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The Effect of Simulated Natural Toxaphene Exposure on the Immune Respone of Mice

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THE EFFECT OF SIMULATED NATURAL TOXAPHENE EXPOSURE ON THE IMMUNE RESPONSE OF MICE

by

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Objectives

The objectives of this investigation were to determine the immunotoxic effects of toxaphene exposure in mice after either ingestion or cutaneous absorption. Immunological competence was determined by studying various parameters of nonspecific, humoral, and cell-mediated immunity. These included 1) total and differential white blood cell counts, 2) organ to body weight ratios, 3) hemagglutination antibody titers, 4) the number of antigen-specific antibody-forming cells, and 5) both mitogen- and antigen-induced lymphocyte proliferation assays. The effect of toxaphene exposure on the overall immunoreactivity was determined by noting any alterations in resistance to infection and disease as measured by the LD_{50} of mice challenged with a Gram negative bacterial pathogen.

Background

The wide range of agricultural applications of toxaphene has resulted in the exposure of humans, livestock, and wildlife to this chlorinated hydrocarbon. Toxaphene is used to control outbreaks of army worms, cut worms, and grasshoppers in cotton, corn, and small grain crops and for sicklepod control in soybeans and peanuts. It is also frequently used as a contact pesticide for livestock ectoparasite

control. These uses account for 95 percent of the 16 million pounds of toxaphene used annually. The routes by which this toxic compound may enter the body where it can accumulate and persist in body fat are provided by ingestion, inhalation, and absorption through the skin.

Due to difficulties in analyzing the complex mixture of toxaphene and its degradation products, very little is known about the exact toxaphene residues in people or in the environment. Since toxaphene is fat-soluble (23), it will accumulate in both animals and fish and can persist for several years in soils and lake sediments. Persistence of toxaphene in the environment presents a constant threat to the health of all warm-blooded animals.

Because toxaphene exists as a complex and largely unidentified mixture of isomers, assessing the risks of this substance in the environment is very difficult. Few studies have been conducted examining the toxic effects of toxaphene or of any other pesticide in the polychloroterpene group. LD_{50} values have been determined for toxaphene doses which will cause death in 50% of the animals tested but information dealing with the overall effects of subtoxic doses is not available.

The United States Environmental Protection Agency placed major restrictions on the use of toxaphene in October, 1982. The decision was based on research data which indicated that toxaphene residues are harmful to "non-target" species such

as fish, birds, and mammals. Additional animal tests suggested a potential for oncogenicity in humans. These tests were typically conducted using toxic levels of toxaphene and again left the effect of subclinical toxaphene exposure open to speculation.

Rationale

Existence of toxaphene residues in animals and humans represents a serious threat to both agricultural and urban communities. Subtoxic toxaphene exposure may affect the hematopoietic and/or lymphoid system rendering exposed animals more vulnerable to disease, infection, and malignancy. A reduction in immunological responsiveness could ultimately result in poor weight gains in livestock as well as pose potential health problems for humans.

Significance

The potential risks of toxaphene exposure on the hematopoietic and/or lymphoid system is an important environmental concern. Both agricultural and urban communities need to be aware of possible health problems which result from toxaphene usage. The general use and effectiveness of broad spectrum pesticides such as toxaphene needs to be studied and greater emphasis given to pest control programs that minimize pesticide use. These measures could help protect humans, livestock, and wildlife against possible damage to the immunological defense system.

LITERATURE REVIEW

Introduction

In recent years the effects of environmental pollutants and industrial chemicals on the immune system have drawn increased interest due to the possible adverse effects these chemicals may have on lymphoid organs, the increase in susceptibility to infection, or the impairment of immunological responsiveness. Few studies have been conducted examining the toxic effects of toxaphene. LD_{50} values have been determined but information dealing with the overall effects of subtoxic doses is minimal.

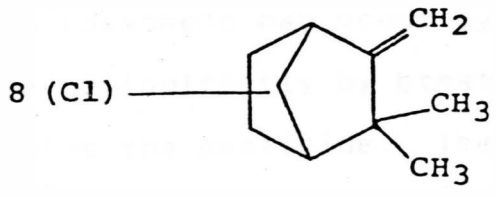
In this review the known toxic effects of toxaphene, an organochlorine insecticide, will be described and the immunotoxic effects of other halogenated hydrocarbons and pesticides will be discussed.

1. Toxaphene

1.1 Chemical and Physical Properties of Toxaphene

Toxaphene is a complex mixture of polychlorinated compounds made by chlorinating camphene to a chlorine content of 67-69%. It's structure is illustrated in Figure 1.

Figure 1.



Toxaphene has not been well characterized but it is known to contain at least 177 C_{10} compounds of 6 to 10 chlorine atoms with no single component of this mixture being the principle active ingredient. Compounds identified in this mixture include several endo-exo isomers of hexa-, hepta-, octa-, and nona- chlorobornanes and chlorobornenes which exhibit a wide variety of biological activity. Some of the 177 components of the toxaphene mixture have actually been shown to exhibit a ten-fold increase in toxicity to mice and houseflies than the common toxaphene mixture (54). 8-Octachlorobornane, one of the compounds present in toxaphene, is known to be highly toxic and because of this, the proportion of this compound present in technical toxaphene could significantly contribute to the toxicity in both target and non-target species (40).

Toxaphene is an amber, waxy solid with a softening point of 82°C. The density at 27°C is 1.66. It is highly soluble in organic solvents, somewhat less soluble in alcohols, vegetable and mineral oils, and insoluble in water.

Toxaphene is available for insecticidal use in the form of wettable powders, dusts, emulsion concentrates, and concentrated solutions in oil.

Exposure to toxaphene may occur by direct application of the pesticide or indirectly by breathing air or consuming food which contains the pesticide. The degree of absorption and toxicity will depend on the amount, the vehicle, and the

route of exposure.

1.2 Metabolism

Extensive metabolism is believed responsible for the low persistence of toxaphene in the environment as compared with other chlorinated hydrocarbon insecticides. It is known that heptachlorobornane, one of the most toxic identified compounds in the usual toxaphene mixture, is metabolized via reductive dechlorination reactions in rats (45). It was later discovered by Chandurker, et al. (5) that oxidative metabolism of toxaphene and two of toxaphene's toxic components, Toxicant B and Toxicant C, by NADPH-dependent mixed-function oxidases from rat liver microsomes also plays an important role in the metabolism and detoxification of toxaphene.

1.3 Agricultural Usage of Toxaphene

Ninety-five percent of the 16 million pounds of toxaphene used annually is for pest control on cotton, wheat, beef cattle, soybeans, and peanuts. In South Dakota 200,000 to 300,000 acres of cropland are treated annually with 1.5-2.0 pounds of toxaphene per acre in an attempt to control grasshoppers, army worms, cut worms, and the bean leaf beetle in corn, alfalfa, soybeans, and small grain crops. Toxaphene is also widely used in South Dakota as a contact pesticide for livestock ectoparasite control.

These applications constitute the major exposure routes by which toxaphene may enter the body where, due to its

liposolubility, it can accumulate and persist in body fat.

1.4 Toxicity of Toxaphene

To assess the acute toxicity of toxaphene, tests have been performed on several species of mammals and birds. LD_{50}'s from these tests are presented in Table 1 (22). As illustrated in Table 1, the toxicity of toxaphene is dependent on the solvent or vehicle employed. As can be seen, toxaphene is more toxic when administered orally in a digestible vegetable oil than in an oil such as kerosene. Likewise, dermally-applied toxaphene is more readily absorbed by the skin when in an oil-based solution than as a dust (22).

In subacute ingestion studies, liver cell changes were occasionally produced in rats fed 50 ppm toxaphene for 6 to 9 months. Definite liver changes were observed in rats fed 200 ppm for the same period (42). Dogs receiving 4 mg toxaphene/kg body weight for as long as 106 days exhibited degenerative changes in the liver parenchyma and in the renal tubules (30). These observations indicate that liver tissue is seriously damaged during toxaphene detoxification.

During subacute dermal studies in rabbits, it was observed that a 30% toxaphene solution dissolved in mineral oil (600 mg/kg/day) produced death after only 2-5 applications (31). A solution of 20% toxaphene in mineral oil (166 mg/kg/day) for 14 days was fatal to 33% of the rabbits treated. In a similar study using guinea pigs, solutions of 20% toxaphene in mineral oil (332 mg/kg/day) for 14 days

Table 1. Acute Toxicity of Toxaphene[a]

Animal	Route	mg./kg. Body Weight	Vehicle
Mouse	oral	112	corn oil
Rat	oral	60	corn oil
Rat	oral	90	peanut oil
Rat	oral	120	kerosene
Guinea pig	oral	270	corn oil
Guinea pig	oral	365	kerosene
Dog	oral	49	corn oil
Dog	oral	> 250	kerosene
Cat	oral	25-40	peanut oil
Rabbit	oral	75-100	peanut oil
Rabbit	oral	250-500	kerosene
Cattle	oral	144	grain
Goat	oral	200	xylene
Sheep	oral	200	xylene
Rat	dermal	930	xylene
Rabbit	dermal	>4000	dust
Rabbit	dermal	<250	peanut oil

[a]Adapted from Hercules, Incorporated (22).

produced death in 73% of the guinea pigs (22). This data indicates that the lethal effect of subacute dermal doses of toxaphene will vary with the species of animal and its ability to detoxify this mixture of compounds.

Chronic oral toxicity studies have been performed on rats, dogs, and monkeys. Rats ingesting 25 ppm toxaphene daily for 2 years exhibited no overt toxic effects; however, at higher levels toxic effects were observed. Slight liver damage occurred at 100 ppm. At higher levels of 1000, 1500, and 1600 ppm, some evidence of central nervous system stimulation and nonspecific liver damage was observed (22,34).

In two separate experiments, dogs were either administered toxaphene daily in a dry diet for 2 years or as a corn oil solution for 4 years. Slight degeneration of the liver occurred as a result of ingesting 40 ppm toxaphene in the dry diet for 2 years whereas 200 ppm toxaphene administered daily for 2 years resulted in moderate degeneration of the liver. Exposure to 5 mg toxaphene in corn oil/kg/day (approximately 200 ppm in diet) for nearly four years caused liver necrosis (34).

