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THE EFFECTS OF TRI-O-TOLYL PHOSPHATE (TOTP) ON THE

IMMUNE SYSTEM OF MICE

by

Craig R. Brinkerhoff

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

of

MASTER OF SCIENCE

in

Toxicology

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1980

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Craig R. Brinkerhoff

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ABSTRACT

The Effects of Tri-O-Tolyl Phosphate (TOTP) on the
Immune System of Mice

by

Craig R. Brinkerhoff, Master of Science
Utah State University, 1980

Major Professor: Dr. R. P. Sharma
Department: Toxicology

TOTP is a prevalent industrial and environmental contaminant which has been shown to be a neurotoxic agent. This study was undertaken to investigate the effects of this compound on the immune system. Several techniques were employed to test the humoral as well as cellular effects. Male Swiss Webster mice were administered by gastric gavage with three doses of TOTP (5, 50, and 500 mg/kg) and one dose TMTP (50 mg/kg) in corn oil once a week for 13 weeks. Control animals were given corn oil alone.

Lymphocyte transformation was determined on cultures of splenic cells obtained from animals sacrificed at 1, 4, 8, and 13 weeks. Relative Proliferation Index (RPI) and Stimulation Index (SI) was calculated for phytohemmagglutinin (PHA), pokeweed (PWM), and lipopolysaccharide (LPS) mitogen treated cultures. Proliferation was measured by uptake of radioisotope labeled thymidine.

Plaque formation as well as delayed hypersensitivity was evaluated at all dose levels after 4 weeks of treatment. Humoral effects were determined utilizing quantitative (Rocket) immuno-electrophoresis. Serum was compared with control serum for IgA, IgG, and IgM fractions.

TOTP was found to cause slight suppression in proliferation of splenic lymphocytes at 8 and 13 weeks although this suppression was non-specific and not dose related. No effects were seen on body and organ weights. Plaque forming cells and delayed hypersensitivity were not affected either by dose or time of treatment. Immunoglobulin fractions tested were similarly not affected by time or dosage. These findings suggest that neither TOTP nor TMTP exhibit immunotoxic effects at levels used.

(47 pages)

INTRODUCTION

Tri-o-tolyl phosphate (TOTP or TOCP) is of interest because it is an adulterant of flavor extracts and cooking oils and a contaminant of tricresyl phosphate which is widely used as industrial oils, plasticizers, lubricants and fuels (Patty 1963). Thus, exposure can occur in manufacturing aryl phosphates through inhalation and skin contact as well as ingestion of contaminated food (Tabershaw and Kleinfeld 1957, Patty 1963). It has also been of concern to those working with these cresyl containing compounds such as workmen on aircraft hydraulic systems (Baldridge et al. 1959, Patty 1963). It is a continuous health hazard because contamination can occur at any point in shipping and handling of these compounds.

TOTP is a compound which closely resembles organophosphate pesticides. This makes TOTP doubly important not only as an environmental contaminant, but as an experimental compound to investigate the toxic effects of phosphate pesticides.

Pesticides occupy a unique position among the many chemicals man encounters daily, in that they are deliberately added to the environment to kill or injure some form of life. Ideally, their actions would be highly specific for undesirable organisms. However, most of the chemicals that are used as pesticides are not highly specific and are generally toxic to many non-target organisms, including man. The mortality rate of man attributed to

poisoning by pesticides has been estimated at 0.65 per million population in the United States, but it has also estimated that there are 100 nonfatal poisonings for each fatal one. Organophosphate pesticides are widely used and their property of relatively rapid degradation in the environment encourages even wider use as replacements for the more persistent organochlorine compounds.

The primary mode of toxic action of all organophosphorus pesticides, to a large degree, has been attributed to the inhibition of cholinesterase enzymes. The immediate cause of death in fatal poisonings being asphyxia resulting from respiratory failure. However, certain pesticide and related organophosphate compounds, including TOTP, possess an additional toxic effect. This effect is characterized by nervous system lesions that result in a form of paralysis which usually appears one to two weeks following exposure. Previous studies have concentrated on esterase inhibition in attempting to elucidate the mechanism of action. To date, no mechanism has been proposed which satisfactorily accounts for the neurotoxic syndrome.

Many studies have been done using TOTP concentrating on neurotoxicity and metabolism, but relatively little has been done concerning the immunological effects. The widespread use of organophosphate chemicals as pesticides and industrial compounds has created much concern as to their possible chronic as well as acute effects on the immunologic response of the exposed individuals.

It has been suggested that neurotoxic effects of chemicals may be related to an autoimmune phenomenon (O'Brien 1967). The

involvement of autoimmunity in TOTP induced neurotoxicity in chickens was studied by Watanabe and Sharma (1977). Their results failed to demonstrate primary involvement of an autoimmune nature.

An increase of chicken lymphatic filtration areas in spleen and liver was reported by Watanabe and Sharma (1976) but other organs did not show a similar response when exposed to TOTP. Also both total plasma levels and gamma-globulin:albumin ratios were greater in treated animals which suggests an immunological stimulation. However, there was no indication of any specific antibody present in plasma or organs (Watanabe and Sharma 1976).

The effects of chemicals on the immune system can be very damaging. Chemicals can produce autoantibodies which can produce dysfunction to parts of the body, or they may cause a malfunction in the normal antibody producing cells, thus greatly reducing the defense mechanisms of the body. The potential for immunological reactions due to exposure to these environmental and industrial chemicals exists. The immune system is a sensitive index of chronic toxicity, and should be considered in the toxicological evaluation of these chemicals.

