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Use of staphylococcal enterotoxin A-induced interleukin 2 production as an indicator of immunotoxicity

Reid, Lynnda Louise, M.S.
The University of Arizona, 1990
USE OF STAPHYLOCOCCAL ENTEROTOXIN A - INDUCED INTERLEUKIN 2 PRODUCTION AS AN INDICATOR OF IMMUNOTOXICITY

by

Lynnda Louise Reid

A Thesis Submitted to the Faculty of the DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY In Partial Fulfillment of the Requirements For the Degree of MASTER OF SCIENCE WITH A MAJOR IN TOXICOLOGY In the Graduate College THE UNIVERSITY OF ARIZONA

1 9 9 0
STATEMENT BY AUTHOR

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SIGNED: Lynda J. Reid

APPROVAL BY THESIS DIRECTORS

This thesis has been approved on the date shown below:

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Professor of Anesthesiology
and Pharmacoogy/Toxicology

Clifton D. Crutchfield
Assistant Professor of Pharmacoogy/Toxicology

July 12, 1970
July 24, 1970
DEDICATION

To my friend and associate Keely Ghirardelli,
whose humor and assistance never failed me.
ACKNOWLEDGEMENTS

For their understanding, encouragement, and support throughout my life, I am deeply indebted to my parents and family.

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LIST OF ABBREVIATIONS

B  benzene
°C degrees Centigrade
Ci  curie
Con A concanavalin A
CsA cyclosporin A
CTLL cytotoxic T lymphocytic leukemia cell line
DMSO dimethyl sulfoxide
dpm disintegrations per minute
FCS fetal calf serum
g gram
x g times gravity
³H-T tritiated thymidine
hr hour
IL-1 interleukin-1
IL-2 interleukin-2
kg kilogram
L liter
M molar
µg microgram (10⁻⁶)
µl microliter (10⁻⁶)
mg milligram (10⁻³)
ml milliliter (10⁻³)
mmole millimole
min  minute
mRNA messenger RNA
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ng nanogram \((10^{-9})\)
NK natural killer T cells
OD change in absorbance
pg picogram \((10^{-12})\)
PHA phytohemagglutinin
RBC red blood cell
SEA Staphylococcal enterotoxin A
SEM standard error of the mean
V vehicle
WBC white blood cell

Prefixes of Measurement:

<table>
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<th>Symbol</th>
<th>Quality</th>
<th>Value</th>
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<tbody>
<tr>
<td>p</td>
<td>pico</td>
<td></td>
<td>(10^{-12})</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
<td></td>
<td>(10^{-9})</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
<td></td>
<td>(10^{-6})</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
<td></td>
<td>(10^{-3})</td>
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Symbols:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>±</td>
<td>plus or minus</td>
</tr>
<tr>
<td>*</td>
<td>significant ((p = &lt; 0.05))</td>
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<tr>
<td>&lt;</td>
<td>less than</td>
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ABSTRACT

An in vitro assay measuring IL-2 production as an index of compromised immune function was developed using primary splenocytes from female C57BL/6 mice pretreated with olive oil, 30 mg/kg Cyclosporin A, or 220 - 880 mg/kg benzene. Splenocytes were mitogen-stimulated to produce IL-2 with SEA, Con A, or PHA. Benzene-induced inhibition of IL-2 production was demonstrated by incubating the splenocyte culture supernatant fraction with an IL-2 dependent T-lymphocytic cell line. Cell proliferation was measured by blastogenesis and MTT reduction as an index of IL-2 mediated response. Benzene and cyclosporin dosed animals demonstrated a significant decrease in splenocyte proliferation. However, cell proliferation in IL-2 dependent cells was inhibited only with the higher doses of benzene indicative of a dose dependent IL-2 mediated suppression. Chemically induced impairment of IL-2 mediated immune function may be detected with this assay.
INTRODUCTION

The immune system is a complex, multifaceted biological system designed to protect the body from potential pathogens which are present in the environment. Interactions between environmental and occupational chemicals and the immune system may result in alterations in the host defense mechanisms, resulting in disease. Immunotoxicity may result in either an enhanced, impaired, or a qualitatively abnormal immune response. An enhanced immune response would result in hypersensitivity, while an impaired response results in immunosuppression, which is characterized by an increased susceptibility to pathogens. Abnormal responses include allergies and autoimmune diseases.

It is the complexity of the immune system and its capability of protecting through multiple pathways which makes detection of immune dysfunction difficult. The complexity of the system derives from an intricate communication network capable of exerting multiple effects based on relatively few cell types. Soluble proteins called interleukins, produced by these cells, appear to regulate many aspects of the immune response (Male, 1987). Interleukin-2 is the best characterized lymphokine and appears to be pivotal in the initiation of a large variety of immune responses (Smith,
An agent that either suppresses interleukin-2 production or function following antigenic stimulation would be considered an immunotoxicant. Immunotoxicants which act in this manner compromise the health of the individual by reducing or eliminating entirely the ability to respond to pathogens.

THE IMMUNE SYSTEM

The function of the immune system is to protect the body from damage caused by invading pathogens - bacteria, viruses, fungi, and parasites. It is capable of recognizing self from the environment (non-self) by markers on cell surfaces called antigens. It is this function which allows the body to identify pathogens as well as transformed neoplastic cells and tissue grafts. Regulatory control of immune response is a function of the interactions of the genetic, cellular, and soluble components of the system (Male, 1987).

Cellular Components of the Immune System:

The primary cellular constituents of the immune system are the leukocytes (white blood cells). Leukocytes are divided into granulocytes, monocytes, and lymphocytes. Monocytes and lymphocytes are responsible for producing the interleukins, soluble factors involved in regulating immune
responses.

Granulocytes are phagocytic cells which function both by destroying microorganisms and aiding the inflammatory process by releasing enzymes (Bowman, 1980).

Monocytes, also known as macrophages, have a variety of functions in the immune response. They are phagocytic cells which can physically remove foreign antigens such as bacteria from the system. While not thought to be specific for any given antigen, their role in concentrating and presenting antigens to lymphocytes is a crucial one. Macrophages also play a role in regulation of the immune response by secreting interleukins, known as monokines, which regulate the type and magnitude of lymphocyte response by enhancing or suppressing cell division or differentiation. (Male, 1987)

Lymphocytes are the key cellular components controlling the immune response, recognizing foreign material by specific surface antigen receptors. During lymphocyte development, recombination and rearrangement of the antigen receptor genes occurs giving the population as a whole a great diversity of antigen receptors. Lymphocytes are separated into two main types based on their maturation process and function. B cells are lymphocytes which develop in foetal liver and subsequently in bone marrow and are responsible for humoral immunity. Mature B cells can be identified by immunoglobulin surface markers which act as their antigen
receptor. Following antigen recognition, B cells divide and differentiate into plasma cells which secrete immunoglobulins under the control of lymphokines released by T cells (Male, 1986).

T cells are lymphocytes which develop and differentiate in the thymus and regulate cellular immune response. T cells have a number of subpopulations which have separate functions and which can be recognized by markers on the cell surface. The major subpopulations include T helper cells which help B cells and cytotoxic T cells recognize antigen. T inducer cell is a term used to describe T helper cells which, following antigen recognition, fulfill their role by secreting interleukins. Cytotoxic T cells are capable of destroying allogeneic cells and virally-infected cells by direct contact through the secretion of toxic molecules which destroy both the cells and the microbes they contain. Natural killer T cells represent approximately 10% of circulating lymphocytes and can act independent of T helper stimulation. Natural killer cells have no antigen receptors and appear always to be in a state of activation (Smith, 1990). T suppressor cells help control the immune response by regulating the action of other T cells and B cells. (Male, 1986)

**Soluble Mediators of the Immune Response:**

Activated T cells and macrophages release proteins
which mediate the response of other immune cells and are essential for the development of the immune response. Factors released by lymphocytes are termed lymphokines and those from monocytes and macrophages are monokines. This group of chemical messengers includes the interleukins, defined as soluble mediators of immunity which are either produced by lymphocytes and/or act on lymphocytes (Dorland, 1982). Eight interleukins have currently been identified but only interleukin-1 and interleukin-2 have been extensively studied. A partial list of interleukins involved in T cell interactions of mice are characterized in Appendix A.

