, and acclimatized for 2 days prior to experimentation. The mice were randomly assigned to 4 experimental groups, each with 5 mice per treatment. The mice were fed a routine lab diet and water ad libitum.

Dosage levels and mode of administration. The solvent control and solvent for methylene chloride was corn oil. All dosing solutions were administered at volume of 0.1 ml/10 g body weight (10 ml/kg). Methylene chloride was administered by gavage to groups of mice at dose levels of 0, 100, 500, and 2,500 mg/kg. Control mice were fed the corn oil (10 ml/kg) only (43). The mice were killed with CO_2 anesthesia at 15, 23, and 30 days after exposure. At the time of sacrifice, animals and the major organs (e. g., liver, thymus, spleen, and kidney) were weighed and spleens were studied for immune function described below.

Splenic mononuclear cells. Spleens were put into a petri dish immediately after the mice were killed and 15 ml of mouse RPMI-1640 were added. The RPMI-1640 was supplemented with 1% sodium pyruvate, 1% 2-mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin (medium). Spleens were minced and a stainless wire mesh screen was placed over them. A plastic barrel from the syringe was used to force the cells through the screen and the cells were transferred to 15 ml centrifuge tubes (25310, Corning Glass Ware, Corning, NY). The dissociated spleen cells were washed twice with 10 ml medium at 200 x g for 5 min. After a second wash, 9 ml of double distilled water was added to the cell pellet to lyse the red blood cells. After 10-15 seconds of mixing, 1 ml of 10 times concentrated PBS was added to the tube to stop the reaction. The cell debris was removed by centrifuging at 200 x g for 5 min and the dissociated spleen cells were resuspended in 5-10 ml medium.

Separation of peripheral blood mononuclear cells. Thirty-five to fifty ml of freshly drawn blood from healthy male volunteers were drawn into syringes which contained 10 units of preservative-free heparin (Sigma Chemical Co., St. Louis, MO), for every 10 ml blood. Plasma was removed by centrifugation (CU 5000; Damon/IEC Division, Needham Hts., MA) at 500 x g for 15 min and about 7 ml of the remaining cells were layered over 3 ml of Ficoll Histopaque-1077 gradient (Sigma) in 15 ml plastic conical tubes (Corning), and centrifuged at 400 x g for 20 min. The PBMC, obtained from the gradient interface, were suspended in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) that was supplemented with 100 units penicillin and 100μ g streptomycin (GIBCO) per ml of medium, and centrifuged at 300 x g for 10 min. The supernatant was decanted and the PBMC were resuspended in medium and centrifuged at 200 x g for 5 min. After the supernatant was decanted, the cells were adjusted to a concentration of 5×10^6 in RPMI-1640 medium.

Exposure to methylene chloride (for both human and mouse in vitro studies). PBMC or murine splenic cells were incubated with different concentrations of methylene

chloride (EM industries Inc., Gibbstown, NJ), which were dissolved in 1% dimethyl sulfoxide (DMSO) and diluted in RPMI-1640. Due to its high volatility, methylene chloride preparations were stored at $4^{o}C$. The PBMC cells were incubated in 24-well flat-bottomed culture plates for 3, 5, and 7, days following exposure to various concentrations of methylene chloride including: 0 (control), 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} M. The splenic cells were incubated with methylene chloride for 6 hours in sealed tubes or plates.

Viability of cells. The number (%) of viable lymphocytes were examined by staining the cells with trypan blue (0.2%). Viable cells excluded the dye while dead cells took up the dye. The percentage of viable cells = (number of viable cells / number of viable cells + number of dead cells) x 100%

Lymphocyte blastogenesis assay. This assay was carried out as described by Oppenheim and Schecter (70). Cultures, 1 x 10^{6} PBMC/ml, were established in triplicate in 96-well flat bottom microculture plates (25860, Corning) and phytohemagglutinin (PHA) (GIBCO), 1, 0.5, and 0.25% of stock solution was added giving a total volume of 0.2 ml per well. PBMC incubated in RPMI-1640 containing 20% fetal calf serum (FCS) (Hyclone, Logan, UT) without PHA were used for control cultures which received the same volume and cell numbers as the wells containing PHA. The cultures were incubated for 3 days at $37^{o}C$ in a humidified atmosphere of 5% CO_2 in air. Twelve hours before harvesting, 0.5 μ Ci of Methyl-$[^{3}H]$thymidine (New England Nuclear, Boston, MA), having a specific activity of 2 Ci/mmol, was added to each well. At the end of the culture period, cells were harvested onto glass fiber filters (Skatron Inc., Sterling, VA) with an semi-automated Titertek harvester (FLOW Laboratories, Inglewook, CA). The filters were dried at $60^{o}C$ for at least 30 minutes and the filter discs which contained the cells were removed and placed into plastic counting vials (Skatron). To each vial was added 2 ml of scintillation fluid SCINTIVERSE (Fisher Scientific, Fair Lawn, NJ). Radioactivity of samples was determined in a scintillation

spectrometer (Parkard Tri Carb. 1500; Packard Instruments, Downers Grove, IL). The murine lymphocyte responses to the mitogen PHA was assayed by culturing 5×10^5 splenic cells with various concentrations of PHA in flat bottom 96 well tissue culture plates. Twenty-four hours before harvest, tritiated-thymidine was added to the cultures and cells were harvested and radioactivity determined as in the human blastogenesis assay.