Monkeys exposed for 2 years to food containing 10-15 ppm (0.64-0.78 mg/kg/day) exhibited no observable signs of intoxication or of damage to the tissues (22).

Additional research has indicated that toxaphene is carcinogenic, causing liver tumors in mice and thyroid tumors in rats (44). Strobane, a terpene polychlorinate closely

related to toxaphene, causes liver hepatomas in male mice.

Another polychlorocamphene insecticide, similar to toxaphene but produced in the U.S.S.R., has been implicated in causing chromosomal abnormalities in humans subjected to high concentrations during occupational exposures. This insecticide has also been implicated in the testicular degeneration and endocrine changes of male rodents. In the female it can cross the placental barrier and damage the fetus (23).

2. Parameters Used to Assess Immunotoxicity

The effects of environmental contaminants on the immune system has been studied extensively in recent years. Aromatic halogenated hydrocarbons, such as polychlorinated biphenyls (PCB's) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and certain pesticides such as DDT have been investigated and shown to induce alterations in immune functions which can alter the host's resistance to infectious agents.

To determine the immunotoxicity of an environmental contaminant, information on a number of immunological parameters is collected after exposure to the suspected toxicant. Weight gain is monitored to detect alterations due to possible decreased food consumption or impaired food utilization which, by itself, can result in a diminished immunologic response. Organ to body weight ratios of lymphatic organs (spleen and thymus) are determined and used to indicate possible immunologic impairment.

The thymus, which is almost fully developed in the last third of fetal life, is responsible for the maturation of a portion of the stem cells produced in the bone marrow. During the maturation process, the stem cells are converted to T cells or thymus-derived lymphocytes which are involved in cellular immunity. Because the thymus provides a vital function in the ontogeny of the immune system, it is used as an indicator for a possible immunological dysfunction.

Unlike the thymus, the spleen is a lymphatic organ which is functional throughout the lifetime of an individual. One important function the spleen serves is as a blood filter which eliminates aged or damaged blood cells as well as systemic or blood-borne particles and infectious agents. After these particles have been trapped by the spleen, a complex interaction involving precursor cells, macrophages, antigen-reactive cells, and antibody-producing cells will normally result in either a humoral and/or cell-mediated immune response. Due to these vital functions, the spleen can therefore be used as an indicator of immunoreactivity.

Total and differential leukocyte counts are normally performed to determine the possibility of chemical-induced immune alterations. The majority of white blood cells, which are the effector cells of the immune system, consist of B and T lymphocytes, polymorphonuclear neutrophils, and monocytes. B and T lymphocytes share a central role in humoral and cell-mediated immune response while polymorphonuclear neutrophils

function as phagocytes. Cell-mediated immunity, a T cell function, is responsible for protective immunity which is effective primarily against fungal, viral, and many intracellular bacterial infections, delayed-type hypersensitivity, and rejection of tumors and foreign tissue. Humoral or antibody-mediated immunity is a B cell function which allows the host to combat bacteria and a number of viruses by enhancing phagocytosis, neutralizing toxins, and preventing viral attachment. Possible alterations in the leukocyte population could include a decrease (leukopenia) or increase (leukocytosis) in the total white blood cell number, a shift in the normal percentage of the different classes of white blood cells, or a combination of both. In any situation, such alterations could lead to a decrease in the disease resistance of the host.

To evaluate the effects of an environmental chemical on humoral or cell-mediated immunity, a number of _in vivo_ and _in vitro_ methods have been employed. The ability of exposed animals to synthesize antibodies following immunization is a measure of humoral immunity. Depending upon the immunogen employed, T cell cooperation may or may not be required. The antibody response to sheep red blood cells or tetanus toxoid is dependent upon the presence of T cells. A normal immune response will be obtained only if there are sufficient T lymphocytes to initially interact with the immunogen and B lymphocytes to subsequently synthesize the antibody. _In vitro_

methods used to quantitate the antibody response include hemagglutination, which can be used to determine both the IgG and IgM antibody titer to the immunogen, and the Jerne plaque assay, which is used to enumerate antigen-specific antibody-forming cells. The cells used in the Jerne plaque assay may be obtained from the peripheral blood or any lymphoid tissue. Resistance to certain bacteria such as Streptococcus pneumoniae can also be used as a test of in vivo humoral immunity.

Antigen- or mitogen-induced lymphocyte proliferation assays can be employed to assess in vitro cell-mediated immune functions. Antigen-induced lymphocyte proliferation is a measure of a specific proliferative response to the immunogen following immunization with the same immunogen. Nonspecific in vitro polyclonal stimulation of lymphocytes occurs following the addition of various mitogens which can be specific for certain classes of lymphocytes. The mitogens frequently used include phytohemagglutinin, concanavalin A, both T cell mitogens, and Escherichia coli lipopolysaccharide, a B cell mitogen. The ability of lymphocytes to proliferate using any of these methods is usually measured by determining the extent of labeled thymidine incorporation into the DNA of dividing cells.

The resistance of animals to infection with Listeria monocytogenes is an in vivo indicator of cell-mediated immunity. Protection against infection from such

intracellular parasites depends primarily on cell-mediated immunity; however, nonspecific phagocytosis also plays a minor role. Cell-mediated immunity can also be measured with delayed-type hypersensitivity skin tests.

Besides these bacteria, other infectious agents including different bacteria, viruses, or even parasites can be used to demonstrate the ability of a host to withstand a challenge infection and therefore assess the competency of its immune system.

3. Immunotoxic Effects of Halogenated Hydrocarbons and Organochlorine Insecticides

A recent review of the literature has indicated that immunological alterations due to toxaphene or any other terpene polychlorinate have not been previously investigated; however, various other halogenated hydrocarbons and organochlorine insecticides have been studied for their effect on the hematopoietic and lymphoid systems.

3.1 Organochlorine Insecticides

The increased usage of pesticides, such as the organochlorine insecticides, in our environment has resulted in an interest as to how these agents persist and accumulate in the environment where they can ultimately affect the wildlife population and the health of man and domestic animals. In the last few years, many studies have been completed to determine the effects of organochlorine insecticides on the immunological reactivity of living organisms.

DDT (dichlorodiphenyltrichloroethane), the most widely studied of the organochlorine pesticides, is able to persist in nature for long periods of time due to its chemical stability. Because of its high lipid solubility, exposure to this chemical results in accumulations of DDT in the fatty tissues of the body. Even though there is a near total ban on this insecticide in the United States, DDT and its metabolites are still present as environmental contaminants. This has been attributed to its chemical stability and slow rate of degradation. DDT is still widely used in other parts of the world.

Dieldrin, lindane, and Mirex are three additional chlorinated insecticides which have been used in the United States. Due to its carcinogenic potential, the use of dieldrin was also banned in the United States with the exception of special-use permits. Lindane, which is the gamma isomer of benzene hexachloride, is a broad spectrum insecticide which is believed to have low toxicity for warm-blooded animals. Lindane has been used in insecticide vaporizers, resulting in cases of accidental poisoning by ingestion of the vaporizer tablets or repeated vapor exposure.

DDT was first reported to be immunosuppressive by Wassermann et al. (64,65) in 1969. In these early studies, rats and rabbits received 200 ppm DDT in drinking water. Rats ingesting the DDT exhibited decreased liver weights, decreased spleen weights and serum gamma globulin levels,

and a reduction in antibodies against ovalbumin. Rabbits receiving the DDT and immunized with Salmonella typhi showed a decrease of serum gamma globulin and a significantly lower anti-Salmonella titer.

Street and Sharma (49) observed suppression of tuberculin delayed hypersensitivity in rabbits after an 8 week oral exposure to DDT. This treatment also resulted in decreased numbers of plasma cells in popliteal lymph nodes, in fewer germinal centers in the spleen, and in atrophy of the thymus cortex.

Gablicks et al. (16) studied the effect of DDT on the immune system of guinea pigs. The guinea pigs received multiple injections of 15 mg DDT per kilogram of body weight at two- or three-day intervals beginning 7 to 14 days before immunization and for 14 to 21 days following immunization. The primary immune response, as measured by the protective immunity against diphtheria toxin, the antitoxin titers, and the increase in gamma globulin fraction, was not altered by this treatment.

In contrast, a study by Shiplov et al. (47) demonstrated that DDT-exposed humans and rabbits injected with a Salmonella typhi vaccine did not exhibit a reduced titer but instead somewhat higher O, H, and Vi agglutinin titers. Similar trends in immunoreactivity were found by Lukic et al. (38) after treating rats with technical grade DDT for 60 days. A daily oral intake of 40 mg DDT per kilogram of body

weight did not cause significant toxic effects. Surprisingly, this dose even enhanced both humoral and cellular immune responses when animals were immunized with bovine serum albumin mixed with Freund's complete adjuvant.

Extensive studies have been performed to assess the effect of DDT and other pesticides on chickens. Chickens which received subclinical oral doses of DDT exhibited increased susceptibility to infection with the protozoan, Histomonas meleagridis (43). In a study conducted by Glick (18), chickens were administered either DDT or Mirex, a chlorinated insecticide used against the imported fire ant, to evaluate the effect of these pesticides on antibody-mediated immunity. Feeding up to 800 ppm DDT significantly depressed the levels of IgG and IgM but did not influence antibody production to bovine serum albumin (BSA) or the plaque-forming cell response to sheep red blood cells. Mirex treatment did not affect any of these immunological indicators. Similar results were obtained in a separate study in which chickens were fed 500 ppm DDT. In this study, ingestion of technical grade DDT did not influence the antibody response to Salmonella pullorum antigen or the antibody response to bovine serum albumin (32). Results of a similar study conducted by Subba Rao and Glick (50) disagreed with the previously observed effects of DDT and Mirex treatment. In this study, it was found that DDT and Mirex treatment could significantly suppress the levels of serum IgG while

markedly increasing the serum IgM level without causing any significant differences in specific antibody production to sheep red blood cells. Because interaction between T and B cells is a prerequisite for a normal antibody response to SRBC and BSA, a reduction in the normal responsiveness of the T cell may account for the depressed IgG and IgM antibody titers of DDT- and Mirex-treated birds observed in the cases previously reported.