Interleukin-1 (IL-1) is a monokine released by antigen stimulated macrophages. IL-1 effects T helper cell activity through several pathways (Male, 1987):

1. Increasing interleukin-2 release and interleukin-2 receptor expression.
2. Raising body temperature, thus enhancing T cell response.
3. Protecting T helper cells from the effect of T suppressor cell functions.

Interleukin-2 (IL-2), released by T helper cells and previously known as T cell growth factor, is a mitogenic lymphokine which induces lymphocyte proliferation and differentiation and appears to be the pivotal component for the generation and regulation of the immune response (Smith, 1988b). Specific IL-2 receptors on the surface of lymphocytes
specifically bind and remove IL-2 via receptor mediated endocytosis (Reed, 1988). Receptor expression is transient following antigen receptor activation, functioning as a control mechanism in immune response (Cantrell, 1984). Natural killer cells, on the other hand, continuously express a portion of the IL-2 receptor, which may explain their constant state of activation (Smith, 1990).

The IL-2/receptor interaction is the molecular event responsible for signaling proliferative clonal expansion and differentiation to specialized functions such as T cell cytolysis and B cell antibody production (Pauza, 1988). The extent of response is dependent on the IL-2 concentration, receptor density, and the duration of the receptor interaction (Smith, 1988a). Thus, IL-2 mediates biological effects by a mechanism that is identical to those attributed to classic peptide hormones (Reed, 1988):

- high affinity
- saturability
- ligand specificity
- target cell specificity.

Antigens:

An antigen is any substance capable of inducing antibody formation and of reacting specifically with those antibodies (Dorland, 1982). Antigens may be soluble (e.g.,
toxins and foreign proteins) or particulate (e.g., bacteria and tissue cells). Most antigens must first be taken up by an antigen-presenting cell (resident or circulating macrophages) which can present it to lymphocytes in an antigenic form (Fudenberg, 1980).

There are a group of antigens which have been termed "super antigens", which are capable of directly interacting with lymphocytes to stimulate an immune response. "Super antigens" are capable of inducing a complete immune response upon primary exposure, whereas most antigens require a secondary exposure to induce a proliferative response. Many venoms and bacterial toxins function this way. An example is staphylococcal enterotoxin A.

The Immune Response:

An immune response results from the interactions between immune cells and antigen. The main steps of this process are:

1) Presentation: The uptake, processing, and presentation of antigen to T and B cells by antigen-presenting cells.

2) Stimulation: The interaction between T helper cells and the various effector cells which include macrophages, B cells, and cytotoxic T cells. These interactions may be mediated by direct cellular interactions, or may be mediated by the interleukins.
3) Regulation: The level and type of response is then regulated by T suppressor cells as well as by the macrophages, T helper cells, interleukins and receptors, and antibody.

4) Inflammation and clearance: The immune system interacts with other cells and enzyme systems to produce inflammatory reactions aimed at eliminating the source of the antigen and minimizing any danger. (Male, 1987)

T cell clonal proliferation after antigen challenge is obligatory for immune responsiveness and immune memory (Smith, 1990). In order for the immune system to launch a wide scale response, it is necessary for the cells which are specific for the stimulating antigen and therefore capable of responding, to reproduce in sufficient quantities to eliminate the antigen. This proliferative response is dependent on IL-2 production and receptor binding (Pauza, 1988). IL-2 secretion and receptor expression on lymphocyte surfaces is stimulated when antigen is bound to the antigen specific receptor site on the cell surface, triggering clonal proliferation and differentiation (figure 1). This response may or may not require antigen presentation, depending on the specific antigen, and can be enhanced by circulating IL-1.
Figure 1: Antigen Presentation and IL-1 stimulated production of IL-2 *in vivo*.
IMMUNOSUPPRESSIVE TOXICANTS

An immunosuppressive toxicant is any substance which is capable of altering the immune system in such a way as to decrease the degree to which it responds to stimulus. The toxic effects may be transient or permanent and often go undetected unless there is a marked decrease in immune function. Clinically, the first signs of immunosuppressive dysfunction are a decreased white blood cell count, immature circulating white blood cells, and/or chronic infections. The most common type of immunosuppressive agents are those which affect the bone marrow, thereby decreasing the number of effective cells in circulation. Benzene is a classic example of this type. Other immunosuppressive agents, such as cyclosporin A which suppresses IL-2 function, act by altering or suppressing one or more components necessary for immune response.

Benzene Toxicity:

Benzene is an important environmental toxicant. It is ubiquitous in the environment and has been identified in soils, fresh and salt water as well as drinking water, both indoor and outdoor air, tobacco smoke and foods. While most acute exposures are of an occupational nature, a far greater number of individuals are exposed to benzene released from gasoline filling stations, from smoking tobacco products, and
from auto exhaust. (Laskin, 1977)

Benzene has been found to be an ideal inducer of immunosuppression. Previous studies using this chemical have demonstrated immunotoxicity in both human and murine in vivo studies and more specifically, to suppress IL-2 production in an in vitro murine T cell study (Post, 1985). Other useful data generated by inhalation studies of acute benzene toxicity include its leukopenic effect, sensitivity for lymphocytes, and suppression of blastogenesis (Aoyama, 1986; Rosenthal, 1985; Wierda, 1981). Chronic exposure causes progressive degeneration of bone marrow leading to aplastic anemia and has been associated with acute myelogenous leukemia (Post, 1985). Benzene has been extensively studied and available human toxicity data are in agreement with effects observed in mouse models (Rusch, 1977).

The mechanism of benzene toxicity on the bone marrow is not completely understood, however; it is thought that toxicity results from its metabolism (Appendix B) to the reactive metabolites phenol, catechol and hydroquinone. These metabolites are thought to accumulate in the bone marrow, resulting in damage to the pluripotential stem cells and/or the early proliferating committed cells in either the erythroid (red cell) or the myeloid (white cell) lines (Rosenthal, 1987; Green, 1981). It has also been postulated that benzene, by metabolism in the lymphocyte to one of its reactive intermediates, can inhibit the production of
lymphokines by interfering with RNA synthesis (Post, 1985).

Cyclosporin A (Cyclosporine, Sandimmune):

Cyclosporin (CsA) is a potent immunosuppressant and unlike benzene, has been shown to exert a specific action on lymphocytes but is not lymphotoxic (Di Padova, 1989). There is no evidence that CsA is toxic to the bone marrow or other immune cells, nor does it have a permanent or mutagenic effect on the lymphocytes. (Borel, 1989) These characteristics make CsA ideal as an immunosuppressive drug, primarily used during tissue and organ transplant procedures, where it has significantly improved survival. CsA is metabolized in the liver (Appendix C) and more than 12 metabolites have been identified. However, CsA appears to be responsible for the immunosuppressive effects observed (Babany, 1989).

CsA has been shown to suppress T cell immune response primarily by blocking the expression of IL-2 (Hess, 1986) by a mechanism acting on the T cell plasma membranes (Colombani, 1985). It has also been shown in in vitro studies to functionally disrupt the IL-2-receptor system which controls proliferation and differentiation of antigen-reactive T cells (Di Padova, 1989). CsA also inhibits the synthesis of mRNA for lymphokines and other proteins required for proliferation (Krönke, 1986). Due to this specific effect on IL-2 mediated immune response, CsA was chosen for the positive immunosuppressive control.
IN VITRO STIMULATION OF LYMPHOCYTE BLASTOGENESIS

Blastogenesis is a term used to describe the mitogenic or antigenic clonal expansion of lymphocytes (Dorland, 1982). Because the immune system has a memory capability, only a relatively few cells capable of recognizing and responding to a specific antigen need remain in circulation. Upon antigen stimulation, these cells produce IL-2 which signals a proliferative response, blastogenesis. In this way, the body can quickly and efficiently produce enough cells necessary to eliminate the antigen. Once the antigen has been removed from the system, the cell number quickly returns to unstimulated levels.