REVIEW OF LITERATURE

Tri-o-tolyl phosphate has been widely used to study the delayed neurotoxicity produced by certain organophosphate compounds. Based on the LD50, the anti-esterase TOTP metabolites were reported to cause paralysis at lower concentrations than TOTP in both mice and chickens (Bleiberg and Johnson 1965). This raises the question of whether unmetabolized TOTP is capable of producing paralysis without prior metabolism in other organs. Watanabe and Sharma (1975) showed that TOTP is capable of directly producing degenerative changes of nervous tissue in culture, although metabolized TOTP and DFP was observed to be more potent than TOTP itself. It is possible that TOTP is metabolized in the tissue culture. Morazain and Rosenberg (1970) reported that spinal cord tissues of chicken was incapable of converting TOTP to its anti-esterase metabolites in vitro, nor do the metabolites accumulate in these tissues.

Toxic Effects

Distribution data obtained from an earlier study involving TOTP indicates significant accumulation of TOTP in the nervous tissue of the chicken. Further, the concentration in these tissues increased throughout a period of two weeks. The general trend in other tissues was to increase over 3-4 days then decline (Watanabe and Sharma 1973).

TOTP has been shown to potentiate the effects of other compounds such as malathion (Murphy et al. 1959). TOTP markedly increased the susceptibility of rats to malathion regardless of route of administration which is explainable on the basis of subsequent experiments which demonstrate that TOTP inhibits detoxification of malathion.

The hydrolysis of acrylate esters and acrylic acid in rat plasma and liver homogenates was significantly inhibited by treatment with TOTP. Apparently tissue carboxyesterases are involved in the detoxification of methyl acrylate and ethyl acrylate and that potentiation of acrylate ester toxicity may occur when carboxy-esterase activity is inhibited (Silver and Murphy 1978).

Animal Studies

Laboratory animals also show delayed paralysis of the upper and lower extremities. Mice were reported to be 1/5 to 1/25 as susceptible to TOTP as cats, dogs, rabbits, etc., with a LD50 dose of 2.8 ml per kg body weight (Hodge and Sterner 1943). Rats were shown to have an oral LD50 of 3000 mg/kg (Hodge and Sterner 1943), and 2500 mg/kg when administered in single doses intraperitoneally (Murphy et al. 1959). Meyers and Mendal in 1953 found that TOTP inhibited aliesterase activity of liver in rats. In the chicken, TOTP causes pathological alterations in the peripheral nervous system and definite lesions in the spinal cord (Bischoff 1959).

Pathologic Studies

Histopathologic studies of the peripheral nerves show that the distal fibers are affected earlier and more severely than proximal fibers. There is also a tendency of the large-diameter fibers to be affected more than small diameter fibers. Damage has also been reported of structures of the central nervous system, particularly the spinal cord where changes were characterized by occasional absence of the nucleolus, migration of the nucleus to the periphery and formation of the fine fatty granules in the cytoplasm of the cell. The results indicated that the multiple neuritis of this paralysis is essentially a degradation of the myelin sheaths of the peripheral nerves with a variable amount of relatively moderate central degenerative changes affecting the anterior horn cells throughout the spinal cord but more often in the lumbar and cervical regions (Smith and Lillie 1931).

This pattern of degeneration has come to indicate that a "dying back" process is occurring in these axons, and this is basically similar to certain distal degenerative conditions long recognized in man. It is assumed that in the "dying back" process there is a malfunction of the nerve cell that causes it to fall in maintaining its enormously long axon due to disturbed metabolism. Thus nutrients are not synthesized and transported at a sufficient rate to maintain the long axon of the peripheral nerves. It is also assumed that the metabolic process is disturbed to some degree in all nerve cells, but, with the doses of agent used or possible,

the degree of disturbance is such as to impair the survival of only those fibers with the greatest demand for the maintenance process (Cavanagh 1969).

The general response to oral or parenteral administration of both alkyl or aryl phosphates has been extensively reviewed. The onset of functional changes begins in the distal parts of the lower limbs, both in man and in all other sensitive animals, with mild sensory disturbances and motor weakness. This phenomena steadily progresses over the next few days, both in severity and in extent, and leads to increasing weakness and flaccidity of the legs and variable amounts of sensory disturbance of the 'stalking' type. The upper limbs may also be involved towards the end of this progressive phase of the poisoning, and again more changes are noted distally than proximally. After about three weeks the peak of the process is reached, and thereafter there is a steady improvement in the functional disturbances. Only in the mildest instances is recovery complete; a degree of residual disturbances may remain, due to variable amounts of damage to long spinal tracts that never recover, despite nearly complete regeneration of peripheral axons (Cavanagh 1969).

Epidemiology

Electrophysiological examinations performed on patients following poisoning with TOTP suggested the existence of the clinicoelectrophysiological forms of neuropathy. (1) The predominantly spinal form is characterized by a clinical picture

resembling that in amyotrophic lateral sclerosis, a long course and persistence of residual signs of neuropathy for several years after ingestion of the toxic substance. (2) The predominantly neuropathic form is mixed, sensorimotor polyneuropathy, predominantly motor and distal, involving especially the terminal parts of longer (peripheral) nerves. The electrophysiological data suggested a mixed process of demyelination and axonal degeneration, and that patients in the later stages of the disease showed that chances of recovery were good for those with PNS signs, but poor for those with CNS signs (Vasilescu 1979).

Case Studies

The first and classic cases of TOTP poisoning reported in the United States resulted from the drinking of liquors containing an illicit extract of Jamaica ginger used to circumvent the prohibition laws. Typically the victims reported onset of symptoms within 10 to 14 days after drinking the extract. Early symptoms included cramping muscular pain in the legs, followed by weakness. In most victims, quadriparesis and often frank quadriplegia with foot and wrist drop developed. Examination usually showed muscular weakness and wasting, with flaccidity and hypoactive reflexes most noticeable in the feet and legs; however, there were often diminished reflexes in the arms and hands. The typical syndrome was identified as a polyneuritis, sometimes called "motor neuritis" because of the paucity of sensory findings (Morgan 1978).

