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Washington, DC 20460

Dear Sir:

REF: 8EHQ-0186-0585S

Based on data appearing in draft reports, we have previously submitted summarized results of immunotoxicology studies sponsored by [ ] (see correspondence of April 14, 1987, and July 10, 1987). Final copies of both the primary and follow-up reports have been received from the investigator, Dr. A. E. Munson of the Medical College of Virginia, and are enclosed.

These studies were initiated to further investigate previously-reported immunotoxic effects of Tinuvin\* 144 (chemical name: bis(1,2,2,6,6-penta-methyl-4-piperidiny1)(3,5-di-tert-butyl-4-hydroxybenzyl)butylpropanedioate; [63843-89-10]). The previous data, generated by Ciba-Geigy Corporation, were the subject of the initial TSCA § 8(e) Notice of Substantial Risk, 8EHQ-0186-0585S.

[ ] considers its interest in this product to be proprietary, even though the data, conclusions, and name of the investigator are not. Therefore, we consider this transmittal letter confidential business information.

Sincerely,

[ ]  
[ ]  
[ ]

\*Tinuvin is a registered trademark of Ciba-Geigy Corporation.



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**FINAL REPORT**

**(NOT FOR DISTRIBUTION)**

**IMMUNOTOXICOLOGY STUDIES ON TINUVIN® 144  
ADMINISTERED BY THE DERMAL ROUTE**

**Protocol Identification  
TVN-14-1-DER**

**July 20, 1987**

**Prepared for:**

[ ]

**Sponsor's Representative:**

[ ]

**Testing Facility:**

**MCV/VCU Immunotoxicology Laboratory  
Box 613, Department of Pharmacology & Toxicology  
Medical College of Virginia/Virginia Commonwealth University  
Richmond, VA 23298**

**Investigators:**

**Albert E. Munson  
Kimber L. White, Jr.  
J. Ann McCay**

**(804) 786-8400**

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ADMINISTERED BY THE DERMAL ROUTE

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**APPENDIX**

Appendix Table A-1  
Protocol TVN-14-1-DER  
Protocol Amendments  
Protocol Deviations

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QUALITY ASSURANCE  
REPORT STATEMENT

"Immunotoxicology Studies on Tinuvin® 144  
Administered By the Dermal Route"

Protocol No.: TVN-14-1-DER  
\_\_\_\_\_ Project No.: 86-19

This study has been reviewed by Mr. Robert Wise, Quality Assurance Manager, in accordance with the United States FDA Good Laboratory Practices Regulations (Federal Register) of October 29, 1984. (21 CFR Part 58).

Approved and  
Submitted by:

Rm Wise  
Quality Assurance Manager

7/20/87  
Date

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### Signature Sheet

The studies cited in this report were conducted in the Immunotoxicology Program at the Medical College of Virginia/Virginia Commonwealth University. The studies were sponsored by \_\_\_\_\_ in \_\_\_\_\_. The conduct of this study was in accordance with Good Laboratory Practices and has been subjected to internal review by the technical staff and the Quality Assurance Unit of Virginia Commonwealth University.

### Signatures

Protocol No.: TVN-14-1-DER  
Project No.: 86-19

Albert E. Munson 7/20/87  
Albert E. Munson

Kimber L. White, Jr. 7/20/87  
Kimber L. White, Jr.

J. Ann McCay 7/20/87  
J. Ann McCay

## Executive Summary

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Studies conducted by the Ciba-Geigy Corporation showed that Tinuvin\* 144 (TVN), administered by gavage to rats, produced changes in the lymphoreticular system, as seen in decreased thymic weight, increased spleen weight, and increased number of peripheral blood leukocytes, with a concomitant reduction in the percent of lymphocytes. The purpose of this study was to determine the potential effects of TVN on the function of the immune system after dermal exposure. The protocol was prepared on October 13, 1986 and revised on November 24, 1986. In life phases of the studies were started on December 5, 1986 and completed on January 17, 1987. The studies were conducted in the MCV/VCU Immunotoxicology Facility at the Medical College of Virginia/Virginia Commonwealth University, Richmond, VA 23298. The Study Director was Albert E. Munson, Ph.D., Professor of Pharmacology and Toxicology. The Co-Study Director was Kimber L. White, Jr., Ph.D. and the Immunology Research Assistant was J. Ann McCay.