Mitogens:

Mitogens are substances which cause cells, particularly lymphocytes, to undergo cell division. While being specific for particular subsets, they are capable of non-specifically stimulating all the cells within that subset. The mitogen concanavalin A (Con A) is derived from the jack bean and stimulates both mature and immature T lymphocytes, as well as primed and unprimed lymphocytes, to undergo blastogenesis. Another example is phytohemagglutinin (PHA), derived from the red kidney bean, which stimulates only primed, mature T lymphocytes. Primed cells are those cells which are in G1, the prereplicative phase of the cell cycle,
while unprimed cells are found in $G_0$, the resting phase (Male, 1986; Smith, 1988a). Mitogens have been used extensively to characterize lymphocyte subpopulations and evaluate immune function. Thurman (1978) demonstrated a dose dependent suppressive response in human lymphocytes exposed to concentrations of benzene (2.2 to 22 mg/ml) for three days. Subsequent incubation with Con A and PHA resulted in a 99.1% decrease and a 99.9% decrease respectively in lymphocyte proliferation at the 22 mg/ml benzene concentration when compared with untreated cells. No suppressive effect was seen in the cells treated with 2.2 or 4.4 mg/ml benzene.

**Staphylococcal Enterotoxin A:**

Staphylococcal enterotoxin A (SEA) is the antigen most commonly associated with food poisoning and is secreted by the bacteria *Staphylococcus aureus* (Pelczar, 1977). SEA-induced immune response has been shown to be dependent on IL-2 mediated blastogenesis (Johnson, 1988). SEA acts as a super antigen, a term used to describe antigens which 1) are capable of directly stimulating T cell blastogenesis without going through antigen processing and presentation by other immune cells, and 2) can elicit an immune response upon primary exposure. SEA when incubated *in vitro* with T lymphocytes (figure 2) is the most potent mitogen known, capable of significant nonspecific induction of T lymphocyte mitosis (Johnson, 1988). This proliferative response is dependent on
the production of IL-2 making this mitogen especially suited to a detection assay designed to evaluate immunosuppression by IL-2 inhibition for a suspected immunotoxicant.
Figure 2: Direct SEA-stimulation of IL-2 production in vitro.
Exposure to environmental pollutants may compromise the immune system by suppressing its function. Immunosuppressants which are not toxic to the functional cells of the immune system are often overlooked as contributors to chronic disease. Often this results only in a diminished ability to effectively deal with common pathogens which may not always lead to disease. IL-2, as the pivotal hormone for induction of an immune response, is a sensitive indicator of functional impairment. An assay which can screen in vitro for IL-2 impairment following an in vivo exposure would substantially aid the identification of immunosuppressive pollutants.

The primary objectives of this research were to:

1) Develop a sensitive assay capable of detecting an immunosuppressive substance which inhibits IL-2 mediated immune response.

2) Demonstrate that an immunosuppressive agent inhibits IL-2 production.

3) Demonstrate SEA-induced IL-2 production and use as an indicator of immunotoxicity.
MATERIALS AND METHODS

The research described here incorporates both *in vivo* and *in vitro* analyses. Lymphocytes harvested from normal and immunosuppressed animals were challenged with mitogens. Their ability to produce IL-2 was subsequently measured using an IL-2 dependent T lymphocytic culture line. Benzene, which has been shown to cause IL-2 suppression *in vitro* (Post, 1985), was chosen as the immunosuppressant. Cyclosporin A, an immunosuppressive drug which specifically inhibits IL-2 mediated immune response, was used as the positive control. Naïve and vehicle dosed animals served as the normal controls. C57BL/6 mice were chosen as the model species for two reasons: previous studies have used this particular strain (Rosenthal, 1985; Wierda, 1981), thus facilitating comparisons, and secondly, the IL-2 dependent cell line used in culture was developed from C57BL/6 mice (Gillis, 1977).

Following dosing, spleen cells were removed from the animals and placed in culture with concanavalin A or SEA to induce IL-2 production. After incubation, the supernatant fraction was removed and incubated with an IL-2 cytotoxic T lymphocytic leukemia (CTLL) cell line. Clonal proliferation of CTLL cells was indicative of the presence or absence of IL-2 and the capability of the lymphocytes isolated from the
spleens of the control, benzene, and CsA dosed animals to respond to mitogenic stimulation.

Two methods of detection were compared: blastogenesis and cell viability as demonstrated by the mitochondrial reduction of MTT. Blastogenesis is an assay based on the incorporation of tritiated thymidine (\(^3\)H-T) by cells undergoing mitogenesis. Mitochondrial reduction of MTT is a quantitative colorimetric assay which measures cell survival and proliferation. MTT is a yellow dye reduced in the mitochondria by succinic dehydrogenase to a purple formazan product. An increase in MTT product can be measured spectrophotometrically and correlates to the number of viable cells (Plumb, 1989).

MATERIALS AND EQUIPMENT

CS7BL/6 Inbred mice: Harlan Laboratories, Walkerville, MD.
Benzol (Benzene): Fluka AG, Burdick & Jackson Laboratories, Inc., Buchs, Switzerland
Cyclosporin A: Dr. G. Mauer, Sandoz, LTD., Basel, Switzerland

Cell Culture Materials:
Cytotoxic T-lymphocytic Leukemia Cells: American Type Culture Collection, Rockville, MD.
Staphylococcal Enterotoxin A: Serva Fine Biochemicals Inc., Garden City, NY.
Concanavalin A - 5 mg/vial: Sigma Chemical Co., St. Louis, MO.
Phytohemagglutinin: Sigma Chemical Co., St. Louis, MO.


Rat T-cell Polyclone (IL-2): Collaborative Research, Inc., Bedford, MA.

M IL-2 (25,000 BRMP units): Collaborative Research.

Fetal Calf Serum (FCS): Irvine Scientific, Santa Anna, CA.

RPMI Medium 1640 with 25 ml Hepes and L-glutamine: GIBCO Laboratories, Santa Clara, CA.

Minimum Essential Medium (MEM) with Earle's salts and L-Glutamine: GIBCO Laboratories.

Gentamicin (10 mg/ml): GIBCO Laboratories.

Deionized Distilled H₂O: GIBCO Laboratories.

MEM Sodium Pyruvate Solution: GIBCO Laboratories.

L-Glutamine: GIBCO Laboratories.

Trypan Blue Stain 0.4%: GIBCO Laboratories.

Reagents:

Ammonium Chloride (NH₄Cl): MCB, Los Angeles, CA.

Dimethyl sulfoxide, 99.9% (DMSO), Spectrophotometric grade: Aldrich Chemical Co., Inc., Milwaukee, WI.

Disodium Phosphate (Na₂HPO₄): EM Science, Cherry Hill, NJ.

Ethylenediamine Tetraacetic Acid (EDTA): Sigma Chemical Co., St. Louis, MO.

Glycine: BioRad, Richmond, CA.

Heparin (1000 usp units/ml): Elkins-Sinn, Inc., Cherry Hill, NJ.

MTT: Sigma Chemical Co., St. Louis, MO.
Potassium Bicarbonate (KHCO₃): Mallinckrodt, Paris, KY.

Safety Solve Scintillation Cocktail: Research Products International Corp., Mt. Prospect, IL.

Sodium Chloride (NaCl): Fisher Scientific, Fair Lawn, NJ.

Sodium Hydroxide (NaOH): Mallinckrodt, Paris, KY.

Sodium Phosphate (NaH₂PO₄): JT Baker Chemical Co., Phillipsburg, NJ.

Tritiated Thymidine (Methyl³H; specific activity = 1.0 Ci/mmol): ICN Radiochemicals, Irvine, CA.

Virgin Olive Oil (100% purity)

Equipment:

Biological Safety Hood: Forma Scientific


Skatron Combi Cell Harvester: Skatron, Inc., Sterling, VA.

96 Well Micro Plate Reader: Skatron, Inc., Sterling, VA.

Sorvall, RT 6000 Refrigerated Centrifuge: Dupont

Fisher Isotemp Oven, 100 series, model 126G

Coulter Model S Plus/IV Cell Counter - Courtesy of Kino Hospital, Tucson, AZ.

Beckman LS 6000 Series Liquid Scintillation Counter: Beckman Instruments, Inc., Fullerton, CA.


Horizontal Rack Shaker: Eberbach Corp., Ann Arbor, MI.

Phase Contrast Microscope: Nikon.
Sterile Supplies:

Acrodisc, 0.2 μm Syringe Filters: Gelman Sciences, Ann Arbor, MI.

Sterile Filter Flasks, 0.2 μm, 500/1000 ml: VWR Scientific, Phoenix, AZ.