Lymphocyte IL-2 production assay. The IL-2 production assay was carried out as described by Gills et al. (71). The interleukin-2 dependent HT-2 cell line was maintained in medium made up of 60% by volume RPMI-1640 supplemented with 2% FCS, penicillin and streptomycin (GIBCO) and 40% supernatant from rat spleen cells as the source of IL-2 prepared in advance by the stimulation of splenic cells in cultures (which were incubated in RPMI-1640 containing Con A at a concentration of 1 μg/ml). Methylene chloride-treated lymphocytes were obtained as described before and incubated in 4 ml culture tubes (American Scientific Products) at a concentration of 2×10^6/ml in 2 ml of RPMI-1640 containing 1% PHA, 5% FCS, 1% 2-mercaptoethanol and sodium pyruvate for 48 hr in humidified atmosphere of 5% CO_2 in air at 37°C. The controls were incubated with same cell numbers and medium without PHA. After 48 hr, the cells were centrifuged at 400 x g for 10 min. The supernatant was saved to test for the biological activity of IL-2.

IL-2 activity was determined in a bioassay based on the IL-2 dependent HT-2 cell proliferation. HT-2 cells were washed and resuspended at a concentration of 4×10^4/ml in RPMI-1640 containing 2% FCS, penicillin, streptomycin, and 1% 2-mercaptoethanol. Each time an unknown sample was assayed, a reference standard of 50 units of human recombinant IL-2 (Genzyme, Boston, MA) in serial two-fold dilutions was used as a positive control. The testing of culture supernatants were carried out in two-fold serial dilutions in RPMI-1640 and prepared in triplicate wells (0.1 ml/well) of a 96-well flat-bottomed microplate (25860, Corning). A volume of 0.1 ml of the

HT-2 cell suspension was added to each well and the cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hr. After this incubation period, 0.4 μ Ci ^{3}H-thymidine (New England Nuclear) was added to each well and cultured for an additional 4 h. The cells were harvested and determined as in lymphocyte blastogenesis assay described before.

NK cell assay (for human study only). The details of the chromium release for determining natural cell killing activity have been described by Warren et al. (72). Human leukemia K-562 cells (ATCC, Rockville, MD) were cultured in RPMI-1640 containing 10% FCS at 37°C, 5% CO_2. The cells were centrifuged at 200 x g for 5 min and incubated at 37°C with 0.1 ml of [51]Cr (sodium chromate, ca. 2 mCi/mmole) for at least 1 h. Methylene chloride treated PBMC (effector cells) were washed one time in RPMI-1640 and resuspended at a concentration of 5 x 10^6/ml in medium supplemented with 20% FCS. A volume of 0.1 ml of PBMC was placed in triplicate wells of a 96-well round-bottomed microplate (25850, Corning) for a 50:1 effector to target cell ratio. In addition, two-fold dilutions of the PBMC were carried out in 20% FCS, giving effector to target cell ratios of 25:1, 12:1 and 6:1. Target cells were incubated in medium with 20% FCS for determination of spontaneous release and maximal release was determined by incubated target cells in 0.5% saponin (Sigma).

After 1 h of incubation, the ^{51}Cr-labeled target cells were washed 3 times in cold medium and resuspended at a concentration of 1 x 10^5/ml in medium. One-tenth ml of the target cell suspension was added to each well and the plates were incubated for 4 h at 37°C in 5% CO_2. Aliquots of 0.1 ml were pipetted from each well into 5 ml plastic vials (American Scientific Products, McGaw Park, IL) and radioactivity was determined with a Packard Model 3320 Tri-Carb Scintillation Spectrometer (Packard Instruments). Percent ^{51}Cr release was calculated by the following formula:

$$\% \text{ release} = \frac{\text{Experimental release - Spontaneous release}}{\text{Maximal release - Spontaneous release}} \times 100$$

Statistical analysis. Data were pooled from triplicate samples and analyzed using Student's t-test and the least significant difference test with a 95% confidence interval for a normal distribution. The observed relative enhancement or suppression of activity for the human natural killer cell assay, blastogenesis assay and IL-2 production are presented in graphic form. Error bars on graphs are representative of the standard error of the mean (SEM).

Results from the assay for IL-2 were derived using a human recombinant interleukin-2 standard curve. This was done by plotting known recombinant IL-2 activity (units/ml) against counts per minute of [^{3}H]-thymidine. The values of the unknown samples were extrapolated from the standard curve of recombinant IL-2, to obtain the concentration of IL-2 activity in the unknown samples. One unit was defined as half the amount of IL-2 activity that would stimulate 50% of maximal total incorporation. The results were then analyzed using the t-test mentioned above.

CHAPTER IV

RESULTS

Murine In Vivo Studies

Body and specific organ mass. Toxicological manifestations in the immune system following xenobiotic exposure in experimental animals may appear as changes in lymphoid organ mass. Therefore, it is essential in immunotoxicity evaluation to weigh the thymus and spleen (1). In general, the animals gained or maintained their mass during the experiments. The body mass of mice exposed to various levels of methylene chloride are given in Table I. No significant difference between mice given methylene chloride and corn oil (solvent) alone was seen with the exception of mice receiving 2,500 mg/kg methylene chloride for 15 days. Specific organ mass on the basis of g/g body mass for thymus and spleen were variable and no significant differences were found between corn oil and methylene chloride treated mice (Table II). The mass of liver was not significantly different between mice given methylene chloride as compared to mice given corn oil only (Table II). In general, no changes in the appearance of these organs were seen in methylene chloride treated mice.

Lymphocyte blastogenesis responses. Responses of spleen cells to several concentrations of the T-cell mitogen PHA, following in vivo administration of 100, 500, and 2,500 μ M of methylene chloride for 15, 23, and 30 days are shown in figure 1, 2, and 3, respectively. The methlene-chloride treated mice had statistically significant ($p < 0.05$) lower blastogenic activity than the corn oil-treated group .