Toxicological variables evaluated included: (1) body and selected organ weights; (2) selected hematological parameters; (3) selected serum chemistry levels; (4) the ability to produce antibody to the T-dependent antigen, sheep erythrocytes; (5) the ability to produce a delayed hypersensitivity response to keyhole limpet hemocyanin; (6) the ability of spleen cells to respond to T-cell mitogens and to allogeneic cells; and (7) the ability of spleen cells to respond to a B-cell mitogen.

TVN, in acetone/olive oil, administered daily for 14 days to the abraded or non-abraded skin of B6C3F1 female mice produced changes in the lymphoreticular system, as manifested in a decrease in thymus weight, an increase in spleen weight, an increase in the number of polymorphonuclear leukocytes, an increase in humoral- and cell-mediated immune responses, and a decrease in lymphocyte proliferative responses; these changes are consistent with an adjuvant effect. There was also a decrease in erythroid elements in one of the studies.

The meaning of an adjuvant action of a chemical in terms of risk assessment can only be speculated. There is a balance in the immune system as it responds to non-self. Allergy is often an imbalance in recognition of non-self antigens and the inability to suppress immunological responses to the antigens. Certain individuals are more prone to developing immune responses to antigens and are considered atopic. An adjuvant could increase the chances of an individual inappropriately responding to environmental antigens and developing allergies to the antigens. Another potential problem in the enhancement of the immune response is in the area of autoimmunity. The presence of an adjuvant over an extended period of time may increase the chances of autoimmunity. A number of infectious processes, particularly viral infections, cause the formation of neo-antigens, which can elicit immunological responses that, in the presence of an augmented immune state, could increase the potential for an immune response to be directed towards self.

The lowest effect level was 7 mg/kg, as seen in the decrease in lymphocyte proliferation to the B-cell mitogen, LPS. Most of the effects were observed at 70 mg/kg.

\*Tinuvin is a registered trademark of the Ciba-Geigy Corporation.

## INTRODUCTION

Studies conducted by the Ciba-Geigy Corporation showed that Tinuvin\* 144 (TVN), administered by gavage to rats, produced changes in the lymphoreticular system, as seen in decreased thymic weight, increased spleen weight, and increased number of peripheral blood leukocytes, with a concomitant reduction in the percent of lymphocytes.

The purpose of this study was to determine the potential effects of TVN on the function of the immune system after dermal exposure. Mice were exposed daily for 14 days to TVN in acetone/olive oil by the dermal route. Separate studies were conducted using intact and abraded skin.

The protocol was prepared on October 13, 1986 and revised on November 24, 1986. In life phases of the studies were started on December 5, 1986 and completed on January 17, 1987.

The studies were conducted in the MCV/VCU Immunotoxicology Facility at the Medical College of Virginia/Virginia Commonwealth University, Richmond, VA 23298. The study director was Albert E. Munson, Ph.D., Professor of Pharmacology and Toxicology. The Co-Study director was Kimber L. White, Jr., Ph.D. and the Immunology Research Assistant was J. Ann McCay.

### Methods of Procedure:

Test Article: The test article, TVN (Bis(1,2,2,6,6-pentamethyl-4-piperidiny)(3,5-di-tert-butyl-4-hydroxybenzyl)butylpropanedioate), CAS Reg # 63843-89-0, Lot # 9102-27, was supplied by the sponsor and was received at the Medical College of Virginia/Virginia Commonwealth University, Richmond, VA on 26 November 1986. TVN was given the MCV Chemical Number MCV-26-11-86-1.

Te. Article Stability: TVN was determined to be soluble in acetone:olive oil in a ratio of 4:1. Since stability data were not available, solutions were prepared daily, immediately prior to application.

Test Article Preparation: TVN was prepared daily as a solution in acetone:olive oil (4:1). The concentrations of the solutions were 70, 7, and 0.7 mg/ml.

Doses Administered: The doses of TVN administered were 70, 7 and 0.7 mg/kg.

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Route of Administration: The test article was administered via dermal exposure by applying the test article directly to the skin using a micropipetter. Animals received 0.01 ml/10 grams body weight. In both sets of studies, animals were shaved on the abdomen one day prior to the start of exposure.