A study by Friend and Trainer (13,14) revealed the enhanced lethal effects of duck hepatitis virus on ducklings after exposure to DDT and dieldrin. While the mortality of the controls was only 6 percent, ducklings exposed to 900 ppm DDT experienced a 47 percent mortality and those exposed to 40 ppm dieldrin suffered a 57 percent mortality.

The effect of exposure to 50 ppm dieldrin in drinking water for 3 months was found to affect serum proteins in rabbits. IgG levels were significantly reduced by the dieldrin treatment but there was no effect on serum IgM levels (66).

In an experiment carried out by Desi et al., rabbits were given lindane by daily oral administration. A significant decrease in the immune response to Salmonella typhi was found in the sera of the animals treated with lindane as compared to the untreated controls (7). In a subsequent study by Dewan et al. (8), the immunosuppressive effect of lindane was assessed in weaning rats over a period of 5

weeks. Agglutinin titers against S. typhi "O" and "H" and S. paratyphi "AH" and "BA" antigens were determined following typhoid-paratyphoid vaccine in control and lindane-fed animals. The titers in treated animals were significantly suppressed as compared to those of the non-treated controls.

These studies indicate that organochlorine insecticides have the potential for suppressing immunological reacticity. Since toxaphene is also an organochlorine insecticides, it may therefore be immunotoxic. Because of the persistence of toxaphene in the environment and its toxic effects on warm-blooded animals, toxaphene presents a constant threat to the health of both humans and livestock. Animals exposed to toxaphene, could possibly be more susceptible to disease, infections, and malignancy. The reduced immunological reactivity could also result in poor weight gains in livestock as well as pose a potential human health problem.

3.2 Polychlorinated Biphenyls (PCB's)

PCB's have been used industrially for waterproofing and flameproofing wood and to improve the properties of protective coatings such as paint, synthetic resins, varnishes, and waxes (27,56). PCB's have also been used as lubricants, heat-transfer agents, and in insulators (56).

Due to their stability and solubility, PCB's are persistent environmental pollutants (contaminants of the environment). The liposolubility of these compounds allows for their storage and subsequent accumulation in animal

adipose tissue which accounts for the increasing concentrations of PCB's in the food chain.

Preliminary toxicity studies, which were initiated to evaluate the hazards of PCB exposure, suggested that these chemicals altered normal immunological functions. Feeding of PCB to chickens resulted in small spleens (12) as well as the atrophy of other lymphoid tissue (57). Dermal application of PCB's resulted in lymphopenia, atrophy of the thymus cortex, and a reduction in the number of germinal centers in the spleens and lymph nodes of rabbits (55). Later, it was observed that a commercial PCB preparation, Aroclor 1254, fed to ducklings for 10 days, increased the susceptibility of the ducklings to duck hepatitis virus (15). However, it has since been observed that increased viral susceptibility following challenge may be due to nonspecific interactions between hepatic toxins and liver pathogens and may thus occur without any actual impairment to the immune system (9).

Because immunosuppression by PCB's was indicated in these early studies, further studies were completed to assess the actual effects of PCB's on the immune system. The immunotoxic effect of chronic ingestion of low concentrations of PCB was assessed in guinea pigs by Vos and de Roij (56). Animals in this study received 10 and 50 ppm Aroclor 1260 in their diets for eight weeks. Following immunization with tetanus toxoid, a significantly reduced number of gamma globulin-containing cells was observed in the draining lymph

nodes of both treatment groups. Even those animals fed 10 ppm PCB had a significant reduction in the concentration of serum gamma globulin. No effect was found on either the spleen weight or the leukocyte counts in these animals. In two subsequent studies using guinea pigs, ingestion of 50 ppm PCB caused reduced numbers of both circulating leukocytes and lymphocytes, thymus atrophy, a reduction in antibody titers, and a decreased number of anti-tetanus toxin-producing cells in the draining lymph nodes after immunization (62,63). These observations indicated a severe suppression of the humoral immune response.

The effect of oral exposure to Aroclors 1221, 1242, and 1254 in rabbits was studied by Koller and Thigpen. They observed a significantly lower serum-neutralizing antibody titer to pseudorabies virus in the experimental groups (29). Wassermann and coworkers (66) observed reduced levels of serum IgG in rabbits who had received 200 ppm Aroclor 1221 for 3 months in drinking water; however, this treatment did not appear to have any effect on serum IgM concentration. Contrary to these studies, Street and Sharma (49) found feeding rabbits low dietary levels of Aroclor 1254 did not result in any significant changes in either the humoral or cell-mediated immune response after sheep red blood cell immunization.

In a similar study in which rats were fed low levels of PCB for seven days, cell-mediated immunity did not appear

to be suppressed (3). In this experiment peripheral blood lymphocytes, isolated from PCB-fed rats, even exhibited an increased mitogenic response upon *in vitro* stimulation by the T cell mitogen, phytohemagglutinin. Previously it had been reported, that tuberculin delayed-type hypersensitivity was significantly suppressed in guinea pigs ingesting 50 ppm PCB (62,63). This study indicated that the PCB treatments suppressed *in vivo* cell-mediated immunity. It appears that PCB exposure does alter cell-mediated immunity; however, the variation in data makes it difficult to state a specific effect.

After a 6 week oral PCB exposure, it was observed by Loose et al. (35,36), that mice exhibited a significantly reduced antibody response to sheep red blood cells. These studies also revealed that such treatment resulted in increased susceptibility to both the toxic effects of gram negative endotoxin and to the lethal effects of *Plasmodium berghei*. A recent series of studies conducted by Thomas and Hinsdill, investigated the effects of low level chronic oral PCB exposure on both monkeys and mice. They found that long term ingestion resulted in significantly reduced humoral immunity in monkeys immunized with sheep red blood cells and that PCB-treated mice challenged with *Salmonella typhimurium* were more susceptible to infection than controls (53). This data supports earlier reports which indicated both a suppression of humoral immunity and decreased disease resistance in

PCB-exposed animals.

Although not intentionally studied, the human health hazards of acute PCB exposure was tragically demonstrated in Yusho, Japan in 1968. At that time, at least 1000 persons were exposed to PCB by eating rice oil contaminated with Kanechlor 400, a mixture of various PCB's which also contained some impurities. Those persons involved in this accident developed chloracne, disorders of the peripheral nervous systems, eye discharges, and hyperpigmentation of the skin, nails, and mucous membranes. As had been previously observed in animal models, these individuals also experienced immunosuppression. Statistics later indicated increased rates of stomach and liver cancers in the victims of the Yusho incident (17). The occurrence of increased cancer rates among these victims confirmed the results of earlier immunotoxicity studies which had been completed using experimental animals. It has been postulated that the immunosuppression was responsible for a less efficient immunosurveillance which then allowed the cancers to proliferate within the body.

3.3 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

TCDD is one of the most toxic compounds known to man. It is a chemical that can be synthesized in minute, but yet toxic amounts during the manufacture of either chlorinated phenolic compounds or chemicals synthesized from chlorophenols, such as the herbicide, 2,4,5-trichlorophenoxyacetic

acid.

In initial toxicological studies, TCDD exposure was shown to induce thymus atrophy in rats, mice and guinea pigs (4,19,21,28,60,61). Atrophy of peripheral lymph nodes was also observed (19,21,61). Due to the laboratory studies which found that TCDD caused thymus atrophy at sublethal doses and the fact that the thymus is the central lymphoid organ, cell-mediated immunity studies were conducted to assess the immune response in TCDD-treated animals. In the initial studies of Vos et al. (61), delayed-type hypersensitivity to tuberculin was the method used to assess *in vivo* cell-mediated immunity in guinea pigs and rats, whereas a local graft versus host reaction was the method used to determine cell-mediated immunity in mice. TCDD was observed in these studies to suppress cell-mediated immunity in guinea pigs; but not rats. Graft versus host activity, in which the weight of the draining popiteal lymph nodes was used to measure the degree of immunological activity, was found to be significantly lower in mice subjected to TCDD than in controls. In a recent study (46), *in vitro* cell-mediated immunity was assessed in mice and rabbits receiving an oral exposure of from 0.01 to 10 ug TCDD/kg/week. Exposure of both species to TCDD at all levels caused an increase in the thymidine uptake by nonstimulated splenic lymphocytes indicating nonspecific stimulation by the presence of TCDD *in vivo*. The blastogenic response to phytomitogens was

decreased at exposure levels greater than 0.1 ug TCDD/kg/week in both the mice and rabbits. In vivo skin reactivity to tuberculin was also reduced in both species in groups receiving either 1 or 10 ug TCDD/kg/week. After immunization with tetanus toxoid in Freund's adjuvant, humoral immunity was also assessed. Dose levels of both 1 and 10 ug TCDD/kg/week reduced the serum anti-tetanus level in both species. The data collected in these experiments provides overwhelming evidence that TCDD exposure causes suppression of both cell-mediated and humoral immunity.

Further studies were conducted to determine if subtoxic levels of TCDD have the capacity to disrupt the host defense system and thereby increase mortality to bacterial challenge. It was determined by Thigpen et al. (51) that low doses of TCDD, administered to mice prior to infection with Salmonella bern resulted in significantly increased mortality and decreased time from infection to death. In a subsequent study, Vos et al. (58) demonstrated that exposure to TCDD reduced the threshold for endotoxic shock in mice, thereby explaining the increased mortality observed in the study by Thigpen et al.

After the effects of TCDD in adult animals had been determined, the effects of pre- and postnatal exposure to TCDD were studied. These studies utilized the offspring after only the mothers had been treated with TCDD. These mice were exposed either pre- and postnatally or postnatally

only (by nursing from TCDD-exposed females). Severe suppression of cell-mediated immunity was determined by measuring *in vitro* mitogen-induced blastogenesis of thymic and splenic lymphocytes, *in vivo* delayed hypersensitivity, spleen cell graft versus host reactivity, and allograft rejection. It was determined in these studies that pre- and postnatal or postnatal exposure via maternal exposure in rats and mice was responsible for severe suppression of cell-mediated immune functions (10,59).