IL-2 production activity. In figure 4 is shown the human recombinant IL-2 standard curve that was represented by plotting counts per minute (CPM) against IL-2 activity (units/ml). Splenic cells of methylene chloride treated mice exhibited a decreased level of IL-2 production as compared to control mice. This alteration in IL-2

TABLE I

Animal mass (g) after methylene chloride exposure

Treatment [a]	Diluent	100	500	2500
Day 0 [b]	20.3	20.7	21.0	20.0
	0.8 [c]	1.1	1.0	1.0
Day 8	23.0	23.5	23.2	21.1
	2.5	1.0	1.0	1.1
Day 15	26.3	25.8	24.8	23.0*
	1.3	1.7	1.0	1.4
Day 23	27.0	26.5	26.3	24.8
	1.0	1.9	0.6	2.6
Day 30	28.2	27.4	27.1	26.0
	0.7	1.5	0.7	1.1

a. Animals were administered by gavage daily with corn oil (diluent) or methylene chloride at doses of 100, 500, or 2,500 mg/kg for 30 days.

b. Mean mass at the beginning of each week and at the time of sacrifice. N=5 for each group.

c. Standard deviation of the mean.

* Significantly decreased ($p < 0.05$).

TABLE II

Specific organ mass (g) after methylene chloride exposure

Treatment[a] :	Diluent	100	500	2500
Mice per Treatment	6	7	7	7
Thymus mass [b]	2.33	3.54	3.36	3.14
	0.58 [c]	0.37	0.81	0.96
Liver mass [d]	68.0	69.2	72.4	73.0
	7.5	6.1	7.3	49
Spleen mass [e]	6.29	5.86	6.21	5.84
	1.03	0.84	0.59	0.61
Kidney mass [f]	27.8	27.1	24.1	23.7
	9.6	5.9	6.5	10.3

a. Mice were administered by gavage daily with diluent (corn oil) alone or methylene chloride at doses of 100, 500, 2,500 mg/kg for 30 days. Parameters were studied at time of sacrifice.

b. Mean thymus mass in units of g/1000g body mass.

c. Standard deviation of the mean.

d. Mean spleen mass in units of g/1000g body mass.

e. Mean liver mass in units of g/1000g body mass.

f. Mean kidney mass in units of g/1000g body mass.