75 cm² Triangular Cell Culture Flasks;
96 Well, Flat bottom, Tissue Culture Clusters;
Stripette Serologic Pipets (5, 10, 25 ml): Costar, Van Nuys, CA.

All other general laboratory supplies used for sterile techniques were autoclaved prior to use. To avoid any possible contamination by endotoxins, no glassware was used. All reagents were made up in sterile, disposable plastic containers and sterile filtered prior to use.

EXPERIMENTAL METHODS

Stock Cytotoxic T Lymphocytic Leukemia (CTLL) Cell Cultures:

Stock CTLL cells cultures were maintained in a 37 \(^\circ\text{C}, 5\% \text{CO}_2\), humidified incubator. Cells were grown at concentrations between 1 x 10⁴ and 1 x 10⁵ cells/ml in a flask containing 20 ml RPMI culture media with 10% fetal calf serum, L-glutamine, sodium pyruvate and gentamicin (Appendix D). Culture media was renewed twice per week and cells were dosed with rat IL-2 media at a ratio of 1:20 every other day to maintain adequate IL-2 concentrations for cell survival.
IL-2 depletion is rapidly followed by a marked loss of cell viability. Cells were subcultured and dosed with rat IL-2 media to assure cells were in exponential growth, 24 hr prior to experimental use. (Freshney, 1987)

**Splenic Leukocyte Collection and Culture:**

Immediately following CO\textsubscript{2} asphyxiation, spleens were aseptically removed from C57BL/6 mice and each spleen placed in a 15 ml sterile, plastic, screw cap, conical centrifuge tube containing 10 ml MEM held at 4 °C. From this point, all procedures were performed under the biological safety hood to maintain the sterility of the samples. The entire contents of the centrifuge tube were placed in a sterile petri dish. Spleen cells were isolated by flushing the spleen with MEM using a 21 gauge hypodermic needle bent at a 45 to 90° angle attached to a 6 ml syringe. Spleens were repeatedly flushed until the spleen appeared devoid of cells (blanched in appearance). The spleen was then discarded and the remaining MEM containing spleen cells replaced in centrifuge tube, sealed, and centrifuged for 10 minutes at 8 °C, 400 x g. The supernatant fraction was removed and discarded and the cells resuspended in 10 ml RBC lysing buffer (Appendix D) and held at 4 °C for 10 min to remove red blood cells. The suspension was again centrifuged as above and cells were washed twice with 10 ml MEM to remove all the buffer. The cells were then resuspended in 10 ml supplemented RPMI plus 10% FCS.
A small aliquot of this suspension was diluted 1:1 with trypan blue stain (Appendix D) to determine cell viability and concentration. Counts were performed using a Bright Line hemocytometer counting chamber and a Nikon phase contrast microscope, 400 x magnification. Viable cells were determined by trypan blue dye exclusion. Cell concentration (cells/ml) was determined using the following formula:

\[
\frac{\text{# viable cells counted} \times \text{dilution factor} \times 10,000}{\text{# squares counted}}
\]

Cell suspensions were adjusted to 2 x 10^6 viable cells/ml.

Aliquots (100 µl/well) of the cell suspension were then transferred to sterile 96 well, flat bottom, microculture plates. A 1:1 dilution was made in each well by adding 100 µl of serum free RPMI supplemented with gentamicin prepared with or without mitogen. Total well volume was 200 µl/well and total cell concentration was 2 x 10^5 cells/well (1 x 10^6 cells/ml). Plates were then incubated at 37 °C, 5% CO₂, under humid conditions to prevent evaporative loss of media. Incubation time was determined by optimization of the assay parameters.

**Experimental Cultures:**

Spleen cell cultures were set up in duplicate sets (figure 3). Tritiated thymidine (³H-T) was added to the first set, incubated 72 hr, and analyzed for blastogenesis. The second set was incubated for 48 hr, centrifuged, and 100 µl
supernatant fraction from each well was transferred to new plates. Stock CTLL cell cultures were washed twice with serum free RPMI supplemented with gentamicin to remove exogenous IL-2. Following final wash, CTLL cells were suspended in supplemented RPMI + 10% FCS at a concentration of $2 \times 10^5$ cells/ml. Aliquots (100 μl/well) of this suspension were then incubated with the supernatant from spleen cultures for 48 hr prior to analysis for blastogenesis. Final concentration of CTLL cells equaled $1 \times 10^4$ cells/well ($1 \times 10^5$ cells/ml), while total well volume equaled 200 μl.

**Mitogen Preparation and Concentrations:**

Mitogens incubated with spleen cell cultures to induce IL-2 production include PHA, Con A and two concentrations of SEA. Working solutions were made using serum free RPMI supplemented with gentamicin as the diluent. Mitogen concentrations were as follows:

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Working Concentration</th>
<th>Final Concentration/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA</td>
<td>2.0 ng/ml</td>
<td>1.0 ng/ml</td>
</tr>
<tr>
<td>SEA</td>
<td>0.2 ng/ml</td>
<td>0.1 ng/ml</td>
</tr>
<tr>
<td>PHA</td>
<td>20 μg/ml</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>Con A</td>
<td>40 μg/ml</td>
<td>20 μg/ml</td>
</tr>
</tbody>
</table>
Figure 3: Experimental Protocol.
Animal Care and Dosing:

Female C57BL/6 mice four to six weeks in age were purchased from Harlan Laboratories, Walkerville, MD. Animals were housed in the Department of Animal Research facilities at the University of Arizona Medical Center and were acclimatized under a biological safety hood on a 12 hr light/dark cycle for at least 5 days prior to use. Animals were separated by dosing groups, 5 - 6 mice per cage with butchers sawdust bedding and fed Teklab standard rodent chow and water ad libitum. Naive control mice were housed under identical conditions as the dosed groups.

Dosing groups included naive controls (C), vehicle controls (V), cyclosporin A 30 mg/kg, and three concentrations of benzene: 880 mg, 440 mg, and 220 mg/kg/day. Each mouse, excepting controls, was injected subcutaneously for 14 days at 24 hr intervals. Mouse weight was monitored and recorded daily prior to dosing (Appendix E). Each animal received a total dose volume of 100 μl/mouse/day. Commercial olive oil was chosen as the dose vehicle to reduce any solvent effects of benzene at the site of injection. CsA was first dissolved in a minimal volume of ethanol (100 μl), then diluted to desired concentration with olive oil. Benzene dilutions were prepared with olive oil. All doses were prepared using an average weight of 20 g/mouse.

Animals were asphyxiated with CO₂ 24 hr following final dosing. The heart was immediately exposed and blood
collected by cardiac puncture with a heparinized syringe and stored at 4 °C for up to 48 hr. The spleen was then excised and placed in 10 ml chilled MEM for subsequent culture procedures.

ANALYTICAL PROCEDURES

Two methods of analysis were used to qualitatively measure cell proliferation: (1) blastogenesis, which measures $^3$H thymidine uptake in dividing cells; and (2) a colorimetric mitochondrial viability assay (MTT), which proportionately reflects cell survival and proliferation.

Blastogenesis Assay:

The blastogenesis assay (adapted from Bradley, Selected Methods in Cellular Immunology, 1980) is a simple method for measuring cellular proliferation. The incorporation of $^3$H-T in the DNA of dividing cells is measured and a quantitative change in blastogenesis detected by comparing the uptake of $^3$H-T in treated cells with controls.

At incubation time zero, 50 μl $^3$H-T (50 μCi/well) was added to each well of cells placed into culture (total well volume = 250 μl). Primary spleen cell cultures were incubated for 72 hr, while CTLL cell cultures were incubated for 48 hr. Following incubation cells were harvested on a
Skatron Compi, multi-well, cell harvester and collected on filter discs. Discs were dried in 60 °C oven for 30 min then placed in scintillation vials containing 3 ml scintillation cocktail. Scintillation vials covered with aluminum foil to exclude light were agitated on an Eberbach horizontal shaker for 30 min and held at 4 °C for a minimum of 8 hr prior to placing in scintillation counter. Results were reported as \(^3\)H dpm/sample.

**MTT Assay:**

Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial enzyme succinic dehydrogenase to a colored formazan product is the basis of this colorimetric viability assay. Change in optical density (O.D.) is measured spectrophotometrically and is dependent on the formation of formazan crystals in viable cells. A linear relationship exists between cell number and MTT-formazan production up to a cell concentration of \(5 \times 10^4\) cells/well (Plumb, 1989; Mosmann, 1983).