The effects of prenatal exposure only were examined in a more recent study (52). The immunological reactivity of offspring from mothers that were fed subtoxic levels of TCDD in their diets four weeks prior to being bred were examined. Offspring from mothers receiving 1 ppb TCDD in their food were more sensitive to an endotoxin challenge. Mice from mothers fed either 2.5 or 5.0 ppb of TCDD also exhibited a significantly reduced spleen anti-sheep red blood cell plaque-forming cell response following immunization. The offspring of mothers fed 5 ppb TCDD had a significantly reduced contact sensitivity response to dinitrofluorobenzene (DNFB). Since only certain T cell-mediated functions were affected, it is believed that maturation of specific T lymphocyte subpopulations may have been hindered in the TCDD-exposed offspring.

After reviewing numerous other studies examining the immunotoxicity of TCDD, it can be concluded that TCDD is an

immunosuppressive compound that affects thymus-dependent immunity, humoral immunity, and has the capacity to disrupt the complete host defense system.

3.4 Polybrominated Biphenyls (PBB's)

PBB's are compounds employed as flame retardants. These compounds are fat soluble and can persist for long periods of time in tissues such as the thymus, liver, brain, and adipose tissues (2).

An initial study in which hexabromobiphenyl (Firemaster BP6) was fed to chickens resulted in atrophy of both the bursa of Fabricus, an organ found only in birds and known to be responsible for B cell maturation, and the spleen. Prompted by this study, Vos and van Genderen (63) conducted an experiment in which guinea pigs were fed low levels of Firemaster BP6 for 45 days prior to immunization with tetanus toxoid. These animals exhibited atrophy of the thymic cortex and depletion of follicles and periarteriolar lymphocyte sheaths in the spleen. A reduction in the antibody titers indicated a suppression of the humoral immune response. In a subsequent study by Farber et al. (11), Firemaster BP6 was fed to dogs. A depletion of lymphocytes, particularly in the T cell zones, occurred in the lymph nodes and the number of plasma cells producing IgG in the popliteal lymph nodes was reduced.

Humoral and cell-mediated immunity was assessed in mice and rats after PBB exposure in a study by Luster et al. (39).

During this experiment, mice and rats were dosed by gavage over a 30 day time period with various levels of Firemaster FF-1 ranging from 0.3 to 30.0 mg/kg body weight per day. In rats, exposure to 3 and 30 mg/kg PBB resulted in a significant reduction in thymus weights. Cell-mediated immunity, as measured by the responsiveness of splenic lymphocytes to stimulation by polyclonal T cell mitogens, was slightly suppressed in both rats and mice receiving 30 mg/kg PBB. The B cell reactivity, as measured by the response of lymphocytes to the B cell mitogen, E. coli lipopolysaccharide, appeared to be somewhat suppressed; however, the antibody response of the mice to sheep red blood cells appeared normal. Because PBB-induced immunosuppression occurred only at levels slightly below those that induce overt signs of clinical toxicity, PBB did not appear to be a significantly immunosuppressive chemical in this study.

Assessing the immunocompetence of a human population accidentally exposed to PBB's has proven to be one of the most interesting studies of the effects of PBB's on immune function. In 1973, cattle feed became heavily contaminated with PBB's when there was an accidental substitution of a PBB for magnesium oxide. This resulted in consumption of farm products containing these polybrominated biphenyls by Michigan dairy farm residents and other urban residents. Following this accident, extensive studies were conducted to determine the effect of PBB's on the immune function of the

humans which had been exposed. In these investigations, Bekesi et al. (1) observed several abnormalities including decreases in the numbers and percentages of peripheral T and B blood lymphocytes with a corresponding increase in undifferentiated lymphocytes. In addition, peripheral blood lymphocytes from PBB-exposed individuals had decreased *in vitro* response to lymphocytes from other individuals as well as reduced levels of mitogen-induced lymphocyte proliferation. With the exception that only a marginal decrease in the B lymphocyte subpopulation was observed (2), the results of Bekesi's studies were subsequently confirmed in both the dairy farm residents and chemical workers accidentally exposed to PBB's.

4. Summary

After evaluating the different immunological studies, it was concluded that the halogenated hydrocarbons, PCB's and PBB's; the pesticides, DDT, Mirex, and lindane; and pesticide contaminant, TCDD, affect immune function. The data presented indicates that many of these chemicals are somewhat selective in their action, suppressing only certain parameters of immune function. Because the immune system has not been previously challenged experimentally by toxaphene or any other polychloroterpene, it is important that data on immune modulation by this chemical be collected and the possible effects determined. Even though the immunological effects of an environmental chemical will vary with

the difference of animal species used and the type, concentration, route, and exposure schedule, it is still possible to extrapolate from an immunosuppressive effect found in laboratory animals to possible adverse effects which may occur in humans, wildlife, and livestock. The mechanism of immunosuppression is not always clearly understood; however, it should be obvious that any impaired immunological reactivity following exposure to an environmental chemical can potentially endanger both man and animals.

MATERIALS AND METHODS

1. Animal Maintenance and Treatment Protocol

1.1 Animals

Mice from an outbred, albino, Charles River strain were used in this study. Mice were bred and maintained in the animal care facilities at the South Dakota State University Microbiology Department where they were housed (no more than 10 per cage), fed (Mouse Chow #5015, Ralston Purina Co., St. Louis, Missouri), and watered ad libitum.

1.2 Experimental Design

The same experimental design was used for studying the effects of toxaphene administered by each of the exposure routes. At the beginning of each toxaphene exposure, three week old mice were divided into groups consisting of a minimum of 6 mice.

Nonspecific indicators of immunologic defense were determined after 14 days treatment.

Prior to the assessment of acquired humoral immunity, mice in the ingestion and dermal absorption groups received an intraperitoneal (IP) immunization of 10^8 SRBC on day 9 of treatment. Treatment was then continued for 5 days with sacrifice on day 14.

Mitogen-induced lymphocyte proliferation assays were performed after mice had received toxaphene treatment for 14 days. Mice used for the antigen-induced lymphocyte

proliferation assay were injected IP with 10^8 SRBC after 14 days treatment and treatment continued for 13 additional days with the toxaphene treatment period extending over a total of 27 days.

1.3 Toxaphene Exposure

1.3.1 Toxaphene Ingestion

The toxaphene (90% solution, Boots Hercules Agrochemicals, Co., Wilmington, Delaware) was dissolved in Mazola corn oil and administered via gastric intubation using a ¼ cc graduated glass tuberculin syringe (Becton-Dickinson, Rutherford, New Jersey) and a balled biomedical animal feeding needle (Popper and Sons, Inc., New Hyde Park, New York). Mice received either corn oil, C_{Ing}, or a toxaphene-corn oil solution, T_{Ing}, at the rate of 0.005 cc/gram of body weight. Preliminary investigations were performed to determine the proper dose levels to be employed. These studies found that immunotoxicity occurred at 30 mg/kg/day. Consequently all subsequent oral treatments were done at this dose.

1.3.2 Cutaneous Absorption of Oil Base Toxaphene

Oil base toxaphene was dissolved in mineral oil (York Pharmacal Co., Brookfield, Missouri) and applied to the abdominal region of each mouse. Mice in this group, T_{Dermal}, received 50 ul of the toxaphene-mineral oil solution (5 mg toxaphene) daily. Control animals, C_{Dermal}, received an equivalent treatment using only 50 ul mineral oil. Proper dose levels had previously been determined in pilot studies.

1.3.3 Cutaneous Absorption of Water Base Toxaphene

A 2% toxaphene-water emulsion was prepared using a concentrated solution of emulsifiable toxaphene. Mice, T_{Emul}, were exposed to the 2% toxaphene emulsion by saturating the fur on the back and abdomen once every five days during the treatment period. Control animals, C_{Emul}, received a similar treatment with water.

2. Methods

2.1 Immunological Methods

2.1.1 Immunization

Sheep red blood cells (SRBC) used for immunization purposes were obtained by jugular venipuncture and collected into a sterile two liter Erlenmeryer flask containing 40 ml of 40% sodium citrate. After collecting the SRBC, they were transferred to sterile screw cap bottles and stored at 4°C. To prepare SRBC for immunization, an aliquot of SRBC was washed three times with 0.85% NaCl and then diluted. Immunized mice received an intraperitoneal immunization of 10^8 sheep red blood cells in 0.25 cc saline.

2.2 Exsanguination and Treatment of Tissues, Blood, and Serum

Using ether-saturated cotton, mice were anesthesized and exsanguinated. Whole blood, collected after severing the subclavian artery, was used for preparing smears for differential white blood cell counts, total leukocyte determinations, and the hematocrit levels. After exsanguination,

mice were terminated by cervical dislocation. Serum was removed after allowing blood to clot at room temperature, clarified by centrifugation, frozen, and stored for further analysis. The mice were weighed after termination and the spleen, thymus, liver, lungs, and kidneys removed and weighed.

2.2.1 Total Leukocyte Counts

Forty microliters of whole blood, collected from each experimental animal, was diluted in 20 ml of Isoton II diluting buffer (Coulter Diagnostics, Inc., Hialeah, Florida) in individual disposable blood dilution vials (American Scientific Products, McGraw Park, Illinois). To lyse the red blood cells in each suspension, six drops of Zap-Isoton II (Coulter Diagnostics, Inc.) was added. A Coulter Counter (Model F, Coulter Electronics, Inc., Hialeah, Florida) was used to determine the total leukocyte number.

2.2.2 Differential Leukocyte Counts

Cells were stained for morphological examination after preparing blood films on 1" x 3" bev-l-edge blood smear slides (Propper Manufacturing Co., Inc.). Camco Quik Stain (American Scientific Products) was employed to differentially stain leukocytes. The percentage of lymphocytes, polymorphonuclear neutrophils, and monocytes was determined by counting 100 cells from a stained blood smear.