15-days of treatment
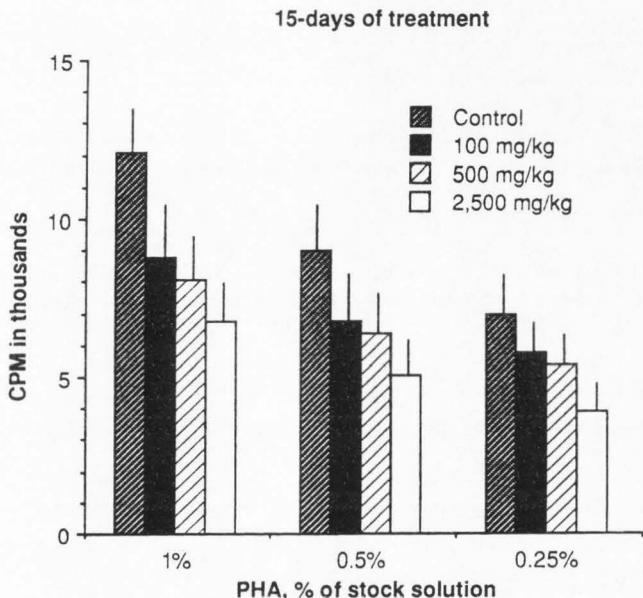
Figure 1. Effect of mice in vivo exposure to methylene chloride on [3H] thymidine uptake in lymphocytes stimulated with various concentrations of phytohemagglutinin (PHA). Values are expressed as mean incorporation of counts per minute of tritiated thymidine and standard error of the mean. PHA responses of CD-1 mice splenic cells treated with various concentrations of methylene chloride for 15 days. A significantly decreased mitogenic activity was found at each PHA concentration (p < 0.05). N=5.
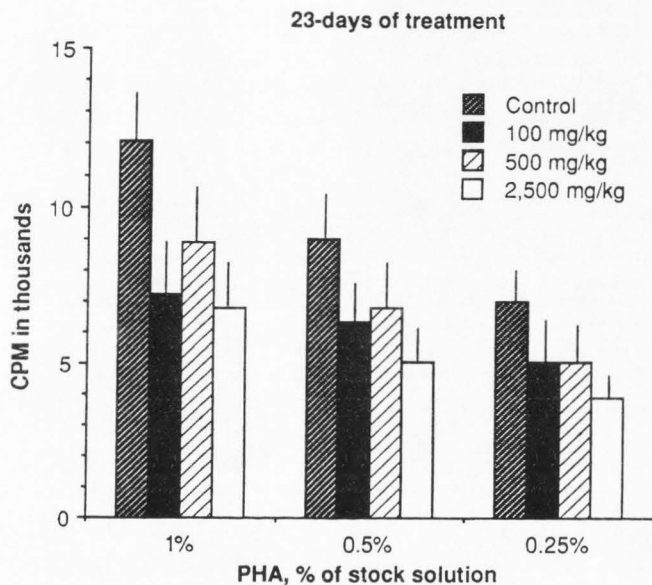
Figure 2. Effect of mice *in vivo* exposure to methylene chloride on [³H] thymidine uptake in lymphocytes stimulated with various concentrations of phytohemagglutinin (PHA). Values are expressed as mean incorporation of counts per minute of tritiated thymidine and standard error of the mean. PHA responses of CD-1 mice splenic cells treated with various concentrations of methylene chloride for 23 days. A significantly decreased mitogenic activity was found at each PHA concentration ($p < 0.05$). N=5.
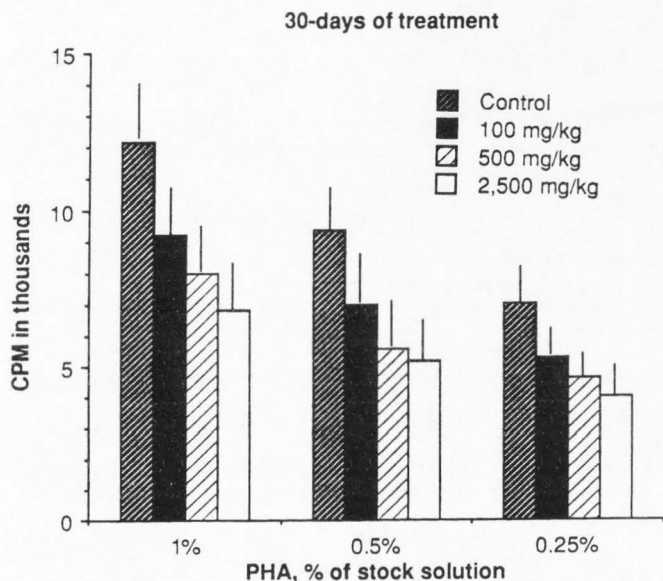
Figure 3. Effect of mice in vivo exposure to methylene chloride on [³H]
thymidine uptake in lymphocytes stimulated with various concentrations of
phytohemagglutinin (PHA). Values are expressed as mean incorporation of counts per
minute of tritiated thymidine and standard error of the mean. PHA responses of CD-1
mice splenic cells treated with various concentrations of methylene chloride for 30
days. A significantly decreased mitogenic activity was found at each PHA
concentration (p < 0.05). N=5.
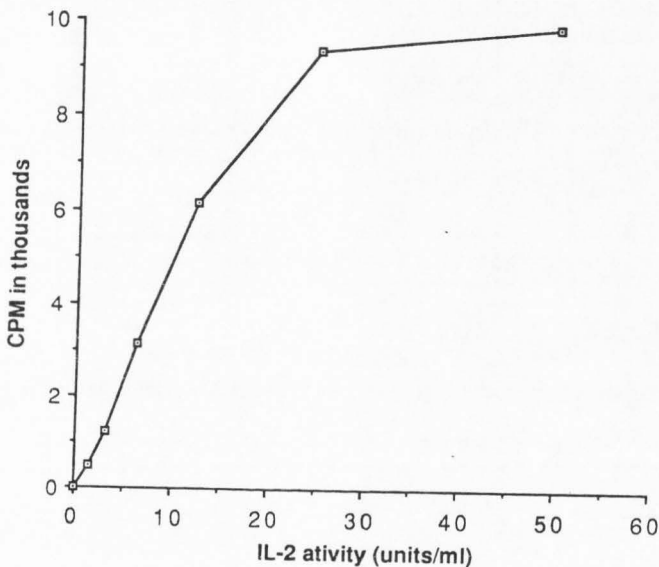
Figure 4. Human recombinant IL-2 standard curve. The proliferative response of HT-2 cells was used to analyze the unknown samples by comparing them with the IL-2 standard curve. Counts per minute (CPM) was plotted against IL-2 activity which was represented as units/ml. Human recombinant IL-2 (50 units/ml) was studied in serial two fold dilutions. To each well was added 0.1 ml of HT-2 cells at 4×10^4/ml in medium. The plate was incubated for 24 hours and during last four hours the cells were pulsed with tritiated thymidine and the incorporation of counts per minute of $[^3H]$-thymidine were counted with a liquid scintillation counter.

significant (p < 0.05) for mice receiving 100, 500, and 2,500 μM methylene chloride for 15, 23, and 30 days. The results are shown in figure 5.

Murine In Vitro Studies

Lymphocyte blastogenesis response. Responses of splenic cells to several concentrations of the T cell mitogen PHA are shown in figures 6. No significant differences were seen between responses of splenic cells that were incubated in RPMI-1640 (diluent) and of those that were treated with methylene chloride for 6 hours.

IL-2 production activity. In figure 7 is the effect of in vitro exposure to methylene chloride on IL-2 production of murine splenic cells. Splenic cells were incubated with various concentrations of methylene chloride for 6 hours. Values were expressed as IL-2 activity (units/ml). No significant differences were seen between responses of splenic cells that were treated with methylene chloride and those that were treated with RPMI-1640 (diluent).

Human In Vitro Studies

Lymphocyte blastogenesis response. Responses of PBMC to several concentrations to the T cell mitogen PHA are shown in figures 8, 9 and 10 respectively. No significant differences were seen between responses of PBMC that were incubated in RPMI and of those that were treated with methylene chloride for 3, 5, and 7 days.