Mass outbreaks of TOTP poisoning have occurred more recently in other countries. A dramatic poisoning which occurred in Morocco in 1959 was a result of contaminated "olive oil" used in cooking. The contaminating lubricating oil sold as such was man-made and was synthesized to withstand the very high temperatures required in turbo-jet aircraft engines (Smith and Spalding 1959). Another serious epidemic occurred in the Quang Tri Province of Viet Nam during 1970-71. The onset of this "Jake walk" was associated with the use of "black market" cooking oil, which on investigation proved to be a tri-o-tolyl phosphate containing U.S. military aviation lubricant supplied to South Vietnamese helicopter units (Dennis 1977).

A case of permanent paralysis in a man engaged in manufacturing tricresyl phosphate was described by Bidstrup and Bonnell (1954). While the final product contained only 1 percent of TOTP, as much as 6-10 percent was present as a contaminant during manufacturing. No exposure data was given, although the patient had worked a total of 5 months in that occupation before symptoms of anorexia, nausea, and aching of the legs had developed (Patty 1963).

Related Phosphate Case Studies

There are several phosphate esters that produce delayed neurotoxic effects. One such case involved Tetra-ethyl pyrophosphate (TEPP) in 1967. Because of a high mean value for the percentage of toxic dose received per hour of work, workers who were involved with the transportation of TEPP were investigated. Although

there were several illnesses associated with this operation, the incidence was considered quite low in view of the relative hazard (Wolfe et al. 1967).

Great Britain reported several cases of paralysis where symptoms resembled very closely to those of TOTP poisoning. The paralysis developed in the third week after exposure to an organic phosphorous compound, bis-(mono-isopropylamino)-fluorophosphine oxide (Mipafox). Like other organic phosphates used as pesticides, Mipafox is an inhibitor of cholinesterase in vitro and in vivo (Bidstrup and Bonnell 1953). Mipafox was also reported to produce demyelination in nerve tissue of rats exposed for 3 to 4 months. Similar results were observed in chickens treated with TOTP (Majno and Karnovsky 1961).

A small crisis arose in the Bayport, Texas area in the early 1970's over the noted cholinesterase inhibitor Phosvel (Leptophos). Velsicol Chemical Corp. was the site of production where some 200 people were exposed. Several cases reported dizziness, blurred vision, numbness, and paralysis in the extremities (Alpern and Gram 1976). Leptophos has been shown to inhibit acetylcholinesterase, and monoamino oxidase. It causes irreversible symptoms of ataxia after 60 days exposure in lambs (El-Sebae et al. 1977).

Immunologic Studies Involving TOTP

Although many studies have been completed using TOTP concentrating on neurotoxicity and metabolism, relatively little has been done concerning the immunological effects. O'Brien (1967)

suggested that neurotoxic effects of chemicals may be related to an autoimmunity phenomenon.

In a previous study on the toxicity of TOTP in the chicken, histopathologic examination of various lymphatic-related organs showed a definite increase of diffuse lymphatic tissue in the spleen and liver of TOTP-treated animals. Other organs did not show a similar response. However, the relative frequency and distribution of diffuse lymphatic tissue throughout the visceral organs were not uniform, which would cause difficulty in any evaluation. In this same study both total plasma protein levels and gamma-globulin:ratios were consistently greater at all sampling periods in the TOTP-treated animals, compared with controls (Watanabe and Sharma 1976). The results presented, although suggestive of an immunologic stimulation in the chicken, did not indicate the presence of any antibody against TOTP present in plasma or other organs (Watanabe and Sharma 1977).

In a subsequent study the involvement of autoimmunity in the pathogenesis of TOTP induced neurotoxicity in chickens was investigated. Immunosuppressive therapy including gamma-radiation and drug treatment was used to determine its effect on the neurotoxic syndrome. Wholebody radiation failed to show any consistent protection from TOTP induced paralysis. Drug therapy also indicated no protection. Thus the study was unable to demonstrate primary involvement of an autoimmune nature in the neurotoxicity produced by TOTP in the chicken (Watanabe and Sharma 1977).

The Immune System

The immune system is composed of the cellular and humoral arms. Thus mediators of immune responses can be either freely diffusable antibody molecules of the immunoglobulin type in humoral immunity, or specifically reactive lymphocytes comprising the cellular type immunity. The immune system responds to many foreign or antigenic substances that enter the body. Their interactions with these antigens cause a production of antibodies (immunostimulation) or a decrease or destruction (immunosuppression) in the antibody response. Macrophages, although not antibody producers, play an important role in immunity because of their phagocytic properties, which also may be stimulated or inhibited by these antigens. The antibody forming response is initiated and regulated primarily by lymphatic cells derived from the bone marrow. Lymphocytes are relatively small, round, cells that are ubiquitous in blood, lymph, and connective tissues, and are of two fundamentally different kinds, B and T cells. The B cells have abundant surface membrane-bound immunoglobulin molecules which are restricted in a given cell to molecules of a particular allotype, accounting for the cell's selective response to a given antigen. Some of these B cells differentiate into mature plasma cells (with the aid of T cell induction), the most active of all lymphoid cells in the synthesis and secretion of Igs. Many also revert back eventually into small cells, which probably function as "memory cells". T cells, in contrast, have far fewer surface Igs and it is not certain that they are restricted in idiootype. The majority of the circulatory

lymphocytes are T cells. They can become specific effector cells for cell-mediated immune responses: they can destroy tumor cells, cause rejection of allografts, and promote the differentiation of resting macrophages into highly bacteriocidal cells capable of destroying microbial pathogens (Davis et al. 1973).