2.2.3 Hematocrit Determination

Packed red blood cell volume was determined by filling heparinized microhematocrit capillary tubes (American Scientific Products) with whole blood and centrifuging for 6 minutes in a hematocrit centrifuge (Clay Adams, Inc., New York).

2.2.4 Direct Hemagglutination

The levels of IgG and IgM SRBC antibody in all serum samples was determined using direct hemagglutination. For the detection of the total antibody level (IgM and IgG), serial two-fold dilutions of the serum were prepared in 96-well conical bottomed microtiter plates (Flow Laboratories, Inc., Hamden, Conneticut) using 0.025 ml saline containing 1:100 v/v normal rabbit serum (NRS) as the diluent. IgG levels were determined by modifying the diluent to contain 0.2 M 2-mercaptoethanol (2-ME, Sigma Chemical Co., St. Louis, Missouri). Since 2-ME is a reducing agent which selectively cleaves the disulfide bridges in the IgM molecule, the titer resistant to this agent was taken to indicate the presence of IgG in the serum. A 0.025 ml aliquot of 2.0% SRBC suspended in saline was mixed with the serial dilutions of the anti-serum and incubated at room temperature for 2-4 hours. Titer was recorded as the reciprocal of the highest dilution of antibody exhibiting agglutination.

2.2.5 Enumeration of Antibody-Forming Cells (AFC) by the Jerne Plaque Assay

The number of cells secreting SRBC specific IgM and IgG was measured using the Cunningham and Szenberg modification (6) of the Jerne plaque assay (25). Spleen cell suspensions were prepared from mice immunized with SRBC five days prior to the assay for AFC. To prepare spleen cell suspensions, the spleen from each animal was removed and placed on top of a stainless steel 60 mesh screen (Small Parts, Inc., Miami, Florida), minced through the screen with a 1 ml plastic syringe plunger, and washed into a 15 ml disposable centrifuge tube with RPMI 1640 medium (Grand Island Biological Co., (GIBCO), Grand Island, New York) supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Maryland) previously heat inactivated (56°C for 30 minutes) and absorbed with SRBC. Using the direct assay to detect IgM AFC, a mixture consisting of 75 ul of the spleen cell suspension, 25 ul SRBC absorbed complement (Difco Laboratories, Detroit, Michigan), 125 ul SRBC (10^9 cells/ml), and 25 ul of the supplemented RPMI 1640 medium was made. The increased number of AFC obtained after substituting 25 ul monospecific rabbit anti-mouse IgG for the 25 ul RPMI 1640 were counted as IgG AFC. Slides were incubated for 30 minutes at 37°C. Data was expressed as the number of IgM or IgG anti-SRBC AFC/10^6 lymphoid cells.

2.2.6 Mitogen-Induced Lymphocyte Proliferation Assay

Animals from the three toxaphene exposure groups were sacrificed and the entire spleen from each animal removed and placed in a 60-mm sterile, disposable petri dish (Falcon, Oxnard, California). The spleens were then individually placed on top of a sterile stainless steel 60 mesh screen (Small Parts, Inc.) and minced through the screen with a 1 ml plastic syringe plunger and washed into a 15 ml sterile disposable centrifuge tube with RPMI medium 1640 with 25 mM HEPES buffer and L-glutamine (GIBCO) supplemented with 5% inactivated fetal calf serum (FCS, GIBCO), 0.5 ml of 2 mM L-glutamine per 100 ml (GIBCO), 100 units/ml penicillin (GIBCO), 100 ug/ml streptomycin (GIBCO), and 26 ug/ml kanamycin (GIBCO).

The cell suspensions were placed on ice for at least 10 minutes to allow large cell aggregates to settle, the supernatant suspension transferred into a second sterile centrifuge tube, washed by centrifugation at 180-200 x g for 10 minutes, and resuspended in 2 ml RPMI 1640 medium supplemented with inactivated 10% FCS and the previously mentioned additives. Cells were enumerated using a Coulter counter and diluted to 9×10^6 cells per ml for the LPS-stimulated cell cultures and 3×10^6 cells per ml for the Con A-stimulated cell cultures. Two hundred microliters of the cell suspension (1.8×10^6 cells/well for LPS-stimulated cultures and 6×10^5 cells/well for the Con A-stimulated cultures) were

dispensed into sterile, flat-bottomed, 96-well tissue culture plates (Flow Laboratories, Inc., McLean, Virginia). Twenty microliters of saline or of either of the mitogens, 250 ug/ml Escherichia coli 055:B5 lipopolysaccharide W (LPS, Difco, Detroit, Michigan) or 100 ug/ml Concanavalin A (Con A, Sigma Chemical Co., St. Louis, Missouri) was added to individual wells of the microtiter plate with each sample being done in triplicate.

Plates were incubated for 48 hours at 37°C in a humidified 5% CO_2-95% air atmosphere before pulsing with 1 uCi of (^{3}H)-thymidine (specific activity 2.0 Ci/mM; Amersham/Searle, Arlington Heights, Illinois) in a volume of 1 ul. Cultures were incubated for an additional 18 hours and then harvested using a Model M12 BRANDEL cell harvestor (Gaithersburg, Maryland) onto Reeves Angel glass fiber filter paper (BRANDEL). The filter paper was placed in scintillation vials, allowed to air dry, and 5 ml of scintillation cocktail added. The scintillation cocktail consisted of 2 liters toluene (Eastman Kodak Company, Rochester, New York), 15.5 g PPO (New England Nuclear, Boston, Massachusetts), 0.5 g POPOP (New England Nuclear), and 1 liter triton X-100 (New England Nuclear). Tridiated thymidine incorporation was measured using a Beckman liquid scintillation counter.

2.2.7 Antigen-Induced Lymphocyte Stimulation Assay

The protocol for the antigen-induced lymphocyte stimulation is identical to that of the mitogen-induced lymphocyte stimulation assay but with the following modifications:

Animals from the three exposure groups received an intraperitoneal immunization with 10^8 SRBC 14 days after the initiation of treatment and 13 days prior to sacrifice.

Cells were prepared as previously described and diluted to 3×10^6 cells per ml (6×10^5 cells per well).

Splenic lymphocytes were stimulated by the addition of 100 ug SRBC ghosts per well, prepared according to the Hanahan and Ekholm procedure (20). Incubation, pulsing, and counting procedures were identical with that of mitogen stimulation.

3. Biological Assay

3.1 LD_{50} Determination

Mice were challenged by the intraperitoneal route with a virulent strain of Salmonella typhimurium obtained from the Microbiology Department at South Dakota State University. Brain Heart Infusion (BHI, Difco Laboratories, Detroit, Michigan) slants were employed as the maintenance medium. Prior to each LD_{50} determination the organism was innoculated initially into BHI broth and incubated for 24 hours at 37°C. A 100 ul aliquot of the initial culture was then added to 5 ml of BHI broth and incubated 12-18 hours at 37°C. To determine the 50% lethal dose (LD_{50}), serial dilutions of the culture were prepared in saline and six mice injected

with 0.1 ml of each suspension. The number of injected organisms was determined by total viable cell counts on BHI agar. Total mortality was determined after 7 days and the number of organisms required to kill 50 percent of the treated mice calculated using the method of Karber (26). The methods of Irwin and Cheeseman (24) were used to calculate standard error and the 95 percent confidence limits.

4. Statistical Analysis

Significance from control values was determined by Student's t-test with significance accepted at $p<0.05$. All statistical analyses were completed using a Texas Instrument 58 calculator.

RESULTS

1. Evaluation of Immunotoxicity Employing Non-Specific Indicators

1.1 Body Weight

Because malnutrition can induce diminished immunological reactivity, the weight gain of control and experimental animals was monitored to detect the possibility of either decreased food consumption or impaired food utilization. Initial body weights and total weight gains of mice from each exposure group are summarized in Table 2.

There were no significant differences in the mean body weights of each set of control and experimental groups prior to treatment, indicating equivalent sized groups. At the termination of the experiment, the weight gain of all experimental groups was less than that of controls. A significant decrease ($p<0.05$) in weight gain was observed in T_{Ing} mice and in the T_{Dermal} group. A highly significant decrease ($p<0.01$) in weight gain was observed for mice in the T_{Emul} group.

1.2 Organ to Body Weight Ratios

The organ to body weight ratios for the thymus and spleen were determined and used to detect any effects of toxaphene on specific lymphoid organs. Organ to body weight ratios were also determined for the liver, kidneys, and lungs. These ratios for each toxaphene exposure group are presented in Table 3. All methods of toxaphene exposure produced a

Table 2. Effect Of Toxaphene On Body Weight and Weight Gain During Toxaphene Exposure Periods

GROUP	BODY WEIGHT[a] grams		WEIGHT GAIN[a] grams/mouse
	Initial	Final	
Ingestion:Control (C_{Ing})	12.4 ± .70	24.7 ± 3.1	12.4 ± 3.4
Ingestion:Toxaphene (T_{Ing}) 30mg/Kg/day	11.8 ± 1.0	21.6[b] ± .67	9.8[b] ± .93
Dermal:Control (C_{Dermal})	23.2 ± 1.6	27.2 ± 3.0	4.0 ± 1.8
Dermal:Toxaphene (T_{Dermal}) 5mg/day	22.7 ± 1.9	24.7 ± 2.2	2.0[b] ± 1.1
Emulsion:Control (C_{Emul})	14.3 ± 2.4	26.6 ± 3.2	12.3 ± 2.3
Emulsion:2% Toxaphene (T_{Emul})	16.1 ± 2.7	23.9[b] ± 1.8	7.8[c] ± 1.7

[a] Mean ± SD.

[b] $p \leq 0.05$.

[c] $p \leq 0.01$.