NK cell activity. In figure 11, 12 and 13 are the results of the NK assay comparing PBMC of control group and PBMC treated with various concentrations of methylene chloride. Although there seems to be a trend of higher NK activity by cells from treated PBMC, the small difference in mean ^{51}Cr-released at four effector to target cell ratios was not significant.
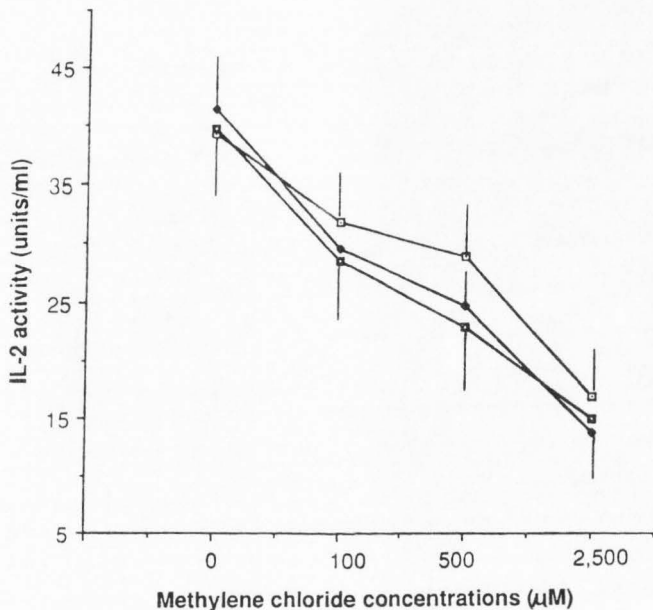
Figure 5. Effect of in vivo exposure to methylene chloride for 15 days (—■—) , 23 days (—●—), and 30 days (—□—) on murine interleukin-2 production activity. Values are expressed as IL-2[a] activity (units/ml). A significantly (p < 0.05) decreased IL-2 production activity was found at each concentration of methylene chloride.

a. IL-2 activity which represented as units/ml was derived from IL-2 standard curve (Fig. 4).
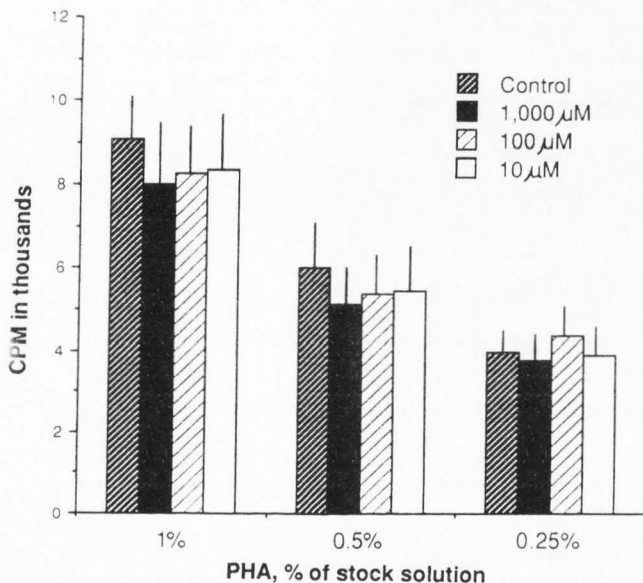
Figure 6. Splenic cells were isolated from male mice and treated with various concentrations of methylene chloride in vitro for 6 hours and assessed for blastogenic response to PHA. Blastogenic response is expressed as mean incorporation in counts per minute of tritiated thymidine and standard error of the mean. No significant differences were found between control splenic cells and splenic cells treated with 10^{-3}, 10^{-4}, and 10^{-5} M of methylene chloride. N=5.
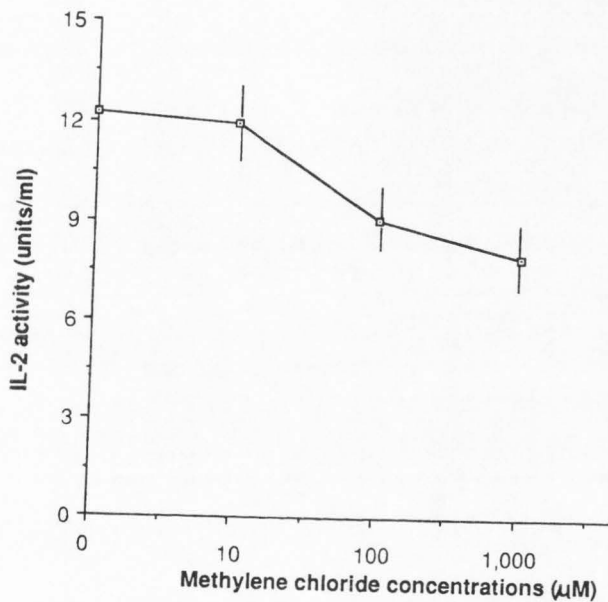
Figure 7. Splenic cells were isloated from male mice, treated with various concentrations of methylene chloride in vitro for 6 hours and assessed for IL-2 production activity. Values were expressed as IL-2 activity (units/ml). No significant difference was found between control splenic cells and cells treated with 10^{-3}, 10^{-4}, and 10^{-5} M of methylent chloride. N=5.

3-days of treatment

Legend:
- Control
- 1,000 μM
- 100 μM
- 10 μM

Y-axis: CPM in thousands (0, 20, 40, 60, 80)