Incubation with certain antigens cause small lymphocytes with the corresponding surface receptors to undergo blast transformation. The cells enlarge; the nucleolus swells; polysomes, rough endoplasmic reticulum, and microtubules develop; and rates of macromolecule synthesis increase markedly. Similar changes are initiated by the binding of certain plant and bacterial mitogens to the surface of B and T cells. These mitogens are specific for various sugars of different surface glycoproteins which are distributed equally on B and T cells. However, the two cell types respond differently. Phytohemagglutinin (PHA), a plant mitogen, stimulates transformation particularly in T cells. Lipo-polysaccharide (LPS), an endotoxin of gram-negative bacteria, is mitogenic for B cells. A plant protein, pokeweed mitogen (PWM), stimulates transformation of both B and T cell types.

Immunotoxicology and Environmental Chemicals

There is no single immunologic test that can completely assess the immune function; the system is a complex variety of interactive cellular compounds whose functions are often studied by monitoring secondary or tertiary events. The existence of a complex "feed back" interaction is only now being revealed and is far from

understood. Toxicologists are in a quandry as to how to assess the immune system since they are inadequately trained in this area and are prone to conduct an immunotoxicity experiment that is not credible to the immunologist. Conversely, the immunologist possesses inadequate knowledge of chemistry or toxicology with the experimental results being exquisite immunological data that defies interpretation from a toxicologists point of view. Team efforts representing the best of both fields are essential (Moore 1979).

MATERIALS AND METHODS

TOTP is a proven neurotoxic and prevalent industrial and environmental chemical. Investigations were conducted to determine if the compound illicitly an effect on the immune system of mice. Several techniques were employed to test the cellular and humoral effects of repeated exposure of the animals to TOTP. The response of splenic lymphocytes to TOTP was determined by the ability of the B and T cells to undergo "blast transformation" or to proliferate in the presence of mitogens. PHA, PWM, and LPS were used for this purpose and tritiated-thymidine added to measure cellular development. The effects on the ability of the organism to produce specific gamma-globulins was determined by measuring their levels in the blood serum by immunoelectrophoresis. For additional parameters plaque formation and delayed hypersensitivity were also observed.

Two forms or isomers of this triester were used. Tri-o-tolyl phosphate was used in three dose levels and tri-m-tolyl phosphate in only the mid-dose level. This was to determine the general characteristic effects of these compounds.

Animal Treatment

Male Sim:(sw)fbr mice (weighing approximately 28 g each at the beginning of the experiment) were obtained from Simonsen Laboratories (Gilroy, California) and divided into five groups after ten days of

acclimation. These animals were housed five per cage, and dosed with TOTP, TMTP or corn oil.

TOTP solution was prepared by mixing 2500 mg of TOTP (Eastman Kodak Co., Rochester, New York) with 50 ml of corn oil. This stock solution, containing 50 mg/ml TOTP was diluted with corn oil to provide the appropriate solutions. TMTP solution for testing was prepared by mixing 250 mg TMTP (Eastman Kodak Co.) with 50 ml of corn oil. The control group received corn oil alone. The concentrations of all solutions were adjusted to provide 0.1 ml per 10 g body weight to mice. The animals were weighed and dosed (gastric gavage method) once a week with 0.0, 5.0, 50, and 500 mg/kg of TOTP and 50 mg/kg of TMTP, for 13 weeks. Five animals were killed by decapitation for a pre-exposure control group. A flow chart for the experimental design follows.

Design of Experiment

<u>Day</u>	<u>Methods and Animals Used</u>
-10	Animals procured and kept for ten days for acclimation period. Total number = 155.
0	Treatment began. Total animals = 150
	Control group (corn oil) = 30
	5 mg/kg (TOTP). 30
	50 mg/kg (TOTP) 30
	500 mg/kg (TOTP). 30
	50 mg/kg (TMTP) 30

- Five animals in the pre-exposure control group were decapitated and tissue collected for testing.
- 7 Lymphocyte transformation, five animals/group were killed and blood and organs collected for testing.
- 14 Challenged animals for Plaque formation with SRBCs. Five animals/group were treated with 0.25 ml of a 20 percent solution.
- 18 Sensitized Delayed Hypersensitivity animals with 0.25 mls of a 10 percent solution of SRBCs. Five animals/group.
- 21 Second challenge of Plaque forming animals with SRBCs.
- 28 Lymphocyte transformation, five animals/group were killed and tissue collected for testing. Plaque forming. Five animals/group were killed and plasma kept for electrophoresis.
- Inoculate in foot-pad of delayed hypersensitivity animals. ¹²⁵I labeled albumin injected IP.
- 29 Delayed hypersensitivity, five animals/group were killed and feet collected for testing.
- 56 Lymphocyte transformation, five animals/group were killed and tissues collected for testing.
- 90 Lymphocyte transformation, five animals/group were killed and tissues removed for testing.

At the time of each kill the blood was collected, cell counts made and serum removed and frozen. Selected organs were collected and weighed. Liver, kidney, and thymus were placed in formalin while the spleens were saved in sterilized saline for lymphocyte cultures.

Spleenic Lymphocyte Cultures

Immediately after their collection, the spleens were washed with sterilized saline solution and mashed with forceps repeatedly in saline to release their cellular contents. The cells were separated from the intact tissue by serially passing the solution through 18, 21, and 25 gauge needles. The solution was allowed to stand for 10 minutes in a glass tube to remove the cell aggregates, after which it was transferred to a different tube removing the sediment and glass adherent materials. This suspension was centrifuged at 2000 rpms for 10 minutes at room temperature. The cell pellet was resuspended in 4 ml of culture media and an aliquot was used for counting the number of lymphocytic cells in a Coulter counter after lysis of erythrocytes.