Table 3. Relative Organ Weights of Mice

Exposed to Toxaphene By Ingestion Or Dermal Application[a]

GROUP	Kidney / Body	Liver / Body	Lung / Body	Spleen / Body	Thymus / Body
Ingestion:Control (C_{Ing})	ND[d]	6.8 ± .65	.80 ±.07	.51 ± .07	.27 ± .07
Ingestion:Toxaphene (T_{Ing}) 30mg/kg/day	ND	8.7[c] ± .49	.88[b] ±.08	.59[b] ± .07	.29 ± .06
Dermal:Control (C_{Dermal})	1.6 ± .21	6.8 ± .44	.68 ±.07	.41 ± .09	.20 ± .05
Dermal:Toxaphene (T_{Dermal}) 5.0mg/day	1.5 ± .20	10.2[c] ± .80	.66 ±.12	.53[b] ± .09	.14 ± .08
Dermal:Control (C_{Emul})	1.6 ± .32	6.7 ± .55	.79 ±.05	.58 ± .10	.26 ± .12
Dermal:2% Emulsion (T_{Emul})	1.7 ± .09	8.6[c] ± .17	.91[c] ±.08	.59 ± .16	.22 ± .05

[a]Mean Percent ± SD.

[b]$p \leq 0.05$.

[c]$p \leq 0.01$.

[d]ND, not determined.

highly significant liver enlargement. Both the T_{Dermal} and T_{Ing} groups had a significant increase in the relative spleen weights; in addition, the T_{Ing} and T_{Emul} groups had significantly larger lungs. The relative weights of the thymus and kidneys were not affected by any route of toxaphene exposure.

1.3 Total and Differential Leukocyte Count

Both total and differential leukocyte counts were performed to determine the effects of toxaphene on the entire white blood cell population and to ascertain if toxaphene preferentially affected any specific class of leukocyte. These results are presented in Table 4. A severe leukopenia was observed in the T_{Ing} group; however, differential counts did not reveal any significant variations in the ratios of leukocyte subsets in this exposure group.

Even though the total leukocyte count was not affected, a significant decrease in the percentage of monocytes was observed in the T_{Dermal} group. The other subset ratios in this exposure group appeared normal.

No significant differences in the differential or total leukocyte counts were observed in the T_{Emul} group.

1.4 Hematocrit Determination

In Table 5, hematocrit values from the three toxaphene exposure groups are compared with their respective controls. Highly significant increases were observed in the packed red blood cell volume in both the T_{Ing} and T_{Emul} groups. The hematocrit value for the T_{Dermal} group was similar to that

Table 4. Differential and Total Leukocyte Counts of Mice Exposed to Toxaphene by Ingestion or Dermal Application[a]

GROUP	Lymphocytes (Percent)	Neutrophils (Percent)	Monocytes (Percent)	Total Leukocytes (x 10^6/ml)
Ingestion:Control (C_{Ing})	74.3 ± 7.5	14.9 ± 5.6	10.6 ± 3.7	17.9 ± 7.5
Ingestion:Toxaphene (T_{Ing}) 30mg/Kg/day	73.4 ± 5.3	17.4 ± 1.1	9.2 ± 6.0	4.1 ± 1.9[c]
Dermal:Control (C_{Dermal})	78.8 ± 4.8	16.8 ± 4.9	4.8 ± 0.96	5.9 ± 2.3
Dermal:Toxaphene (T_{Dermal}) 5.0mg/day	83.2 ± 4.6	14.6 ± 5.5	2.2 ± 1.6[b]	7.3 ± 2.0
Dermal:Control (C_{Emul})	80.8 ± 3.3	17.2 ± 3.6	2.0 ± 2.1	11.5 ± 3.7
Dermal:2% Emulsion (T_{Emul})	82.4 ± 7.2	16.4 ± 6.9	1.2 ± 1.3	14.3 ± 4.3

[a] Mean ± SD.

[b] $p \leq 0.05$.

[c] $p \leq 0.01$.

Table 5. Hematocrits Of Mice Exposed To Toxaphene By Ingestion Or Oermal Application[a]

GROUP	Hematocrit
Ingestion:Control (C_{Ing})	37.8 ± 1.8
Ingestion:Toxaphene (T_{Ing}) 30mg/Kg/day	41.8 ± 1.3[b]
Oermal:Control (C_{Dermal})	41.0 ± 4.0
Dermal:Toxaphene (T_{Dermal}) 5.0mg/day	40.4 ± 2.1
Dermal:Control (C_{Emul})	42.2 ± 1.6
Dermal:2% Emulsion (T_{Emul})	45.8 ± 1.6[b]

[a]Mean percent ± SE.

[b]$p \leq 0.01$.

of the control.

2. Immunotoxic Effect of Toxaphene on Humoral Immunity

2.1 Direct Hemagglutination

There was no significant difference in total antibody titers after a primary immune response to sheep red blood cells between control and toxaphene-treated mice in the two dermal toxaphene exposure groups. However, ingestion of 30 mg toxaphene per kilogram body weight significantly enhanced total antibody production (Table 6). Since the IgG titer was approximately the same after 2-ME treatment of the serum, it can be concluded that IgM was the antibody that was primarily affected by the toxaphene treatment.

2.2 Jerne Plaque Assay

Using the Jerne plaque assay, antigen-specific antibody-forming cells (AFC), isolated from the spleen, were enumerated. The response of toxaphene-treated mice to the immunogen, SRBC, was significantly suppressed in two of the three toxaphene treatment groups. Toxaphene ingestion resulted in significant suppression of the total (IgM and IgG) and the IgM AFC number in the spleen (Figure 2). The number of cells producing IgG antibody and the total AFC number was significantly reduced in mice receiving dermal exposure to 5.0 mg toxaphene per day (Figure 3). No significant alteration in humoral immunity was observed in the group receiving treatment with the 2% toxaphene emulsion (Table 7).

Table 6. Hemagglutination Antibody Titers In Normal And Toxaphene-Treated Mice[a]

GROUP	Total Anti-SRBC Titers (IgM + IgG)	Beta-mercaptoethanol Resistant Antibody (IgG)
Ingestion:Control (C_{Ing})	20 (9-43)	<4
Ingestion:Toxaphene (T_{Ing}) 30mg/Kg/day	342[b] (247-473)	6 (4-9)
Dermal:Control (C_{Dermal})	215 (201-231)	20 (15-26)
Dermal:Toxaphene (T_{Dermal}) 5.0mg/day	151 (118-192)	17 (14-22)
Dermal:Control (C_{Emul})	183 (149-223)	10 (9-12)
Dermal:2% Emulsion (T_{Emul})	198 (178-219)	10 (9-12)

[a]Geometric Mean (95% Confidence Intervals)

[b]$p \leq 0.01$.

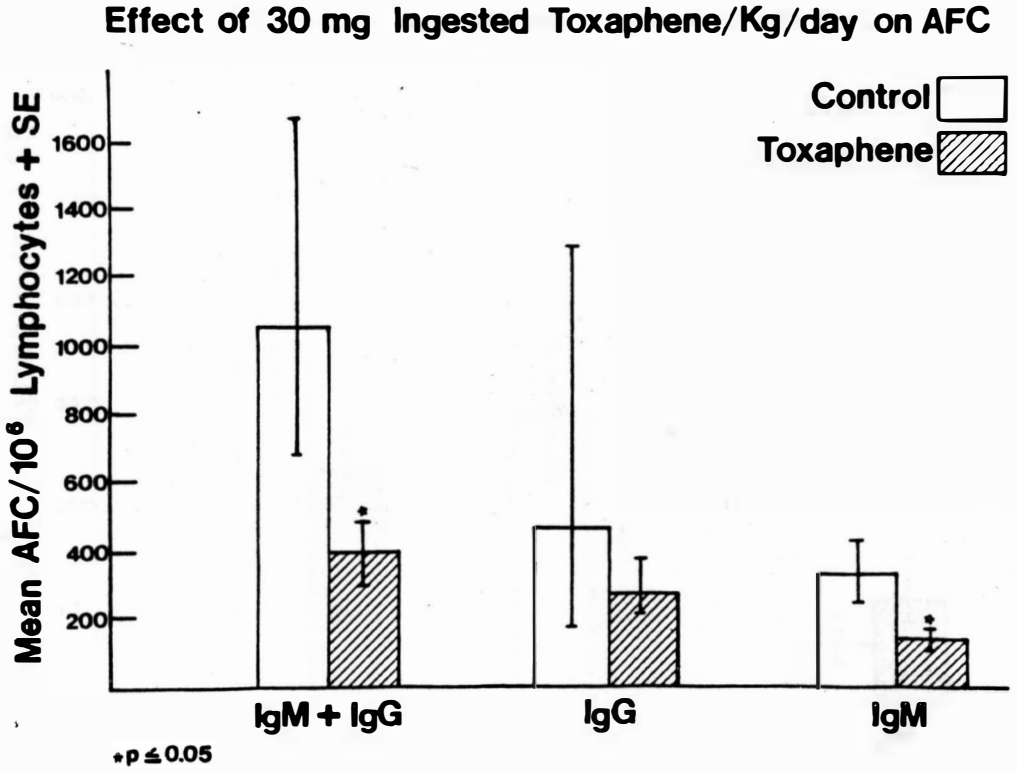


Figure 2. IgM and IgG anti-SRBC antibody-forming cells (AFC) in the spleen of control and toxaphene-treated mice. Each bar and vertical line represent values for the mean number AFC $\pm$ standard error of 6 mice per group 5 days after IP immunization with 10^8 SRBC.