X-axis: PHA, % of stock solution (1%, 0.5%, 0.25%)
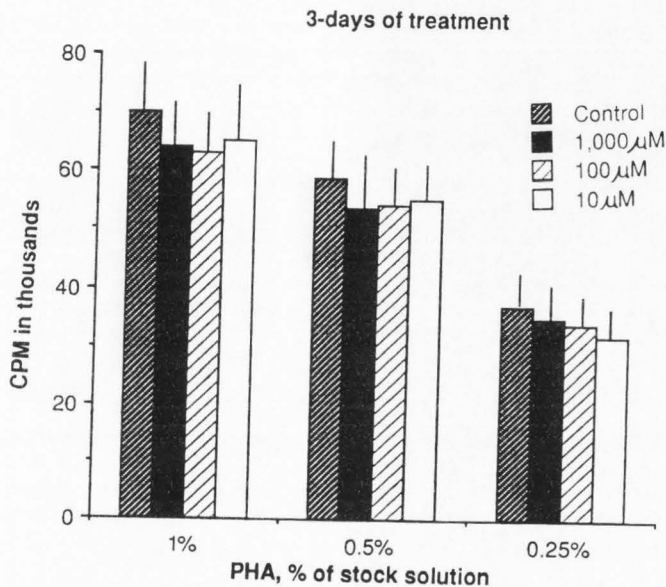
Figure 8. Mean incorporation in counts per minute of tritiated thymidine and standard error of the mean of peripheral blood mononuclear cells. No significant differences were found between response of control PBMC and of PBMC treated with 10^{-3}, 10^{-4}, and 10^{-5} M of methylene chloride for 3 days. N=7.
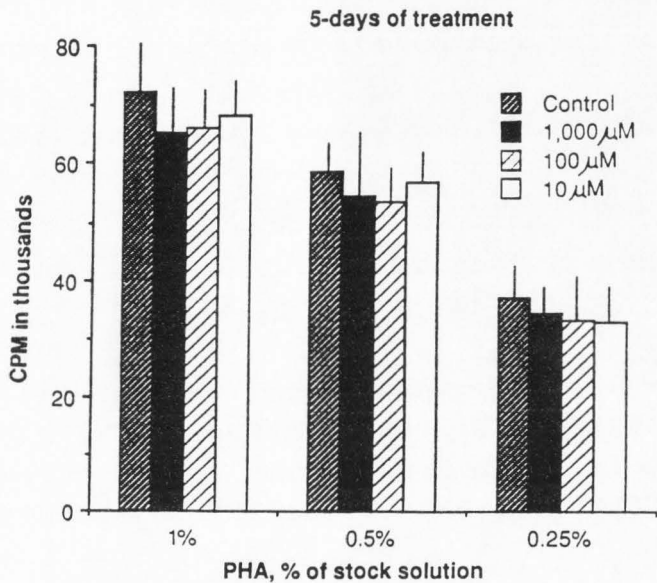
Figure 9. Mean incorporation in counts per minute of tritiated thymidine and standard error of the mean of peripheral blood mononuclear cells. No significant differences were found between response of control PBMC and of PBMC treated with 10^{-3}, 10^{-4}, and 10^{-5} M of methylene chloride for 5 days. N=7.

7-days of treatment
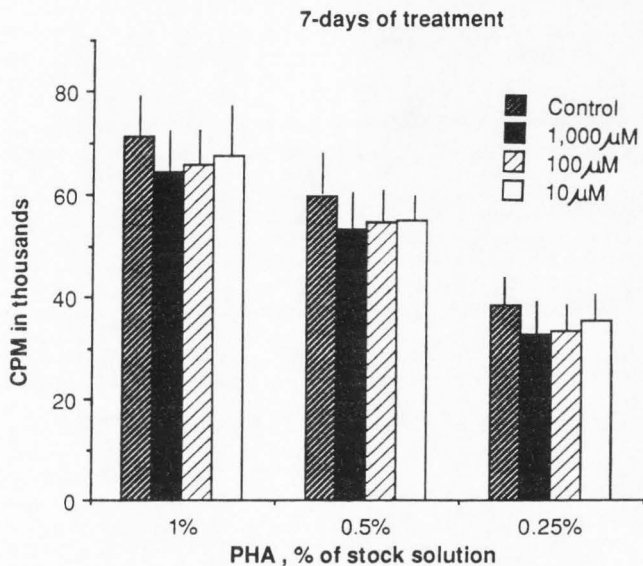
Figure 10. Mean incorporation in counts per minute of tritiated thymidine and standard error of the mean of peripheral blood mononuclear cells. No significant differences were found between response of control PBMC and of PBMC treated with 10^{-3}, 10^{-4}, and 10^{-5} M of methylene chloride for 7 days. N=7.

3-days of treatment

Legend:
- Control
- 1,000 μM
- 100 μM
- 10 μM

Y-axis: Mean % 51 Cr release

X-axis: Effector to target cell ratio (50:1, 25:1, 12:1, 6:1)

Fligure 11. Natural killer cell activity represented as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells. No significant differences were found between results obtained using PBMC incubated in medium and PBMC treated with various concentration of methylene chloride for 3 days. N=7.