Lymphocyte transformation was determined on the culture by a method modified from that of Sharma and Gehring (1979). The culture media used was RPMI-1640 (Grand Island Biologicals, Grand Island, N.Y.) containing 10 percent heat-inactivated fetal calf serum and 100 units of penicillin and 100 µg of streptomycin per ml. The media was supplemented with 3 mg/ml of L-glutamine. The suspension was adjusted to provide approximately 8×10^6 cells/ml and 50 µl of this layered into each well of a micro-tissue-culture plate (Microtest II. Falcon 3042). To each well, 50 µl of additional media was added and 50 µl of media either with or without mitogen was also added. Mitogens PHA, PWM, and LPS were obtained from GIBCO. The plates were incubated

for 52 hours at 37°C in humidified 5 percent CO₂ in air atmosphere. After this incubation period, 50 µl of media containing 0.5 µCi of tritiated-thymidine (New England Nuclear) was added to each well and the incubation continued for an additional 16 hours. The cells were harvested and vigorously washed with physiological saline using a model M12 cell harvester and collected on glass fiber discs (Whatman, Grade 934-AH). These discs were placed directly in regular counting vials, 6 ml of scintillation solution added and radio-activity counted in a scintillation spectrometer (Packard, Tri-Carb 2660). All cultures were run in triplicates. The Relative Proliferation Index (RPI) (Koller et al. 1970) for PHA, PWM, and LPS were calculated by dividing the net cpm of the test groups (mitogen-stimulated) by the net cpm of the control group (unstimulated). A Stimulation Index (SI) was also calculated by dividing the cpm of the mitogen stimulated cells by the unstimulated cells for each animal.

Plaque Formation

Five mice in each dosage level were randomly selected and inoculated intraperitoneally with 0.25 ml of a 20 percent suspension of sheep red blood cells 14 and 7 days before sacrifice. The spleen from each mouse was removed with forceps and forced through 18, 21, and 26 gauge needles. The spleen cell suspension was counted in the Coulter counter and adjusted to approximately 1×10^7 cells per ml. The number of plaque forming cells (PFC) was measured according to a method of Cunningham and Szenberg

(1968). Four pieces of double-sided tape, each $\frac{1}{4}$ in. wide were laid across a clean microscope slide dividing it into three areas. One large precleaned cover slip was pressed firmly onto the tape to form three shallow chambers. A mixture containing 45 percent splenic cells, 45 percent of a 10 percent suspension of SRBCs, and 10 percent pooled guinea-pig complement (Miles Laboratories, Elkhart, Indiana) in deluent, was delivered by a pasture pipette into each chamber. The chambers were sealed with a mixture of petroleum jelly and mineral oil, and incubated at 37°C for 30 minutes. The plaques were counted using a microscope. The number of plaques per million splenic cells was calculated from the number of plaques observed per microliter of the initial mixture.

Delayed Hypersensitivity: Radioisotope Food-Pad Assay

Five mice per dose level were randomly selected and immunized with 0.25 ml of SRBCs (10 percent). Ten days later each group was injected in the right food pad with 0.02 ml of the same dilution of SRBCs. Immediately thereafter, ^{125}I -labeled bovine serum albumin was injected i.p. into the mice. The radioiodine-labeled protein was prepared as described by Boone, Irving, and Rubenstein (1971). The usual dose per mouse was 5.0 μCi in 0.10 ml. The mice were killed 24 hours later and the test and control feet cut off at the junction of the lower and middle thirds of the tibia and counted in a gamma spectrometer (Abbott Laboratories, Auto-logic 7407). Results were expressed as the "foot-count ratio": counts per minute given by the test food divided by counts per minute given by the control feet (Vadas et al. 1975).

Quantitative Immunoelectrophoresis

Blood taken from animals that had been challenged with SRBCs was centrifuged and serum drawn off. This serum was used to test for IgA, IgG, and IgM immunoglobulins. The standard serum run against the test serum was collected from five untreated mice and pooled.

The method used was modified from Bjerrum et al., (1973). Agarose (1 percent) obtained from Bio-Rad (Richmond, Va.) was prepared and mixed with carbamylated anti-globulins. Goat antimouse immunoglobulins (Miles Lab., Elkhart, Indiana) was used. This mixture was poured onto plates (10 cm x 10 cm) to provide a thickness of approximately 1.5 mm.

Wells were formed in the plates and samples of each mouse serum was added to each in 2 μ l quantities. Each plate also contained samples of the standard. All samples, standard as well as test, were run in duplicate. Serum was diluted to a 1:10 ratio. The plates were immediately placed in the electrophoresis cell (Model 1400 horizontal gel type, Bio-Rad Lab.) containing TEMED buffer (pH 5). Electrophoresis was conducted (toward the cathode) for three hours (fast, 300 volt) or over night (slow, 100 volts).

After electrophoresis, the gel plates were fixed in 7 percent TCA for 15 minutes and then washed in water for 5 minutes. The gels were then pressed for 10 minutes and dried thoroughly. Dried gels were stained with Amino Black for an additional 15 minutes and then decolorized for a few minutes in a destaining solution.

Plates were then dried and rockets identified. Heights of the standard serum and test serum were compared and the percent of standard for the test serum calculated.

Statistical Manipulation and Data Presentation

Lymphocyte transformation data is presented in tabular form as Relative Proliferation and Stimulation Indices for PHA, PWM, and LPS in splenic cultures in relationship to time and amount of TOTP exposure. RPI's are also presented in graphical form for each mitogen. The effects of TOTP on body and organ growth was tabulated and presented as g/100 g of body weight. Plaque forming cells were presented as PFCs per million splenic lymphocytes, and delayed hypersensitivity as foot-count ratio. A comparison of whole blood WBCs and RBCs was also calculated. Electrophoretic rocket heights were taken and test serum presented as percent of standard in relation to TOTP dosage level. All data was tested for significant changes from the control groups by using a two-tailed Dunnett's t test (Dunnett 1955).