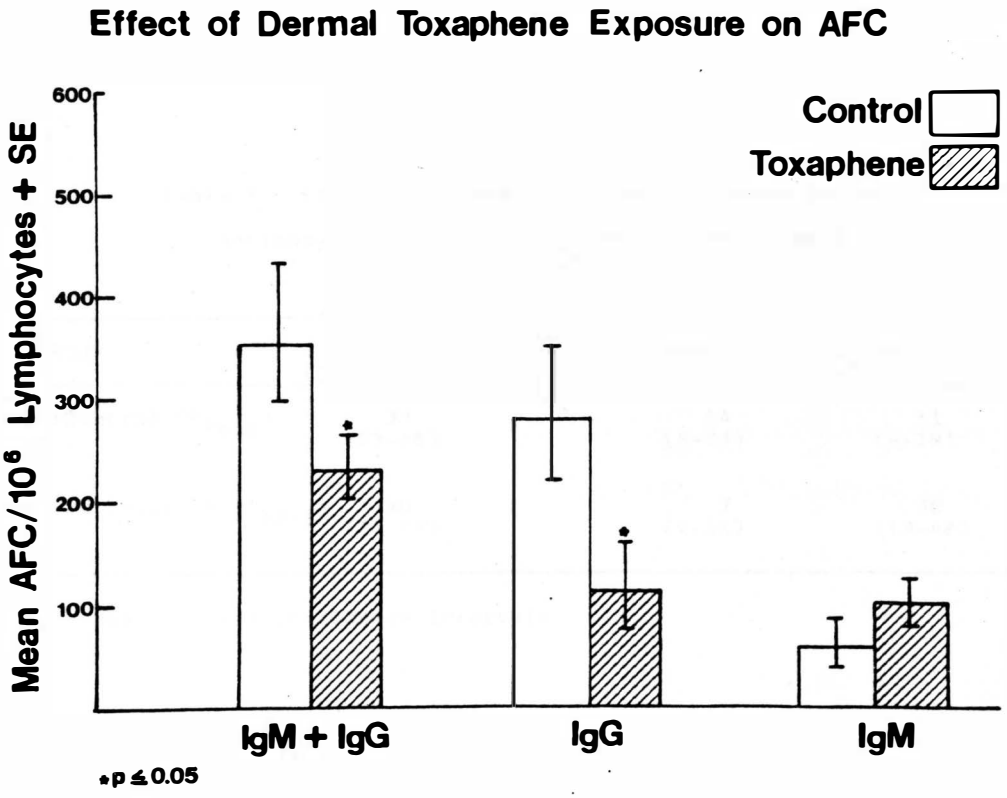


Figure 3. IgM and IgG anti-SRBC antibody-forming cells (AFC) in the spleen of control and toxaphene-treated (5 mg/day) mice. Each bar and vertical line represent values for the mean number AFC ± standard error of 5 mice per group 5 days after IP immunization with 10^8 SRBC.

Table 7. Effect Of Dermal Toxaphene Exposure On The Antibody-Forming Cell Response Of The Spleen[a]

GROUP	Total AFC (IgM and IgG)	IgM AFC	IgG AFC
Dermal:Control (C_{Emul})	33 (23-48)	14 (8-23)	11 (6-19)
Dermal:2% Emulsion (T_{Emul})	40 (27-59)	7 (4-11)	30 (19-48)

[a]Geometric Mean and 95% Confidence Intervals.

[b]$p \leq 0.05$.

[c]$p \leq 0.01$.

3. Immunotoxic Effect of Toxaphene on Cell-Mediated Immunity

3.1 Mitogen-Induced Lymphocyte Proliferation

The relative proliferation, as measured by the amount of ^{3}H-thymidine incorporated into cellular DNA, of splenic lymphocytes from toxaphene-exposed mice after polyclonal stimulation by the mitogens, Con A and LPS, is seen in Figure 4. The responsiveness of spleen cells to the B cell mitogen, LPS, was significantly altered in both of the dermal exposure groups. LPS-induced lymphocyte proliferation was significantly enhanced in animals exposed daily to 5.0 mg technical grade toxaphene. However, in the animals receiving a periodic total body exposure to the 2% toxaphene emulsion, LPS-induced lymphocyte proliferation was significantly reduced. The reactivity of splenic lymphocytes, after Con A stimulation, was not significantly altered in either group receiving dermal toxaphene exposure. No significant alterations in lymphocyte proliferation by either Con A or LPS was observed in the T_{Ing} group.

3.2 Antigen-Induced Lymphocyte Proliferation

The splenic lymphocytes of immunized mice from all treatment groups were isolated 13 days post-immunization. Figure 4 demonstrates the blastogenic activity of these cells after antigenic stimulation with SRBC ghosts. Either method of dermal toxaphene exposure suppressed antigen-specific cell-mediated immunity as indicated by the significant decrease in SRBC ghost-induced lymphocyte proliferation.

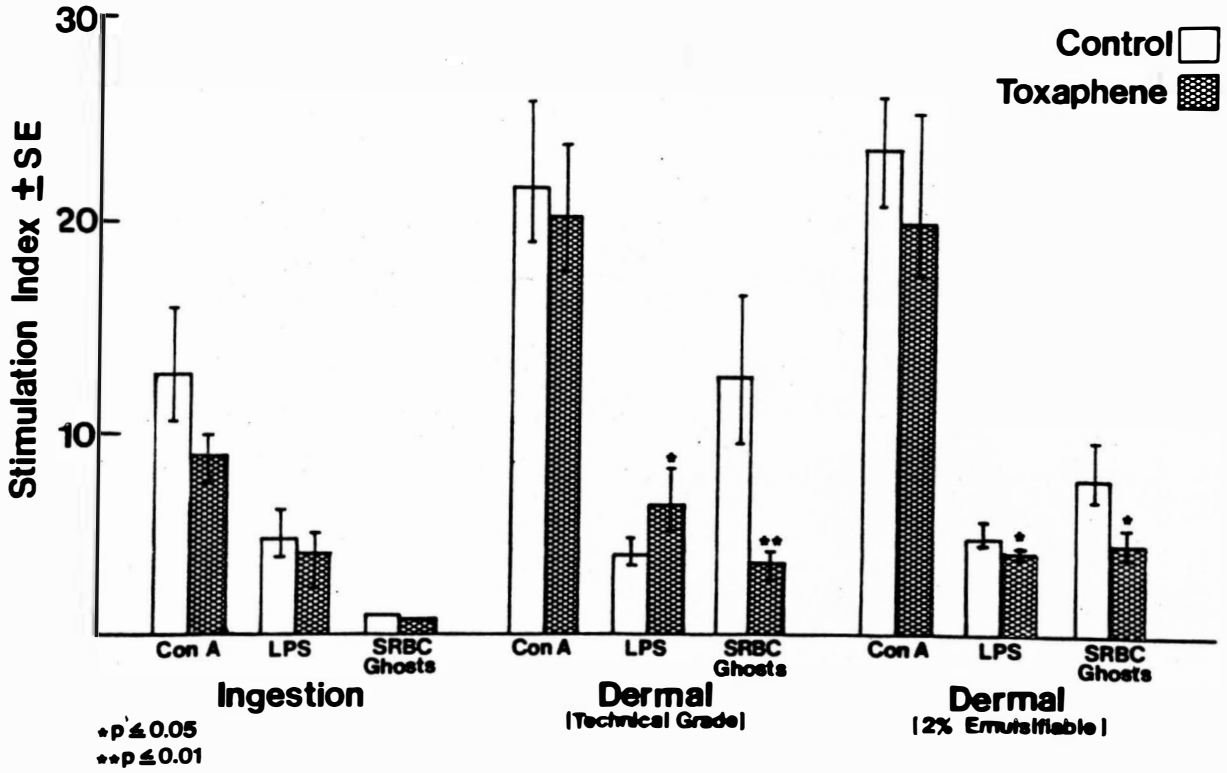


Figure 4. Stimulation index (cpm treatment/cpm control) of splenic lymphocytes isolated from control and toxaphene-treated mice for lymphocyte proliferation assays. The bars and vertical lines represent the mean stimulation index and standard error of six animals.

There did not appear to be any significant alterations in antigen-induced proliferation of splenic lymphocytes isolated from the T_{Ing} group.

4. LD_{50} Determination

To assess the effect of sublethal quantities of toxaphene on disease resistance, both control and toxaphene-treated mice were challenged with Salmonella typhimurium.

An increased susceptibility to Salmonella typhimurium was demonstrated in mice from the T_{Ing} group. The LD_{50} values of Salmonella typhimurium in this group were 4500 (1000-20,000) as compared to 14,000 (4000-53,000) for that of the respective controls.

A decreased susceptibility to Salmonella typhimurium or a lower LD_{50} value was demonstrated in mice receiving the 2% emulsifiable toxaphene treatment. The LD_{50} for mice receiving this toxaphene exposure was 15,000 (2500-89,000) as compared with the control value of 7000 (1000-43,000).

There was no difference in the LD_{50} of the T_{Dermal} and C_{Dermal} groups. The corresponding LD_{50} values were 3000 (500-24,000) and 5000 (1000-28,000), respectively.

DISCUSSION

1. Nonspecific Indicators of Immunity

In order to assess whether a given compound is directly responsible for any observed immunosuppression or immunoenhancement, various nonspecific factors must be evaluated to exclude the possibility that the alterations in immunity are not due to indirect effects of the compound.

1.1 Body Weight and Weight Gain

It is believed that the immunological response is a more sensitive parameter for general malnutrition than is a decrease in body weight (63). To ensure that toxaphene exposure did not induce a state of malnutrition, the weight gain of mice was monitored. Even though the mice exposed to toxaphene appeared clinically normal, the weight gain of toxaphene-treated mice was statistically less than that of the controls in each of the three toxaphene exposure groups. However, since both the relative spleen and thymus weights of exposed animals were statistically greater than or equal to those of control animals, it was assumed that these mice were not suffering from malnutrition.

In a previous study (33), food consumption and weight gains of mice force-fed corn oil, the diluent used in the toxaphene ingestion experiment, were monitored and compared with mice receiving saline by gavage. No significant difference ($p<0.05$) was observed on either food consumption or weight gain. This indicated that corn oil alone did not appear to

depress weight gain by inadvertently suppressing food intake.

1.2 Organ to Body Weight Ratios

Gross examination of lymphoid tissues is usually completed at the termination of a routine immunotoxicity study. Alterations in immunoreactivity may be indicated by visible changes as well as alterations in the organ weights, relative to the body weights, of the animals exposed to a given chemical.