It was necessary to experimentally determine optimal experimental conditions for the CTLL cell line. Modifications in the basic assay methods used by Mosmann et al (1983) achieved higher absorbance readings at 570 nm, enhancing the sensitivity of the assay. Acidified isopropyl alcohol was replaced by dimethyl sulfoxide (DMSO) which proved to be a more effective solvent in solubilizing the formazan crystals
(Carmichael, 1987). Plumb et al (1989) found that raising the pH of the solubilized product to 10.5 with the addition of glycine buffer (Appendix D) caused a significant increase in absorbance at 570 nm and overcame the variability caused by the presence of culture media. Other parameters experimentally determined and presented later include incubation time, plated cell number/well, and a comparison of mitogen stimulation at concentrations used for primary spleen cultures and carried over in the supernatant incubated with the CTLL cells.

MTT (50 μl/well) at a concentration of 2 mg/ml (Appendix D) was added to cell cultures 4 hr prior to analysis. Plates were then centrifuged at 400 x g for 5 min and the supernatant fraction was removed, leaving approximately 30 μl culture media plus cells in each well. DMSO (200 μl/well) and glycine buffer (20 μl/well) were added to each well and plates were shaken for 5 minutes to ensure complete dissolution of cells and formazan crystals. Plates were then read on a Skatron multiwell scanning spectrophotometer (ELISA) reader at a wavelength of 570 nm and a reference wavelength of 630 nm. Results were subtracted from blank media wells and expressed as nm of absorbance. Plates were kept on the shaker after addition of DMSO and read within 15 minutes.
Statistical Analysis:

The N values represent individual animals. Each data point represents the mean ± SEM for each dose group with 3-4 replicates per sample. Comparisons were made using ANOVA for multiple comparisons with $p = < 0.05$ considered significant.
RESULTS

OPTIMIZATION OF MTT ASSAY USING CTLL CELLS

No significant difference was found in the MTT assay by altering the concentration of MTT (1 to 5 mg/ml) or time (4, 6 or 8 hr) MTT was incubated with CTLL cells prior to analysis (figures 4 and 5). Optimal conditions were therefore set at 2 mg/ml MTT added 4 hr prior to analysis to conserve reagent and time.

CTLL growth rates vary based on cell density and the availability of IL-2. The number of cells/well plated was determined so that cells were in exponential growth phase at the time of harvest (figure 6). It was also important to choose a cell concentration and incubation time which demonstrated a significant difference in proliferation between antigen-stimulated cells and untreated cells. Basing optimal cell concentration/well and incubation time on exponential growth and an observable difference in O.D. between treated and untreated cells, 20,000 cells/well incubated for 48 hr was chosen for future experimental procedures (figure 6, graph C). Cell concentrations greater than 20,000 cells/well demonstrated a rapid depletion of IL-2, resulting in short exponential growth times. Cell concentrations less than
20,000 cells/well exhibited good exponential growth phases after 40 hr of culture, but the differences in O.D. would require a prolonged incubation time or a large study population to verify significance.

CTLL cell response to mitogens demonstrated the mitogenic superiority of SEA in stimulating blastogenesis. The higher concentration of SEA (1.0 ng/ml) appeared to be slightly toxic to the CTLL cells resulting in a decreased proliferative response when compared with the lower (0.1 ng/ml) SEA concentration. However, SEA was still found to stimulate a quantitatively higher proliferative response compared to the conventional mitogens Con A and PHA. Cells incubated in the absence of IL-2 were unable to proliferate in the presence of mitogen.
Figure 4: Reduction of different concentrations of MTT in CTLL cells. Concentrations ranging from 1 - 5 mg/ml MTT were incubated with CTLL cell concentrations ranging from 10 - 500 x 10^3 cells/ml for a total of 4 hr. Each experimental point is representative of the mean ± 6 cultures.
Figure 5: Incubation times of MTT with CTLL cells. CTLL cell concentration ranging from 10 - 500 x 10^3 cells/ml were incubated with 2 mg/ml MTT for 4 - 8 hr. Each experimental point is representative of the mean ± SEM of 6 cultures.
Graphs A - F represent six concentrations of CTLL cells incubated for 0 to 64 hr. CTLL cells were incubated with rat T cell polyclone (IL-2 media) and RPMI media (control) or one of the following mitogen concentrations: SEA 1.0 ng/ml, SEA 0.1 ng/ml, Con A 20 µg/ml, PHA 10 µg/ml. MTT was added to cultures 4 hr prior to analysis. Samples were treated with DMSO and glycine buffer read on a multiwell scanning spectrophotometer at a wavelength of 570 nm and a reference wavelength of 360 nm at eight hour intervals. Each point represents the mean ± SEM of 3 wells per culture plate.

Exponential growth and a significant increase in O.D. from control for antigen stimulated cells were considered important in selection of optimal conditions. Graph C represents the optimal parameters selected for future studies: 20,000 cells/well (100,000 cells/ml), incubated for 48 hr.
Graph A: 100,000 CTLL cells/well.

Graph B: 50,000 CTLL cells/well.

Graph C: 20,000 CTLL cells/well.
Graph D: 10,000 CTLL cells/well.

Graph E: 5,000 CTLL cells/well.

Graph F: 2,000 CTLL cells/well.
**ANIMAL STUDIES**

**Benzene Immune Suppression: 3 Day Study**

A preliminary experiment to test the effect of benzene concentrations, antigen concentrations, and incubation parameters was performed. Animals (n = 2/dose group) were dosed intraperitoneal for three days with either olive oil or 880 mg/kg/day benzene. Animals were killed and splenocyte cultures prepared. Proliferation of primary spleen cells was determined by blastogenesis (figure 7) while CTLL proliferation was analyzed by MTT reduction (figure 8).

From this preliminary study several conclusions were drawn. First, SEA-induced IL-2 production was significantly increased when compared to the response from Con A and PHA stimulated cells (figure 7). Secondly, exposure to 880 mg/kg/day benzene for three days resulted in decreased mitogen-stimulated proliferation in both the primary spleen cells and the CTLL cells. Decreased CTLL proliferation resulted from decreased IL-2 concentrations in the spleen culture supernatant fraction (figure 8). Lastly, analogous proliferative responses were observed using the MTT and blastogenesis assays. The primary spleen cells and CTLL cells both demonstrated a comparable mitogen stimulated proliferative response in naive, vehicle and benzene treated animals. However, the blastogenesis assay proved to be a much more sensitive proliferative assay. A 250 fold increase
in proliferation was seen between the control and SEA induced cells where only an 8 fold increase was demonstrated by MTT. Based on this observation, it was decided to continue the experiments using the blastogenesis assay to measure the proliferative response for both the primary spleen cell and CTLL cell cultures.
Figure 7: Splenocyte blastogenesis from C57BL/6 mice exposed to benzene. Mice were pretreated with 880 mg/kg benzene or olive oil for 3 days prior to sacrifice. Spleen cells were removed and cultured at a concentration of $2 \times 10^5$ cells/well and incubated with mitogens for 48 hr. Blastogenesis was measured followed by an additional 24 hr incubation with $^3$H-T. Each experimental point represents 2 animals with 6 replicate cell cultures/animal.
Figure 8: IL-2 dependent blastogenesis in CTLL cells: 3 day benzene study. Spleen cells were removed from C57BL/6 mice pretreated with 880 mg/kg benzene or olive oil and cultured with mitogens to stimulate IL-2 production. Following 48 hr incubation, the supernatant fraction was removed and incubated with CTLL cells (2 x 10^4 cells/well) for 48 hr. CTLL cell proliferation, measured by reduction of MTT in the mitochondria of viable cells, was indicative of the presence of IL-2 in the spleen cell supernatant. Each experimental point represents 2 animals with 6 replicate cell cultures/animal.
Cyclosporin / Benzene Immune Suppression: 14 Day Study

Animals were dosed subcutaneously for 14 days with olive oil, cyclosporin or benzene (n = 4-6/dose group). Weight gain was monitored daily as an early indicator of dose intolerance. A steady weight gain was seen in all groups with a dose response effect observed in benzene treated animals (figure 9). There was a substantially greater weight gain in the dosed groups as compared with naive controls. This was attributed to the large increase in lipid intake represented by the olive oil vehicle. Individual animal weights are found in appendix D. Average delivered dose per group was as follows:

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>Average Weight/Mouse</th>
<th>Average Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive Controls</td>
<td>16.5 ± 1.4 g</td>
<td>—</td>
</tr>
<tr>
<td>Olive Oil Vehicle</td>
<td>18.2 ± 1.5 g</td>
<td>—</td>
</tr>
<tr>
<td>CsA  30 mg/kg</td>
<td>17.0 ± 1.3 g</td>
<td>25.5 mg/kg/day</td>
</tr>
<tr>
<td>Benzene 880 mg/kg</td>
<td>17.2 ± 0.8 g</td>
<td>756.8 mg/kg/day</td>
</tr>
<tr>
<td>Benzene 440 mg/kg</td>
<td>17.8 ± 1.2 g</td>
<td>391.6 mg/kg/day</td>
</tr>
<tr>
<td>Benzene 220 mg/kg</td>
<td>17.2 ± 1.6 g</td>
<td>189.2 mg/kg/day</td>
</tr>
</tbody>
</table>

Blood removed from animals was analyzed as an indices of bone marrow and lymphocyte toxicity. White blood cell (WBC) counts, red blood cell (RBC) counts, and percent of lymphocytes were compared by dosing group. Animals treated with benzene showed significantly decreased WBC (figure 10) and percent lymphocytes (figure 11). No significant decrease was seen in circulating RBC (figure 12). However, peripheral blood smears viewed microscopically (1000x, oil immersion) were indicative of bone marrow dysfunction. Polychromasia
Figure 9: Average weight gain of C57BL/6 mice during chronic exposure to olive oil, CsA, or benzene. Animals were weighed daily prior to dosing. Each point represents the mean ± SEM for each group (n = 4-6).
Figure 10: The effect of benzene on circulating WBC counts in C57BL/6 mice. Blood samples were collected by cardiac puncture following 14 days pretreatment with olive oil, CsA, or benzene. Each point is representative of a single sample from each animal used in the study. (* significantly reduced: p < 0.05)
Figure 11: Percent of lymphocytes of total circulating WBC in blood from mice pretreated with olive oil, CsA, or benzene. Blood was collected by cardiac puncture following 14 days chronic exposure. Each point is representative of a single sample from each animal in the study. (* significantly reduced: p = < 0.05)
Figure 12: The effect of benzene on circulating RBC counts in C57BL/6 mice. Blood samples were collected by cardiac puncture following 14 days pretreatment with olive oil, CsA, or benzene. Each point is representative of a single sample from each animal used in the study.
may be indicative of anemia and early release of cells from the bone marrow. Megakaryocytosis, the presence of platelet precursors in peripheral blood, was also found and indicates bone marrow dysfunction (Wintrobe, 1974).

Spleens were aseptically removed immediately following cardiac puncture. Gross examination revealed marked atrophy of spleens from all mice treated with benzene, which is indicative of toxicity. Splenocyte recovery from spleens exposed to benzene was reduced by 70% to 90%.

Blastogenesis assays performed on splenocyte cultures demonstrated a significant reduction in mitogen-stimulated proliferation in animals treated with CsA and benzene as compared with vehicle controls (figure 13).

It was interesting to note that SEA-induced proliferation of splenocytes from mice dosed with 220 mg/kg benzene was significantly reduced when compared with vehicle controls, however, a significant increase was measured when compared with naive controls. This seemed indicative of a different mechanism of toxicity. This was substantiated by the CTLL blastogenesis assay (figure 14) which demonstrated sufficient IL-2 in the supernatant fraction from spleen cells taken from mice dosed with 220 mg/kg benzene to support cell growth similar to that in the naive and vehicle control groups. The CTLL blastogenesis assay showed significant decreases in IL-2 production only in the 880 mg/kg and 440 mg/kg dosed animals.
Figure 13: Blastogenesis of primary spleen cells from C57BL/6 mice following chronic administration of olive oil, CsA, or benzene. Female mice were pretreated with olive oil, 30 mg/kg CsA or 3 doses of benzene (880, 440, or 220 mg/kg). Spleen cells were removed and incubated (2 x 10^5 cells/well) with culture media (control), SEA or Con A plus 3H thymidine for 48 hr. Cells were harvested and placed in 3 ml scintillation fluid and held at 4 °C for 8 hr prior to analysis. Each bar represents the mean ± SEM of the animals in each dose group (4 replicate wells/culture).

* = significant from vehicle control, p < 0.05.

n = 4 - 6 mice/group.
Figure 14: IL-2 dependent blastogenesis in CTLL cells:
14 day CsA and benzene study. C57BL/6 mice were
pretreated with olive oil, 30 mg/kg CsA or 3 doses
of benzene (880, 440, or 220 mg/kg benzene).
Spleen cells were removed and incubated with
mitogens to stimulate IL-2 production. The
supernatant fraction was removed after 48 hr and
incubated with CTLL cells (2 x 10⁶ cells/well) plus
°H-T. Cells were harvested after 48 hr incubation,
placed in 3 ml scintillation fluid, and held at
4 °C for 8 hr prior to analysis. Each bar
represents the mean ± SEM of the animals in each
dose group (4 replicate wells/culture).

* = significant from vehicle control, p < 0.05.
n = 4 - 6 mice/group.
The immune stimulation and IL-2 detection methods described in this research were applied to 4-phenylcyclohexene (4PC), an environmental toxicant currently being investigated as an indoor air pollutant. 4PC, a volatile compound emitted from new carpet, is a byproduct in the manufacturing of the latex backing of carpet involving the carboxylic-styrene-butadiene polymerization process (Crabbe, 1984).

At the present time, there is no evidence to implicate 4PC as an immunotoxicant. However, some individuals have experienced symptoms similar to those found with an allergic or hypersensitive reaction when exposed to 4PC. In the last several years, there have been a growing number of health complaints following the installation of new carpeting. Reported symptoms include: eye, nose, and throat irritation, headache, sinus irritation, respiratory irritation, and fatigue (Crabbe, 1984; Walsh, 1986; NIIP, 1986). Toxicological studies on 4-PC are inconclusive. However, slight eye irritation in rabbits and cellular damage and hemorrhage in the lungs of rats exposed by intratracheal administration have been reported (Walsh, 1986) and may be related with eye irritation and respiratory symptoms exhibited...
by humans exposed to 4-PC at much lower concentrations.

INVESTIGATION OF 4-PHENYLCYLOHEXENE AS AN IMMUNOTOXICANT

Dosing and Analysis:

Whole spleens from female B6C3F1 mice were donated by Lisa Parola and Dr. Steve Hooser, Department of Pharmacology and Toxicology, University of Arizona. Animals were treated with 4PC or sesame seed oil (dose vehicle) for 30 days for reproductive toxicity testing. Dosing groups included vehicle (sesame seed oil) controls, and two concentrations of 4PC, 3.0 mmole and 6.0 mmole. Total prepared dose volume for each animal was 2.5 ml/kg/day. In addition to daily intraperitoneal dosing and weight checks, a vaginal smear was prepared. (Parola, 1990) Animals were killed by CO₂ asphyxiation 24 to 72 hours following the final dose. (For the purposes of the reproductive studies, all mice were in estrus at the time of death.)