Dermal Abrasion: Animals used in the abraded studies were shaved one day prior to administration of the test article. Additionally, on each of the exposure days, the cornified layer of the epidermis was removed with a Model 403 nylon brush attached to a Dremel Moto-Flex Tool Model 332. The rotating (13,000 rpms) nylon bristles were applied to the skin for 3-5 seconds immediately prior to the administration of the test article. This procedure caused a reproducible dermal abrasion without discomfort to the mice.

Vehicle Control: Acetone:olive oil (4:1) was used as the vehicle. Vehicle control animals were administered 0.01 ml/10 grams of body weight by dermal exposure. Vehicle control animals were dosed at the same time as the treatment groups.

Positive Control: Cyclophosphamide (Sigma Chemical Co.) was used as a positive control for the immunological studies. Positive control animals received 50 mg/kg of cyclophosphamide (CPS) on days 11 through 14 by i.p. injection. Animals received 0.1 ml/10 grams body weight. CPS was prepared in physiological saline and stored at -20°C. It was thawed quickly, just prior to use, and administered within 10 minutes of thawing.

Study Duration: All animals received the test article for 14 consecutive days. On day 15, one day after the last exposure, animals were evaluated for the appropriate immunological and toxicological parameters.

Species Selection: Female B6C3F1 mice were used in this study. The mouse is currently the species of choice in conducting immunological studies. The B6C3F1 mouse is the National Toxicology Program (NTP)-designated mouse strain and a large data base exists on this strain. The major components of the immune system in the mouse and man are the same; agents that perturb the immune system in man perturb the immune system in the mouse in a similar manner. The mouse has previously been found to be sensitive to the effects of various drugs and environmental chemicals on the immune system.

Test System: Female mice were purchased from Charles River Breeding Laboratories. The mice were hepatitis and Sendai free. Animals arrived at 5-6 weeks of age (17-19 grams). Upon arrival the mice were quarantined for at least one week prior to commencement of treatment. All mice were randomized using a computer-generated randomization procedure. Mice were housed 4 animals per cage in plastic shoebox cages with sawdust (hardwood) bedding and maintained on Ziegler Rat and Mouse Ration (35-555 NIH 31) and tap water ad libitum from water bottles. Certified diet is suitable to preclude contamination which might interfere with the outcome of the study. The cages were covered with filtered bonnets and placed on a rack such that individual dose groups were not all at one level, i.e., the cages were ordered from top to bottom. Mouse cages were cleaned and sanitized twice a week. Animals were housed in the Strauss Building (MCV/VCU), where the temperature was maintained at 18-26°C and the relative humidity

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between 30 and 70%. The light/dark cycle was maintained on 12-hour intervals.

**Experimental Design.** Following randomization, animals were assigned to an immunological study as either abraded or non-abraded animals. Within each study, animals were assigned to one of five groups. Group 1 animals were the vehicle control group, identification code VH, and received vehicle only as their treatment. Group 2 animals received the low dose of TVN, 0.7 mg/kg, and had the identification code D1. Group 3 animals were treated with 7 mg/kg of TVN and were identified as D2. The animals assigned to group 4 received the high dose of TVN, 70 mg/kg, and had the identification code D3. The positive control animals, group 5, received CPS, 50 mg/kg, and had PC as their identification code. A total of 304 female B6C3F1 mice were used in completing this protocol and repeat studies (Tables 6R, 18R, 19R, and 21R). Eight animals were used in each of the experimental groups, except the positive control (CPS) groups, which had 4 animals each.

**Parameters Investigated:**

**1. General**

- a. **Body Weight.** Animals were weighed on the first day of treatment, and on days 8 and 15 of the study. Animal weights are presented as body weight in grams and as change in body weight at 1 and 2 weeks.
- b. **Observations.** Animals were observed daily at the time of dosing for any pharmacotoxicological signs (SOP/TOX/028). Any remarkable effects were recorded on the study log sheet, present in the exposure room, in accordance with the SOP (SOP/TOX/016).