Mice in the toxaphene-treated groups appeared to have suffered from generalized toxicity. This conclusion is based on the observation that the relative liver weights of mice exposed to toxaphene were significantly greater than controls. Because the liver is the primary detoxifying organ of the body, this increase in the relative liver weight is not surprising. The increase in the relative liver weight indicates that the toxaphene was able to exert some biological effect on the liver despite the relatively short period of administration. Similar pesticide-induced effects on the liver are not uncommon and have been observed in mice administered TCDD (60), guinea pigs exposed to PCB (62), and chickens fed DDT (18).

The relative spleen and thymus weights were not diminished by any of the three toxaphene treatments. This indicates that toxic damage to the lymphopoietic system was not a major effect of toxaphene treatment.

An unexpected observation was the increased lung to body

weight ratios in the T_{Ing} and T_{Emul} exposure groups. This might have resulted from the occurrence of lung edema resulting from the aspiration of toxaphene into the lungs of the T_{Ing} exposure group during force-feeding or inhalation of toxaphene by the T_{Emul} group following treatment.

1.3 Effect of Toxaphene on White Blood Cells

A highly significant leukopenia, the decrease in the total number of circulating white blood cells, was observed in T_{Ing} mice. This observation suggests that toxaphene may be toxic to the majority of the white blood cell population. The reduction in white blood cells resulted from decreases in the normal numbers of all or some of the subsets of white blood cells and could therefore result in immunosuppression due to impaired antigen recognition, production of antibodies, or the phagocytosis of foreign antigens.

Monocytes, a specific subset of leukocytes, were more affected by dermal exposure to technical grade toxaphene than any other method of exposure. A significant decrease in this cell type would seriously affect the phagocytic efficiency of foreign materials in the body and thereby suppress host resistance to bacterial infection (51).

1.4 Effect of Toxaphene on Hematocrit Values

Because exposure of the hematopoietic system to a toxic chemical such as toxaphene may cause anemia, hematocrit levels were determined to detect any possible toxaphene-induced abnormalities. The packed cell volume of the T_{Ing}

and T_{Emul} treated mice was significantly higher than in the controls. The hematocrit values observed in the T_{Dermal} group were unaffected. These results indicate that anemia, as measured by packed red blood cell volume, was not a consequence of toxaphene exposure.

2. Specific Indicators of Immunity

2.1 Humoral Immunity

2.1.1 Hemagglutination Titers

Immunotoxicity is frequently measured by quantitating serum antibody titers. The ability of exposed animals to synthesize antibodies following immunization is a direct measure of humoral (B cell) immunity. However, depending upon the immunogen used, antibody production may also be an indirect measure of T cell reactivity since T cell cooperation is required for many substances. The antibody response to SRBC is such an immunogen. A normal immune response to SRBC will be obtained only if there are sufficient numbers of macrophages to process the immunogen and enough T cells to interact with the processed immunogen and subsequently present it to the B lymphocytes which ultimately synthesize the antibody.

A highly significant increase in SRBC antibody was observed in mice of the T_{Ing} group. This could result from either an adjuvant effect or the cytotoxicity of toxaphene for antibody-containing lymphocytes. In view of the toxaphene-induced leukopenia this latter explanation is

probably the correct explanation. No changes were observed in the antibody titers of mice in either the T_{Dermal} or T_{Emul} groups when compared to the controls. This indicates that there were sufficient macrophages, T, and B lymphocytes present to interact and produce a normal immunologic response. Such an explanation is supported by the observation that these groups did not suffer from any significant leukopenia.

2.1.2 Jerne Plaque Assay

Antigen-specific antibody synthesis was examined at the cellular level by the Jerne plaque assay. This technique is able to enumerate antibody-forming cells (AFC). The antibody response, as measured by this technique, is a more sensitive method for detecting immunosuppression than direct hemagglutination. This procedure is able to directly determine the number of IgM AFC. By modifying the technique, it can also be used to indirectly quantitate plasma cells secreting any of the other immunoglobulin classes.

A decrease in both IgM AFC and total AFC was found in toxaphene fed mice. This observation substantiates the theory presented for the highly significant increase in SRBC antibody as measured by hemagglutination. The decrease in both IgM AFC and total AFC, found in toxaphene fed mice, suggests that toxaphene is cytotoxic for antibody-containing lymphocytes. Because these data indicate that ingestion of toxaphene has a significant effect on IgM AFC, it can be

concluded that levels of serum IgM will also be diminished during an immune response. Since IgM is the first antibody produced during the primary immune response, an animal exposed to toxaphene would not be as capable of eliminating an antigen as an animal not exposed to toxaphene.

The T_{Dermal} treatment group also exhibited suppression of humoral immunity as measured by the Jerne plaque assay. This treatment group experienced suppression of both the total AFC number and the number of IgG AFC in the spleen. These results suggest that dermal toxaphene exposure may inhibit maturation of the immune response. Since T cells are responsible for the switch from IgM to IgG production by B cells, this indicates that either the function of T cells or the ratio of macrophages, T, and B lymphocytes may be somewhat affected. This is suspected since the total number of spleen cells obtained from toxaphene treated mice was essentially the same as the number obtained from controls.

Dermal exposure to the 2% toxaphene emulsion produced no significant alterations in humoral immunity as detected by the Jerne plaque assay.

2.2 Cellular Immunity

2.2.1 Mitogen-Induced Lymphocyte Proliferation

In vitro culture techniques are used to assess the immune competent cell response to specific mitogens. The lymphoproliferative response of mouse lymphocytes to T or B

cell mitogens is a sensitive indicator of immune depression resulting from cell depletion, maturation impairment or the induction of suppressor cells (10). T cells are selectively stimulated by the mitogen Concanavalin A. LPS is a mitogen used to evaluate B cell responsiveness. Blastogenesis was determined by the amount of ^{3}H-thymidine incorporated into cellular DNA.

The *in vitro* blastogenic response of splenic B cells to LPS was observed to be significantly enhanced in the T_{Dermal} exposure group. An increased LPS response was not expected due to the previously discussed results of the Jerne plaque assay which suggested suppression of humoral immunity. From these results it may be concluded that T_{Dermal} exposure may suppress the immunological activity of effector cells which are required in a normal humoral immune response.

LPS-induced lymphocyte proliferation was significantly decreased following dermal exposure to the 2% toxaphene emulsion. A dysfunction in humoral immunity had not been indicated in earlier studies in which normal antibody titers and antibody-forming cell numbers were observed.

The responsiveness of lymphocytes, from mice in either group receiving dermal toxaphene exposure, to Con A was not significantly altered in this investigation. This suggests that the subpopulations of T cells, which are stimulated by Con A, are capable of normal proliferation following toxaphene exposure.

2.2.2 Antigen-Induced Lymphocyte Proliferation

The SRBC-induced lymphocyte proliferation assay was an additional method used to test for defects in T cell reactivity. There was a highly significant decrease in SRBC proliferation in both T_{Dermal} and T_{Emul} mice indicating a malfunction in cell-mediated immunity in both groups. It was significant to note that the only immunological parameters affected by emulsifiable toxaphene exposure were the suppression of both SRBC antigen-specific and LPS-induced lymphocyte proliferation. This selectivity indicates that only certain subsets of B and T lymphocytes were affected by this treatment.

3. LD_{50} Determination

Determining host resistance to infection following exposure to chemicals that are suspect in altering normal immune functions, is an important parameter in studying immune competence. Thigpen et al. (51) demonstrated that low levels of TCDD which did not produce clinical or pathological change resulted in enhanced susceptibility to Salmonella bern. In this study it was demonstrated that levels of toxaphene which do not produce clinical or pathological signs still have the capacity to alter the host defense system. The data presented establishes that mice ingesting daily doses of toxaphene and subsequently infected with Salmonella typhimurium have a significantly higher rate of mortality. Host defense factors which could

be responsible for the increased susceptibility to S. typhimurium include: 1) a defect in cell-mediated immunity due to a loss of accessory cells or by inhibition of cell functions; 2) suppression of the humoral immune response; 3) inhibition of phagocytosis; and/or 4) a reduction or loss of functional phagocytic cells (51).

The decreased susceptibility to S. typhimurium in the T_{Emul} group is a surprising phenomenon. The low levels of emulsifiable toxaphene administered in this experiment may have acted as an adjuvant and significantly enhanced one or more immune parameters specifically responsible for defense against a bacterial challenge with S. typhimurium.

Because humans may be exposed to environmental contaminants such as toxaphene these findings which prove that immunosuppression occurs following toxaphene exposure are very important. A serious threat on the health of humans is actually being indicated. Livestock which are treated with toxaphene for ectoparasite control may become immunosuppressed rendering them more vulnerable to infection, diseases, and malignancy. This reduced immunological reactivity could ultimately result in poor weight gains in livestock and increased production costs.

4. Summary of Immunotoxic Effects of Toxaphene

Toxaphene is an immunosuppressive agent when administered by either direct dermal contact or ingestion. Depending upon the route of exposure, toxaphene has the ability to

inhibit humoral immunity, cell-mediated immunity, or both.

Significant suppression in both the humoral and cell-mediated immune responses resulted from daily dermal exposure of 5.0 mg oil-base toxaphene. Ingestion of 30 mg/kg/day of oil-soluble toxaphene also resulted in suppression of humoral immunity; however, an observable effect on cell-mediated immunity was not noticed. The impaired immunological responsiveness observed in the ingestion and dermal application studies occurred at relatively high concentrations of toxaphene not normally found in nature but because toxaphene is known to accumulate and persist in adipose tissue its continued use could represent a potential problem.

The simulated "dip" exposure with a 2% solution of emulsifiable toxaphene resulted in suppression of cell-mediated immunity without an observable effect on humoral immunity. The 2% toxaphene emulsion applied dermally to research animals in this experiment is identical to the concentration employed as a contact pesticide for livestock ectoparasite control. Because this toxaphene exposure caused suppression of cell-mediated immunity in mice it is possible that cell-mediated immunity would also be suppressed in livestock rendering them vulnerable to infection, disease, or malignancy. Any subclinical infection could affect weight gains and result in an increase in production costs.

It has been shown in this investigation that toxaphene is an immunosuppressant. These findings indicate that

extreme caution is advised for all application and storage procedures.

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