RESULTS

In mice, TOTP and TMTP exposure for 13 weeks produced no significant effects on growth, or weights of spleen, liver, kidney, and thymus (Table 1). At the time of termination, several mice in the high dose level had a slight discoloration of the ears, tail, and feet as well as the internal organs. However, no significant difference was noted in any of the parameters tested.

Splenic Lymphocyte Cultures

Relative Proliferation and Stimulation Indices of lymphatic uptake of thymidine were calculated for splenic cultures using PHA, PWM, and LPS for mitogenic induction (Table 2). The stimulation index showed an increase of thymidine uptake by only PWM at 4 weeks, there was a decrease at 8 and 13 weeks for both PHA and PWM but not LPS.

The relative proliferation index indicated no significant change from the control for PHA (Figure 1). PWM, however, showed a decrease at 8 weeks for the 500 mg/kg TOTP and 50 mg/kg TMTP, and at 13 weeks for all dose levels (Figure 2). LPS induction was affected only at the 4 week period from the low and mid-dose levels (Figure 3). Thus an overall trend points toward a decrease in mitogenic stimulation as time of treatment increases.

Table 1. Organ and body weights of mice treated with different dose levels of TOTP and TMTP.

Week	Dose	Body Weight (g)	Organ Weights and Their Relation to Body Weight							
			Spleen		Liver		Kidney		Thymus	
			(g)	(g/100g)	(g)	(g/100g)	(g)	(g/100g)	(g)	(g/100g)
0	0	31.1(.24)	0.11(.01)	0.40(.05)	1.91(.13)	6.57(.51)	0.51(.02)	1.75(.13)	0.08(.02)	0.29(.04)
1	0	31.5(.54)	0.15(.04)	0.48(.12)	2.04(.07)	6.50(.06)	0.50(.03)	1.59(.02)	0.05(.01)	0.18(.01)
	5	31.8(2.7)	0.16(.02)	0.52(.06)	2.00(.12)	6.40(.25)	0.51(.02)	1.63(.11)	0.08(.02)	0.26(.09)
	50	28.8(2.5)	0.21(.04)	0.82(.26)	1.80(.26)	6.26(.21)	0.50(.03)	1.75(0.8)	0.05(.01)	0.18(.03)
	500	32.4(1.5)	0.18(.03)	0.56(.13)	2.35(.11)	7.32(.59)	0.56(.03)	1.67(.06)	0.07(.01)	0.18(.04)
	50M	33.0(.45)	0.12(.01)	0.37(.03)	2.00(.07)	5.91(.17)	0.52(.02)	1.59(.07)	0.06(.01)	0.18(.02)
4	0	35.3(1.5)	0.13(.01)	0.36(.02)	2.18(.08)	6.18(.08)	0.56(.02)	1.57(.03)	0.07(.01)	0.19(.02)
	5	35.1(1.4)	0.14(.03)	0.41(.03)	2.01(.13)	5.71(.20)	0.56(.04)	1.59(.04)	0.07(.01)	0.19(.03)
	50	38.2(1.7)	0.16(.01)	0.41(.02)	2.33(.12)	6.09(.12)	0.57(.04)	1.49(.07)	0.07(.01)	0.18(.01)
	500	32.5(1.9)	0.15(.02)	0.45(.05)	2.04(.14)	6.29(.25)	0.15(.02)	1.57(.02)	0.06(.01)	0.16(.02)
	50M	36.5(2.5)	0.27(.07)	0.78(.25)*	2.14(.13)	5.88(.07)	0.57(.03)	1.58(.03)	0.05(.01)	0.12(.02)
8	0	40.4(2.7)	0.20(.02)	0.48(.04)	2.40(.21)	5.93(.19)	0.59(.05)	1.45(.05)	0.06(.01)	0.16(.02)
	5	36.9(2.6)	0.23(.02)	0.62(.04)	2.40(.27)	6.41(.30)	0.55(.04)	1.49(.03)	0.05(.01)	0.13(.02)
	50	35.1(2.2)	0.20(.02)	0.56(.02)	1.94(.18)	5.51(.23)	0.51(.04)	1.46(.07)	0.07(.01)	0.20(.01)
	500	37.7(2.0)	0.23(.05)	0.61(.15)	2.97(.38)	7.92(1.0)*	0.59(.03)	1.57(.06)	0.05(.01)	0.14(.02)
	50M	39.3(2.5)	0.20(.02)	0.52(.03)	2.48(.15)	6.32(.17)	0.56(.04)	1.44(.08)	0.06(.01)	0.14(.01)
13	0	42.7(2.4)	0.25(.05)	0.38(.09)	2.60(.09)	6.14(.31)	0.71(.03)	1.56(.04)	0.06(.01)	0.19(.01)
	5	44.4(.55)	0.18(.02)	0.41(.06)	2.90(.08)	5.71(.17)	0.76(.02)	1.59(.05)	0.05(.01)	0.19(.02)
	50	43.3(1.8)	0.22(.02)	0.51(.04)	2.68(.16)	6.18(.16)	0.70(.07)	1.49(.09)	0.06(.01)	0.18(.01)
	500	46.5(3.0)	0.23(.03)	0.43(.05)	2.88(.30)	6.14(.26)	0.74(.09)	1.57(.10)	0.06(.01)	0.16(.01)
	50M	43.7(1.3)	0.23(.02)	0.52(.06)	2.78(.15)	6.35(.22)	0.68(.03)	1.55(.05)	0.06(.01)	0.13(.02)

*P<0.05 using Dunnett's test for significance between treated and control mean values. Parenthesis = standard error of the mean.

M=TMTP.

Table 2. Lymphocytic proliferation response of mice exposed to TOTP and TMT.