5-days of treatment

Legend:
- Control
- 1,000 μM
- 100 μM
- 10 μM

Mean % ^{51}Cr release

Effector to target cell ratio: 50:1, 25:1, 12:1, 6:1
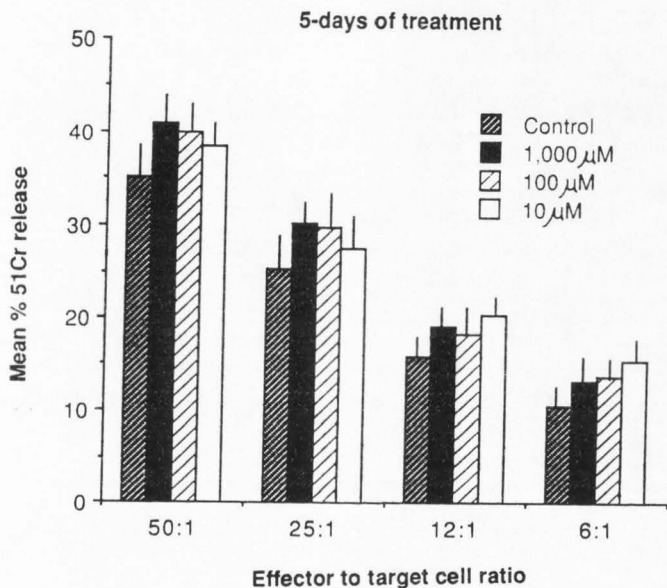
Figure 12. Natural killer cell activity represented as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells. No significant differences were found between results obtained using PBMC incubated in medium and PBMC treated with various concentration of methylene chloride for 5 days. N=7.
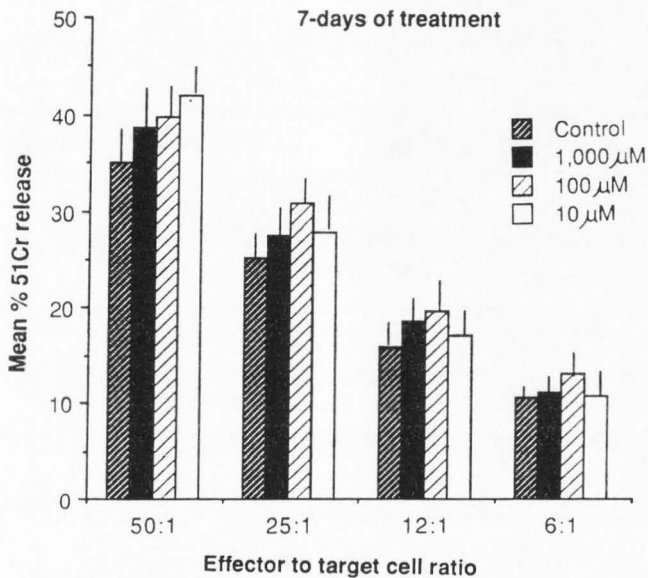
Figure 13. Natural killer cell activity represented as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells. No significant differences were found between results obtained using PBMC incubated in medium and PBMC treated with various concentration of methylene chloride for 7 days. N=7.

IL-2 production activity. In figure 14 are data suggesting slightly increased human IL-2 production following in vitro exposure of PBMC to various concentrations of methylene chloride. Although there seems to be a trend of higher IL-2 production activity by cells from treated PBMC, the small difference is not significant.
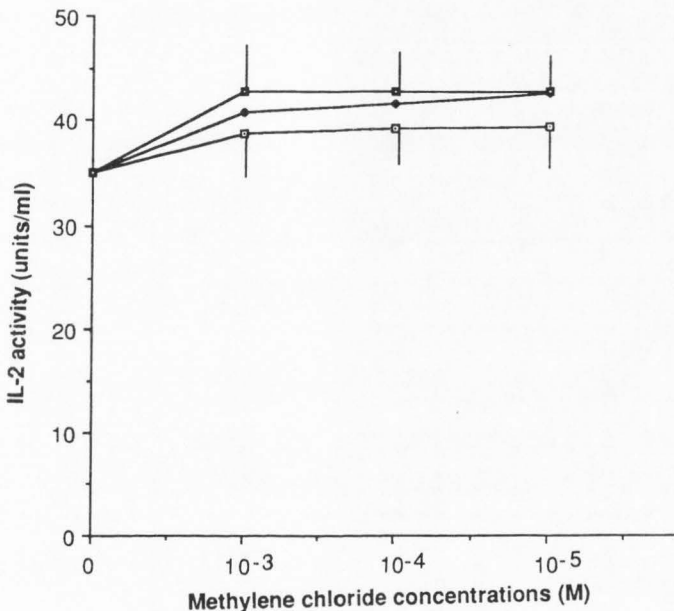
Figure 14. Effect of in vitro exposure to methylene chloride on IL-2 production of human PBMC. PBMC were incubated with 0, 10^{-3}, 10^{-4}, and 10^{-5} M methylene chloride and assessed for IL-2 production following 3 days (—□—), 5 days (—●—), and 7 days (—□—) of this treatment. Values are expressed as IL-2 activity (units/ml) and standard error of the mean. No significant differences were found between response of control PBMC and of PBMC treated with 10^{-3}, 10^{-4} and 10^{-5} M of methylene chloride. N=7.
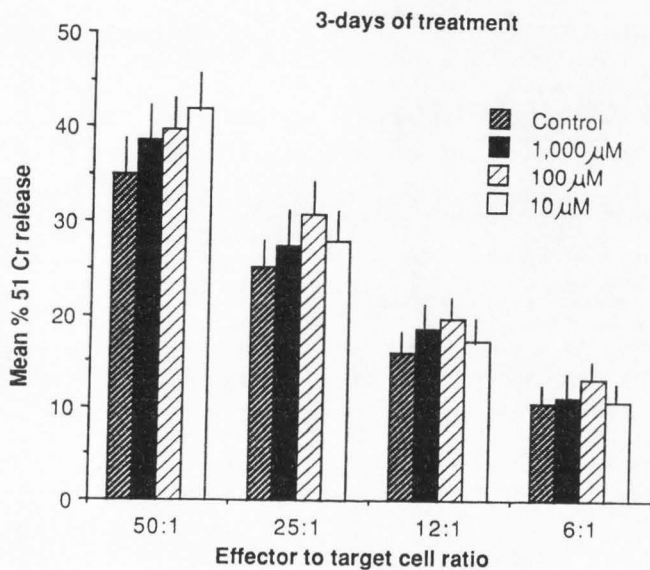
CHAPTER V

DISCUSSION

Many toxicities have been associated with methylene chloride exposure (see literature review in Chapter II). For example, this exposure may cause various neurochemical changes in the cerebral cortex and hypertrophy or proliferation of astroglial cells. However, only a very limited number of studies have been conducted on immunotoxicites associated with this chemical. Exposure to bromoform may suppress phagocytosis function (18), exposure to chloroform has been reported to decrease T-dependent antibody response anti-SRBC in both male and female mice (18), and exposure to bromodichloromethane has been found to depress delayed-type hypersensitivity (19).