Blood analysis for leukocytes and red blood cells was not performed on these animals as the blood was needed for the reproductive studies. All other procedures, including primary spleen culture preparation and incubation, mitogen preparations, CTLL culture preparation, blastogenesis assay and MTT assay, were performed exactly as described previously.
Results:

At the doses examined, 4PC did not significantly alter the ability of primary spleen cells to proliferate or to produce IL-2 in sufficient quantities to support proliferation of CTLL cells in culture (figures 15, 16 and 17). Blastogenesis assays performed on splenocytes and CTLL cells were comparable and demonstrated the increased sensitivity of measuring $^3$H-T uptake compared with the reduction of MTT to detect a difference in the blastogenic response. A hyperproliferative response was not observed, but these procedures were not designed specifically to rule out a hypersensitive immune response.
Figure 15: The effect of 4PC on blastogenesis of primary spleen cells in B6C3F1 female mice. Animals were pretreated for a total of 30 days with sesame seed oil or 4PC prior to sacrifice. Spleen cells were removed and incubated (2 x 10^5 cells/well) with culture media (control), SEA or Con A plus 3H thymidine for 48 hr. Cells were harvested and placed in 3 ml scintillation fluid and held at 4 °C for 8 hr prior to analysis. Each bar represents the mean ± SEM of the animals in each dose group, n = 5 with 4 replicate wells/culture.
Figure 16: IL-2 dependent blastogenesis in CTLL cells: 4PC study. Female B6C3F1 mice were pretreated for 30 days with sesame seed oil vehicle, 3 mmole 4PC or 6 mmole 4PC. Spleen cells were removed and incubated with mitogens to stimulate IL-2 production. The supernatant was removed after 48 hr and incubated with CTLL cells (2 x 10⁶ cells/well) plus ³H-T. Cells were harvested after 48 hr incubation, placed in 3 ml scintillation fluid, and held at 4 °C for 8 hr prior to analysis. Each bar represents the mean ± SEM of the animals in each dose group, n = 5 with 4 replicate wells/culture.
Figure 17: IL-2 dependent growth in CTLL cells: 4PC study analysis by MTT viability assay. Female B6C3F1 mice were pretreated for 30 days with sesame seed oil vehicle, 3 mmole 4PC or 6 mmole 4PC. Spleen cells were removed and incubated with mitogens to stimulate IL-2 production. The supernatant fraction was removed after 48 hr and incubated with CTLL cells (2 x 10^4 cells/well). 2 mg/ml MTT was incubated with each well for 4 hr prior to analysis. Plates were developed with DMSO and glycine buffer (pH 10.5) and read spectrophotometrically at 570 nm. Each bar represents the mean ± SEM of the animals in each group, n = 5 with 4 replicate wells/culture.
DISCUSSION

A number of studies have examined the immunotoxic effects of benzene, primarily using mitogens such as Con A and PHA to measure the ability of lymphocytes to proliferate in vitro (Wierda, 1981; Pfeifer and Irons, 1982; Rozen, 1984). Inhibition of RNA synthesis and IL-2 formation was shown following in vitro incubation of benzene and its metabolites hydroquinone and p-benzoquinone with mouse spleen lymphocyte cultures (Post, 1985). My studies, which combined in vivo benzene exposure with in vitro mitogen stimulation of isolated spleen lymphocytes, were performed to assess inhibition of IL-2 production. The results demonstrate a dose-dependent suppression of IL-2 production. This study supports previous work demonstrating the immunotoxicity of CsA as an immunosuppressant which functions by altering IL-2 function rather than as a toxicant to the immune cells or bone marrow. CsA was able to significantly reduce mitogen-stimulated blastogenesis of primary spleen cultures without evidence of myelotoxicity. This did not appear to result totally from inhibition of IL-2 production, since sufficient IL-2 was present in the supernatant from the spleen cell cultures to support proliferation of the IL-2 dependent CTLL cell line. The probable mechanism of immune suppression exhibited in the
mitogen-stimulated spleen cultures was through inhibition of receptor expression or binding.

Circulating blood cell profiles and spleen appearance greatly enhanced the studies by supplying confirmatory information and helping to define immunotoxic effects. Common immunotoxic effects which may alter immune response include cytotoxicity resulting in the death of immune cells, morphological changes in the bone marrow, alterations in antigen and/or interleukin receptors, and altered protein and mRNA synthesis. The tests performed on the blood taken from mice dosed for 14 days confirmed the toxic effect of benzene on the bone marrow, resulting in anemia and leukopenia. The reduction of WBC and total lymphocytes combined with the presence of megakaryocytes and polychromatic RBC in circulating blood were indicative of an alteration in bone marrow cellularity and integrity (Wintrobe, 1974). No abnormal or immature leukocytes were noted and therefore damage to stem cells can not be confirmed. Blood examined from mice dosed with benzene for 3 days or CsA for 14 days showed no such evidence of bone marrow toxicity.

The blood profile presented at 14 days appeared very similar for all three doses of benzene. It was interesting to note therefore, that although the spleens were markedly atrophied and the cultured splenocytes from the mice dosed with benzene 220 mg/kg demonstrated significant blastogenic suppression similar to those dosed with 880 mg/kg and 440
mg/kg benzene, the CTLL cells incubated with the supernatant fraction from the primary cultures were capable of proliferation. The ability of the CTLL cells to proliferate was indicative of the presence of IL-2 in the spleen cell supernatant fraction and a mechanism of toxicity at the lower concentration which was not dependent on IL-2 suppression. This pattern was similar to that observed in the CsA dosed animals, suppression of antigen stimulated blastogenesis of the primary cells, but not of the IL-2 dependent CTLL cells. The primary mechanism of action in CsA immune toxicity has been shown to affect the transcription of mRNA for IL-2, although, in some systems CsA has been shown to affect the expression of the IL-2 receptor (Male, 1987; Di Padova, 1989). Suppression or alteration of receptor function would explain the suppression of primary cell culture blastogenesis and the presence of sufficient IL-2 in the culture supernatant fraction to support CTLL cell blastogenesis as described in this research.

A second possible explanation may involve the ability of the immune system to utilize multiple pathways to maintain homeostasis. It would appear that not only benzene concentration, but the duration of exposure determines the degree of immunosuppression by affecting multiple parameters. The spleen cells taken from animals dosed for 3 days with 880 mg/kg benzene and those dosed for 14 days with 220 mg/kg benzene did not demonstrate suppression of IL-2 as did those
dosed with 880 mg/kg and 440 mg/kg benzene for 14 days. This was demonstrated by the ability of the CTLL cells to proliferate when incubated with spleen cell supernatant fractions. Immunosuppression with 880 mg/kg for 3 days appeared to be a cytotoxic reaction without significantly altering spleen morphology, circulating leukocyte numbers or IL-2 production. At 14 days, the same dose caused spleen atrophy, a highly significant \( p = 0.0020 \) decrease in WBC, anemia and suppression of SEA-induced IL-2 proliferation as evidenced by CTLL proliferation \( p = 0.0175 \). In comparison, spleens from the lowest dose of benzene, 220 mg/kg, also showed marked atrophy, anemia, significant decrease in WBC \( p = 0.0041 \), but no significant decrease in splenocyte blastogenesis as compared with naive animals \( p = 0.17 \) or of IL-2 by SEA induced spleen cells (CTLL proliferation, \( p = 0.30 \)).

It was also noted that either the stress produced by long term dosing or the vehicle, olive oil, appears to potentiate an immune response. When animals were dosed for 3 days, no significant difference was apparent between the naive animals and the vehicle control animals. However, after 14 days dosing, a significant \( p = 0.0159 \) increase in SEA-stimulated blastogenesis was seen in the spleen cell cultures. This was carried over into the CTLL cell assay and reflects a real increase in IL-2 production.

An initial study comparing olive oil and corn oil
as vehicles showed no significant difference between naive controls, olive oil dosed animals and corn oil dosed animals (Appendix E). A similar effect was seen in the 4PC studies where sesame seed oil was used as the dose vehicle (Appendix E). This would tend to support the theory that the stress resulting from repeated injections is responsible for the potentiation of the immune response seen in treated animals over the response seen in naive animals and not the dose vehicles. This potentiation strengthens the results which showed dramatic reduction of SEA-induced proliferation in CsA and benzene dosed animals as compared to animals dosed solely with olive oil.

Incorporation of $^3$H thymidine during cell proliferation proved to be a more sensitive method than the MTT cell viability assay. The increased sensitivity made it possible to detect significant differences using a smaller study population. The MTT assay would prove to be a more valuable tool when testing for cytotoxic effects in a totally in vitro system. Its primary advantages appear to be its ability to test large quantities of samples in a very short time. Microculture plates with a capacity for 96 samples can be read within seconds. It also has the advantage of not requiring radionucleotides or numerous washing steps.

It was evident from these studies that SEA was a superior inducer of both primary spleen cells and the CTLL culture cell line than the conventional mitogens Con A and
PHA. A slight decrease in blastogenesis exhibited by the CTLL cell growth curves when incubated directly with 1.0 ng/ml SEA as compared with the lower concentration of 0.1 ng/ml demonstrated a cytotoxic cell-specific effect (figure 7). This inverse relationship between SEA concentration and proliferation was not evident in the primary spleen cultures nor in the CTLL cell cultures exposed through incubation with splenocyte supernatant fraction containing a lower concentration of SEA.