**2. Standard Toxicology Section**

- a. **Terminal body weights and selected organ weights were recorded.** Body weights were recorded 1 day after the last exposure (day 15). One day after the last exposure, the mice were anesthetized, bled (cardiac puncture), and necropsied. Animals were examined for gross pathology and the following organs removed, cleaned of connective tissue and weighed: brain, thymus, lungs, liver, spleen and kidney (SOP/TOX/016). The organs (except for the spleen) were placed in 10% neutral formalin and are being maintained in the MCV/VCU Archives.
- b. **Hematology.** Selected hematological parameters were assessed. One day after the last exposure, blood was collected by cardiac puncture into a wetted heparinized syringe. A blood smear was prepared at the time of blood collection and, after dehydration with methanol, stained with Wright's and Giemsa. The following parameters were assessed: erythrocyte and leukocyte number, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Leukocytes were differentiated into lymphocytes, polymorphonuclear leukocytes (polys), monocytes and eosinophils. Total lymphocytes, polymorphonuclear leukocytes, monocytes and eosinophils were calculated by multiplying

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the total leukocytes count by the percent of each cell type, which was determined by the differential count. (SOP/HEM/004,006,010,012,014).

c. Serum Chemistries. Blood was collected into a 1-ml syringe and dispensed into a glass tube for clotting. Serum was harvested after 2 hours of clotting and the following serum chemistries were measured using an ABA-100 Bichromatic Analyzer: SGPT, BUN, glucose, albumin, and total protein. (SOP/CHM/006,012,016,032,034).

### 3. Immunology

a. Spleen IgM antibody response to a T-dependent antigen, sRBC (SOP/PFC/006). The primary IgM response to sheep erythrocytes (sRBC) was enumerated using a modified hemolytic plaque assay of Jerne (Bullock and Moller, Eur. J. Immunol. 3: 172, 1973). Mice receiving daily dermal doses of their appropriate treatment were sensitized with  $2 \times 10^8$  sRBC i.v. on day 11 of exposure. On day 15, animals were sacrificed; spleen cells were prepared by mincing the spleen between two frosted microscope slides and diluted to 3 ml. An aliquot of cells was added to a test tube containing guinea pig complement, sheep erythrocytes and warm agar. After thorough mixing, the test tube mixture was plated in a petri dish, covered with a microscope cover slip and incubated at  $37^\circ\text{C}$  for 3 hours. Cell counts were performed on the 3 ml sample and the number of cells/spleen, AFC/spleen and AFC/ $10^6$  spleen cells determined. The plaques which developed were counted using a Bellco plaque viewer. A plaque results from the lysis of sheep erythrocytes and is elicited as a result of the interaction of complement and antibodies directed against sheep erythrocytes which are produced in response to the i.v. sensitization. Each plaque is generated from a single IgM antibody-producing B-cell and thus the number of antibody forming cells (AFC) present in the whole spleen can be calculated. The data are expressed as specific activity (AFC/ $10^6$  spleen cells) and AFC per spleen.

As background, sheep erythrocytes are a T-dependent antigen and thus T-cells, B-cells and macrophages are required to function properly in order to obtain an AFC response. If a xenobiotic affects any of these cell types to a significant degree, an altered response will be observed. As a result, the T-dependent IgM response to sRBC is one of the most sensitive immunotoxicological assays currently in use. A significant modulation in the IgM AFC response, when appropriately compared to untreated controls, indicates that the test agent is capable of modifying the humoral immune response in the whole animal and thus has the potential for immunotoxicity.

Listed below are publications in which the above assay has been utilized by scientists in the MCV/VCU Immunotoxicology Program:

Munson, A.E., Sanders, V.M., Douglas, K.A., Sain, L.E., Kauffmann, B.M. and White, K.L., Jr.: In vivo assessment of immunotoxicity. Environ. Health Persp. 43:41-52, 1982.

Holsapple, M.P., McNerney, P.J., Barnes, D.W. and White, K.L., Jr.: Suppression of humoral antibody production by exposure to 1,2,3,6,7,8-hexachlorodibenzo-p-dioxin. J.

15 6

**Pharmacol. Exp. Ther.** 231:518-526, 1984.

White, K.L., Jr., Lysy, H.H. and Holsapple, M.P.: Immunosuppression by polycyclic aromatic hydrocarbons: A structure activity relationship in B6C3F1 and DBA/2 mice. **Immunopharmacology** 2:155-164, 1985.