Week	Dose mg/kg	PMA			P-M			LPS		
		SI	RPI	SI	SI	RPI	SI	SI	RPI	RPI
0	0	1.47(.11)			2.00(.17)			4.11(.42)		
1	0	1.37(.18)			1.52(.17)			4.26(.83)		
	5	1.76(.16)	2.44(.43)		3.04(.49)	3.92(1.5)*		6.51(.85)		1.50(.18)
	50	1.73(.18)	2.39(.65)		2.62(.43)	2.70(.68)		5.89(.51)		1.43(.20)
	500	1.78(.31)	2.61(.93)		2.43(.09)	2.40(.47)		4.41(.73)		1.10(.19)
4	0	1.42(.27)	1.82(.96)		1.30(.38)	1.40(.27)		5.01(.89)		1.59(.24)
	5	2.37(.45)			2.33(.33)			5.39(.43)		
	50	2.10(.42)	1.06(.23)		2.79(.79)	0.96(.15)		5.08(.41)		0.69(.05)*
	500	2.54(.56)	0.52(.13)		3.85(.17)*	1.10(.06)		5.02(.98)		0.47(.09)*
8	0	1.75(.33)	0.61(.13)		3.05(.91)	1.00(.04)		6.44(.79)		0.83(.10)
	50	2.30(.41)	0.62(.32)		2.56(.08)	0.68(.17)		4.14(.76)		0.43(.13)*
	500									
	500	2.32(.25)	0.61(.09)		3.36(.38)	0.27(.11)		3.52(.22)		0.91(.16)
13	0	1.77(.23)	0.56(.09)		2.49(.33)	0.61(.07)		3.24(.24)		0.61(.11)
	50	2.04(.30)	0.69(.28)		1.93(.50)*	0.51(.19)*		3.26(.39)		0.92(.20)
	500	2.10(.23)	0.56(.13)		2.71(.31)	0.51(.07)*		4.43(.93)		1.20(.08)
	500	4.17(.83)			5.75(.63)			6.49(1.0)		
13	0	2.00(.28)*	0.69(.23)		2.19(.38)*	0.51(.11)*		2.45(.45)		0.60(.23)
	50	2.07(.49)*	0.55(.23)		3.77(.66)	0.61(.18)*		3.77(.66)		1.37(.36)
	500	2.32(.49)	0.58(.23)		2.15(.34)*	0.47(.06)*		9.87(.86)		1.37(.36)
	500	2.32(.53)	0.78(.29)		2.14(.34)*			2.71(.41)		0.70(.15)

*Significantly different from control using Dunnett's test, $p < 0.05$; numbers in parenthesis = standard error of the mean.
N=8/10.

Figure 1. Relative proliferation index of splenic lymphocyte cultures to PHA. RPI = net cpm of test groups divided by net cpm of control groups. Vertical bars represent standard error of mean and * indicates a significant change from the control at $P < 0.05$. M = TMTP.

Figure 2. Relative proliferation index of splenic lymphocyte cultures to PWM. RPI = net cpm of test groups divided by net cpm of control group. Vertical bars represent standard error of mean and * indicates significant difference from control at $P < 0.05$. M = TMTP.

Figure 3. Relative proliferation index of splenic lymphocyte cultures to LPS. RPI = net cpm of test groups divided by net cpm of control groups. Vertical bars represent standard error of mean and * indicates value significantly different from control at $P < 0.05$. M = TMT.

Plaque Formation

Plaque forming cells were calculated per million splenic cells (Table 3). Because of relatively high standard error of the means no change was noted from the controls using Dunnett's test for significance.

Delayed Hypersensitivity

Delayed hypersensitivity inflammatory response was tested by use of radioisotopic label. Foot count ratio was tabulated by dividing the counts of the test foot with those of the control foot. Results of this were negative with no significant difference from the control (Table 3).

Quantitative Immunoelectrophoresis and Hematology

Serum immunoglobuline, IgA, IgG, and IgM in mice at different levels of TOTP exposure are shown in Table 4. These were calculated as percent of pooled serum used as standard. The control serum was obtained from mice of the same strain without treatment and pooled. No significantly different changes were noted in the treated groups as compared to the control group.

No apparent change took place in whole blood erythrocyte and lymphocyte counts as shown in Table 5. There was an increase in RECs at the mid dose levels of TOTP and also TMTP after 1 week and at the mid dose level of TOTP after 13 weeks. No change was found in the white cell count at any dosage or time interval.

Table 3. Plaque forming cells and delayed hypersensitivity foot count ratio.

Dose, mg/kg	PF cells	count ratio
0	918 (294)	1.15 (.03)
5	1079 (164)	1.12 (.02)
50	629 (137)	1.37 (.17)
500	596 (78)	1.33 (.12)
50M	527 (118)	1.17 (.07)

Note: Plaque forming cells tabulated per million splenic lymphocytes. Foot count ratio was with ^{125}I label. Standard error of the mean in parenthesis. M = TMTP.

Table 4. Quantitative immunoelectrophoresis of mouse serum immunoglobulins.

Dose, mg/kg	IgA	IgG	IgM
0	118 (5)	92 (9)	95 (1)
5	88 (2)	102 (11)	103 (2)
50	103 (4)	83 (5)	102 (2)
500	99 (9)	101 (9)	99 (2)
50M	112 (12)	87 (5)	95 (4)

Note: Calculated as percent of standard pooled mouse serum. Standard error of the mean in parenthesis. M = TMTP.

Table 5. Whole blood erythrocyte and lymphocyte counts.