There was no evidence found to support an immunotoxic response following long term exposure to 4PC. However, such a response in a hypersensitive population cannot be ruled out.
Proliferation of CTLL cells incubated with supernatant fraction from SEA-stimulated lymphocyte cultures is a sensitive method of detecting IL-2 mediated immune response. This proliferative response can be measured by either the incorporation of tritiated thymidine by cells undergoing mitosis or by MTT reduction in the mitochondria of viable cells. Of the two, blastogenesis was found to be the more sensitive method of detection.

Benzene was found to inhibit IL-2 production at doses of 880 mg/kg/day and 440 mg/kg/day following 14 days of dosing. Reducing 880 mg/kg/day benzene dosing to 3 days or reducing the concentration to 220 mg/kg/day for 14 days results in suppression of blastogenesis but not of IL-2 production.

SEA at concentrations as low as $10^{-13}$ to $10^{-15}$ M were capable of inducing a greater immune response than conventionally used mitogens such as Con A and PHA.

Using the methods developed in this study, it is possible to test for immunotoxicity which alters immune response through suppression of IL-2 following exposure to environmental pollutants. Since any source of lymphocytes is acceptable, including peripheral blood, this assay presents a viable analytical tool for investigating human exposures to suspected immuno-suppressive agents.
PROPOSALS FOR FUTURE WORK

These studies represent only a qualitative analysis of IL-2 production and function. A quantitative determination of IL-2 concentrations necessary to support CTLL proliferation would greatly enhance the present study.

There are currently available, anti-IL-2 receptor antibodies which can be used to block IL-2 mediated effects. By using these antibodies, it is possible to rule out interfering substances which may cause a false proliferative response. One such example is interleukin 4, which also stimulates resting T cells and activated B cells. Using these antibodies, it would also be possible to design experiments to assess IL-2 receptor expression and function.

Another IL-2 dependent T cell lines needs to be examined. The CTLL cells extremely difficult to maintain in culture over a prolonged period of time. Most other investigators do not try to maintain the cells but bring them up fresh from frozen cultures for each experiment. This requires approximately 2 weeks.

Dosing mice for 3 days proved to be too short of an exposure time to see mechanistic changes, however, 14 days proved to be excessive at the dose concentrations used. It would be interesting to not only try another experiment using the same parameters for 7 days, but also to look at doses less than 220 mg/kg benzene. White cell counts and total
lymphocyte percent are much better indices of acute toxicity than monitoring animal weights. At least one other immunotoxicant should also be examined.

Lastly, the ultimate goal of an assay of this type is to be able to specifically test for IL-2 mediated immunosuppression in an exposed individual. By using concentration techniques, venus blood samples can be used as a lymphocyte source.
## APPENDIX A

### INTERLEUKINS INVOLVED IN T CELL INTERACTIONS OF MICE

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Target Cell</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin 1</td>
<td>Activated accessory macrophage</td>
<td>T cells, many other cells</td>
<td>Enhances T cell proliferation, IL2 production, B cell activation</td>
</tr>
<tr>
<td>Interleukin 2 (T cell growth factor)</td>
<td>Activated T cells</td>
<td>Activated T cells</td>
<td>Stimulates T cell proliferation and differentiation of activated T cells, binds to a specific receptor that is expressed after activation</td>
</tr>
<tr>
<td>Interleukin 3</td>
<td>Activated T cells</td>
<td>Activated T cells</td>
<td>Induces 20a-hydroxysteroid dehydrogenase in spleen cells, stimulates differentiation of activated T cells</td>
</tr>
<tr>
<td>Interleukin 4 (BSF1)</td>
<td>Cultured thymocytes</td>
<td>T and activated B cells</td>
<td>Stimulates resting T cells, anti-Ig activated B cells</td>
</tr>
<tr>
<td>Interferon α</td>
<td>T cells</td>
<td>NK cells</td>
<td>Stimulates IL1 production and NK activity, suppresses growth of certain viruses</td>
</tr>
<tr>
<td>Interferon γ (immune interferon)</td>
<td>T cells</td>
<td>T cells, NK cells</td>
<td>Stimulates NK and TK activity, enhances B cell differentiation (see Table 10-4)</td>
</tr>
<tr>
<td>T suppressor factor</td>
<td>T cells</td>
<td>T&lt;sub&gt;s&lt;/sub&gt; cells</td>
<td>Inhibits T helper cells</td>
</tr>
</tbody>
</table>

Figure B1: Biotransformation of Benzene (Oak Ridge National Laboratory, 1989).
Figure B2: Urinary metabolites of benzene (Oak Ridge National Laboratory, 1989).
APPENDIX C

METABOLISM AND DISPOSITION OF CsA

CsA is metabolized by the hepatic microsomal biotransformation enzymes. The predominant metabolites being a monohydroxylation of the C-17 and N-demethylation of the C-21 moiety (Lemaire, 1985). Most evidence points towards the parent CsA compound as the moiety responsible for the majority of the immunosuppressive effect (Duncan, 1988). The immediate distribution of $^3$H-CsA in mice was equal between the solid organs and whole blood (Backman, 1988). Within four hours, the kidney begins to concentrate CsA, so that by 24 hours following exposure, the total concentration of CsA in the kidney is 9 to 23 times greater than in the blood (Belitsky, 1986).
Molecular Formula
\( \text{C}_{62}\text{H}_{111}\text{N}_{11}\text{O}_{12} \)

<table>
<thead>
<tr>
<th>Primary Metabolite</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>aMetabolite 1</td>
<td>1</td>
</tr>
<tr>
<td>(Hydroxycyclosporine)</td>
<td></td>
</tr>
<tr>
<td>aMetabolite 17</td>
<td>17</td>
</tr>
<tr>
<td>(Hydroxycyclosporine)</td>
<td></td>
</tr>
<tr>
<td>aMetabolite 21</td>
<td>21</td>
</tr>
<tr>
<td>(N-Demethylcyclosporine)</td>
<td></td>
</tr>
</tbody>
</table>

Figure C: Cyclosporin A structure and sites of metabolism (Maurer, 1983).
APPENDIX D

REAGENT PREPARATIONS

RPMI Culture Media:
- Fetal Calf Serum 10%
- Gentamicin 50 mg/L
- Sodium Pyruvate 2 g/L
- L-Glutamine (200 mM stock) 10 ml/L

Sterile filter and store at 4 °C.

RBC Lysing Buffer:
- Sterile H2O 100 ml
- EDTA 3.7 mg
- NH₄Cl 829 mg
- KHCO₃ 100 mg

Sterile filter and store at 4 °C

Trypan Blue Stain:
If a lighter background is desired, stain may be diluted with saline. Stain must be sterile filtered prior to use.

MTT (2 mg/ml):
Make up desired volume in PBS and sterile filter. Make fresh for each use and protect from light.

Phosphate Buffered Saline (PBS) pH 7.4: 10x stock
- 0.2 M NaH₂PO₄ (27.6 g/L) 190 ml
- 0.2 M Na₂HPO₄ (28.4 g/L) 910 ml
- NaCl 87.7 gm

Sterile filter and store at R.T.

Glycine Buffer:
- 0.1 M glycine + 0.1 M NaCl
  equilibrate to pH 10.5 with 0.1 N NaOH
Figure E1: Naive Controls.
Figure E2: Vehicle Controls, 100 μl Olive Oil/Day.
Figure E3: Cyclosporin A - 30 mg/kg/day.
Figure E4: Benzene - 880 mg/kg/day.
Figure E5: Benzene - 440 mg/kg/day.
Figure E6: 220 mg/kg/day.
APPENDIX F

COMPARISON OF OIL VEHICLE CONTROLS

<table>
<thead>
<tr>
<th>Vehicle Controls</th>
<th>Olive Oil</th>
<th>Corn Oil</th>
<th>Olive Oil</th>
<th>Sesame Seed Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure Time</td>
<td>3 days</td>
<td>3 days</td>
<td>14 days</td>
<td>30 days</td>
</tr>
</tbody>
</table>

![Graph showing comparison of oil vehicle controls](image-url)
REFERENCES