A murine model was used in the present study in attempt to obtain additional information on the in vivo effects of methylene chloride on the immune system. A murine model has been used successfully in studies of immunotoxicities associated with many chemicals (17, 18, 19). The present studies investigated effects of the administration of methylene chloride on murine body and specific organ mass and blastogenic activity and IL-2 production of murine splenic cells.

The ability of T lymphocytes to proliferate in response to antigen and mitogen is thought to reflect in vivo functional capability. PHA binds to both B and T lymphocytes, but stimulates blast transformation only in T cells. Thus, reduced proliferative response to PHA of splenic cells from mice treated with methylene chloride indicates that this chemical alters T cell function. However, it is not clear whether it is methylene chloride or its active intermediates which suppresses lymphocyte blastogenic activity. The metabolism of methylene chloride which involves alkylation of DNA by a reactive metabolite and incorporation into the normal pathways of DNA biosynthesis (73) has been studied both in human subjects and in animals. Formation of reactive intermediates may alter or inhibit the synthesis of various proteins which are

important for lymphocyte DNA replication. Through these possible mechanisms, methylene or its metabolites may interfere with normal lymphocyte DNA synthesis and cause lower proliferation response.

Much evidence indicates that IL-2, one of the most studied lymphokines, is produced by the helper T cells and plays an important role in the generation of cytotoxic T cells. IL-2 also enhances helper T-cell activities that promote B-cell function and stimulates NK cell activity. Another important role for IL-2 in the modulation of immune function is to act on another set of T lymphocytes to produce gamma-interferon (73), another lymphokine regulating a variety of host functions, including antiviral and immunoregulatory responses (74) and NK-cell cytotoxicity (75). The current findings show that exposure to methylene chloride in vivo has a suppressive effect on both T-cell blastogenic activity and IL-2 production. It is well known that IL-2 is a strong mitogen for both cytotoxic T and helper T cells. Lotze and Rosenberg (73) have reported that mitogen-stimulated T-cell proliferation depends on the production of IL-2. Therefore, the lower T-cell blastogenic activity from methylene chloride treated mice is possibly due to defective IL-2 production.

Another possibility for the inhibitory effect of methylene chloride might be an indirect effect on endocrine and/or the CNS. Since endocrine mechanisms influence the immune system (76), and the CNS is also involved in the modulation of immune responses (77). It is possible that a chemical affecting one of these two systems may have an indirect effect on the suppression of immune functions.

investigation were also carried out to determine if exposure of murine splenic cells or human PBMC to methylene chloride in tissue culture altered certain of their immune parameters in vitro. The present study assessed blastogenic activity, IL-2 production, and NK cell activity of murine splenic cells and PBMC following in vitro incubation with methylene chloride. The findings indicated that exposure of mouse

splenic cells to methylene chloride in tissue culture did not have a suppressive effect on the blastogenic activity and IL-2 production activity.

CHAPTER VI

CONCLUSIONS

A number of toxicities have been associated with exposure to methylene chloride including renal necrosis, pulmonary injury, CNS damage, and hepatotoxicity. In order to examine the basis of possible immunotoxicities associated with methylene chloride exposure, several tests of cellular immune function were performed using a murine model and PBMC from healthy human subjects.

In the murine model, methylene chloride, even when given at high doses, was tolerated relatively well. Body mass, specific organ mass for thymus, kidney, spleen, and liver were found to be normal. However, CD-1 mice given methylene chloride demonstrated a significantly decreased blastogenic response to PHA and depressed IL-2 production. Decreased mitogenic response reflects depressed T cell function and is associated with reduced IL-2 production. Although the exact mechanism of methylene chloride induced suppression of immune function is not known, it has been suggested that this chemical and/or its intermediates may cause toxicities by altering several cellular functions, such as cell membrane, enzymes, hormones, and binding receptors. Methylene chloride also can prevent or modify the synthesis of various proteins, antibodies and various enzymes which are important for the immune functions. Indirectly, methylene chloride may affect the endocrine system and/or the CNS which in turn may produce substances which effect the immune system. That products of the endocrine system and CNS have influence on the immune system is thoroughly established. On the contrary, upon exposure of murine splenic cells to methylene chloride in vitro no significant depression of T cell blastogenic response and IL-2 production activity was found.

Immune function of PBMC from healthy human subjects was investigated at 3, 5, and 7 days after in vitro exposure to various concentrations of methylene chloride. No significant depression was found in NK cell activity, blastogenic response to the T-cell mitogen PHA or IL-2 production.

The present study showed depression of immune functions following in vivo exposure to methylene chloride but no decrease in selected immune parameters were observed following in vitro exposure to this chemical both in human PBMC and murine splenic cells. But this difference in findings were anticipated since there are limitations associated with in vitro test systems. First, metabolic alteration of methylene chloride in vitro may not be the same as it is in vivo, since methylene chloride metabolism requires microsomal cytochrome P-450 monooxygenase which is likely to be either absent or in trace amounts in lymphoid cell suspension. Secondly, as mentioned above, methylene chloride may be having an indirect effect on the immune system through some other system such as the endocrine system or the CNS which could not be detected in vitro.

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