Week	Dose, mg/kg	WBC x 10^3 /mm ³	RBC x 10^6 /mm ³
0	0	8.6 (1.6)	8.2 (0.4)
1	0	7.0 (3.7)	5.6 (0.4)
	5	5.6 (1.3)	6.9 (0.7)
	50	13.8 (4.4)	7.9 (0.8)*
	500	4.9 (0.3)	7.0 (0.5)
	50M	4.1 (0.3)	7.9 (0.3)*
4	0	3.0 (0.5)	4.9 (0.6)
	5	4.0 (0.4)	5.6 (0.3)
	50	3.6 (0.8)	5.5 (0.2)
	500	3.5 (0.8)	5.3 (0.1)
	50M	3.2 (0.2)	5.2 (0.5)
8	0	9.5 (1.7)	8.7 (0.7)
	5	5.1 (0.7)	6.7 (0.2)
	50	7.7 (0.9)	7.8 (0.8)
	500	13.1 (4.6)	7.5 (0.3)
	50M	15.0 (2.8)	6.9 (0.2)
13	0	6.1 (1.3)	5.6 (0.2)
	5	5.8 (0.7)	6.0 (0.2)
	50	8.8 (2.4)	6.8 (0.1)*
	500	3.9 (0.6)	5.5 (0.3)
	50M	8.0 (2.8)	5.0 (0.5)

*Significantly different from control using Dunnett's test,
 $P < 0.05$. Numbers in parenthesis = standard error of the mean.
M = TMTP.

Note: WBC and RBC are in counts/cubic millimeter.

DISCUSSION

Tri-o-tolyl phosphate, a prevalent industrial and environmental chemical, is similar in structure as well as toxic action to many organophosphate compounds. TOTP is a very toxic nerve agent and has been involved in several poisonings in man (Morgan 1978). Not only TOTP itself but its metabolites have been found to accumulate and produce degenerative effects in nervous tissue (Watanabe and Sharma 1975).

Many possible explanations have been postulated for its mode of toxic action. Inhibition of cholinesterases, direct degradation of the myelin sheath, failure to maintain the nerve's basic metabolism, and effects related to autoimmunity have been reported (Silver and Murphy 1978, Smith and Lillie 1931, Cavanagh 1969, O'Brien 1967).

This project was undertaken to study the effects of TOTP on the ability of lymphatic cells to proliferate and produce antibodies. A 90-day oral administration to TOTP up to 500 mg/kg in mouse produced no effect on growth rate or organ weight of the animals.

Splenic lymphocytes were cultured in vitro with three known mitogens for 52 hrs, and their ability to proliferate measured by addition of radioisotope labeled thymidine. TOTP had relatively little effect on the lymphocytes when stimulated with PHA. However, at both 4 and 13 weeks the PWM and LPS assays indicated that TOTP at all doses and especially the mid and high levels caused a

suppression of lymphocyte activity. TMTP had only slight suppressive action according to PWM. Although this action was non-specific and not dose related it coincides with an earlier study done by Watanabe and Sharma (1976) where lymphocytic tissue was noted to be more diffuse after treatment with TOTP in the chicken.

The results of the plaque formation study showed no significant trends for the various treatment groups which suggested that lymphatic activity was not affected by TOTP. Likewise, the delayed hypersensitivity test showed similar results in that no significant relationship was seen between doses of TOTP and in foot count ratios. Therefore, the hypersensitivity reaction does not appear to be affected by this organophosphate compound. It was reported by Watanabe and Sharma (1976) that plasma protein levels and gamma-globulin:albumin ratios were consistently greater for TOTP treated animals versus control. Our results, however, failed to show any consistent increase or decrease in IgA, IgG, and IgM immunoglobulin levels when compared to standard serum. This suggests no immunotoxicologic involvement of TOTP on serum proteins.

Exposure to certain environmental contaminants has resulted in marked effects on antibody synthesis (Koller et al. 1974, 1975). In a recent study by Koller et al. (1976) leptophos was shown to cause organophosphate poisoning, but did not significantly alter antibody formation to a specific antigen (SRBC). Primary and secondary immune responses were tested for by the hemolytic plaque assay, therefore leptophos apparently does not interfere with the humoral tissue system. Several organophosphate pesticides have been reported to affect the immune system. Rats exposed to methylnitrophos,

chlorophos, and triufon were reported to have decreased hemagglutination titers, complement levels (Shtenberg et al. 1974), and size of splenic lymphoid follicles (Dinoeva 1974). Following exposure to anthio the ability of neutrophils to phagocytize was diminished in goats (Aripdzhanov 1973). Methylparathion (Street and Sharma 1975) decreased the number of globulin producing cells in the popiteal lymph nodes of rabbits but had no effect on the hemolysis of hemagglutination titer to SRBC antigen. Conflicting data concerning the effects of organophosphate pesticides on immunity indicate the action of the pesticide may be dependent upon several factors such as the compound configuration, method of exposure, and type of antigen. It is also conceivable that only certain organophosphates affect immunity or that only certain species of animals are susceptible.

In conclusion, at the dose levels used in this study, TOTP produced no significant effects on the immune system of mice as determined by several in vitro assays of immunocompetence. Organophosphate poisoning was evident by moderate discoloration and scruffy appearance of mice that received 500 mg/kg TOTP. This was interpreted to be clinical signs of organophosphate stress.

SUMMARY

Male Swiss Webster mice exposed to 5, 50, 500 mg/kg TOTP and 50 mg/kg TMTP for 13 weeks showed no significant difference in body and organ weights in relation to dosage and time of treatment. Splenic lymphocytes (obtained at 1, 4, 8, and 13 weeks) cultured in vitro with mitogens PHA, PWM, and LPS showed a stimulation for all dose levels at 4 weeks. Thereafter a suppression was observed especially with PWM and LPS stimulated cultures. Although there was a definite suppressive trend at 8 and 13 weeks it was not dose related and not always statistically significant and therefore non-specific.

Other parameters tested; plaque formation by splenic cells, delayed hypersensitivity, and immunoelectrophoresis, showed no significant changes from the control groups.

Thus at the treatment levels used, TOTP, produced no significant effects on the immune system of mice as determined by these in vivo and in vitro assays of immunocompetence.

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