White, K.L., Jr., Sanders, V.M., Barnes, D.W., Shopp, G.M., Jr., and Munson, A.E.: Immunotoxicological investigations in the mouse: general approach and methods. **Drug Chem. Toxicol.** 3:299-332, 1985.

b. Spleen cell proliferation response to mitogens (SOP/MT/006). The spleen cell mitogenicity assay is an *in vitro* assay which evaluates the ability of B-cells and T-cells to undergo blastogenesis and proliferation following stimulation with a cell type-specific mitogen. The mice were exposed *in vivo* to the test compound for 14 days. On day 5, animals were sacrificed and spleen cells prepared under sterile conditions by mincing the spleens between two frosted microscope slides. Spleen cells were counted and adjusted to a concentration of  $2 \times 10^6$ /ml. One hundred  $\mu$ l of spleen cells at this concentration ( $2 \times 10^5$  cells) were added to each well of a microtiter plate. The appropriate concentration of mitogen was added to each well and the cells incubated for 3 days. Four concentrations of the T-cell mitogens, concanavalin A and phytohemagglutinin, and two concentrations of the B-cell mitogen, lipopolysaccharide, were used. Multiple concentrations of mitogens were used to assure that an optimal concentration has been included. The optimal mitogen concentration varies from lot to lot of mice. Two mitogens, concanavalin A and phytohemagglutinin, were used to evaluate the response of T-cells. For concanavalin A, 0.5, 1.0, 5.0 and 10  $\mu$ g/well were used and for phytohemagglutinin, 0.1, 0.5, 1 and 5  $\mu$ g/well were used. For the T-cell mitogens, there is a pronounced bell-shaped curve, with the optimal concentration usually being 1.0 or 5.0  $\mu$ g for concanavalin A and 0.5 or 1.0  $\mu$ g for phytohemagglutinin. The proliferative response of B-cells was evaluated using the mitogen, lipopolysaccharide, from *S. typhosa* 0901, at 10 and 100  $\mu$ g/well. There is not a clear-cut, bell-shaped curve for lipopolysaccharide and, therefore, only two concentrations of this mitogen were used. For interpretation, only the optimal concentration of mitogen is used to compare the chemical exposure group to the appropriate control group. During the last 18 hours of incubation, 2  $\mu$ Ci  $^3$ H-thymidine were present in the culture. Cells were harvested and counted in an LKB liquid scintillation counter; the data were collected directly onto computer diskettes for analysis. The incorporation of  $^3$ H-thymidine into the proliferating cells was used as the endpoint of the assay and the data are expressed as cpm/culture. Because the TVN could cause blastogenesis and proliferation, a media control was used and a stimulation index was calculated; this stimulation index is the cpm for the mitogen-induced response divided by the cpm of the medium for each animal for each mitogen concentration.

As background, blastogenesis (cellular enlargement with increase in endoplasmic reticulum) and proliferation (increase in DNA synthesis and cell division) are integral parts of the immune response of both B- and T-cells. If a xenobiotic adversely affects these responses, an altered immune response can result. A significant change in the spleen cell

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proliferative response to mitogens, when appropriately compared to untreated controls, indicates that the test agent is capable of modulating blastogenesis and/or proliferation and thus has potential for modifying the immune response.

Listed below are publications in which the above assay has been utilized:

Munson, A.E., Sanders, V.M., Douglas, K.A., Sain, L.E., Kauffmann, B.M. and White, K.L., Jr.: In vivo assessment of immunotoxicity. Environ. Health Persp. 43:41-52, 1982.

Holsapple, M.P., Munson, A.E., McCay, J.A., and Bick, P.H.: Suppression of cell-mediated immunocompetence after subchronic exposure to diethylstilbestrol in female B6C3F1 mice. J. Pharmacol. Exp. Ther. 227:130-138, 1983.

Holsapple, M.P., McNERney, P.J., Tucker, A.N. and White, K.L., Jr.: Effect of N-Nitrosodimethylamine on humoral immunity. J. Pharmacol. Exp. Ther. 229:493-500, 1984.

White, K.L., Jr., Sanders, V.M., Barnes, D.W., Shopp, G.M., Jr., and Munson, A.E.: Immunotoxicological investigations in the mouse: general approach and methods. Drug Chem. Toxicol. 8:299-332, 1985.

c. Mixed leukocyte response to DBA/2 spleen cells (SOP/MLR/002). The mixed leukocyte response (MLR) is an in vitro assay which evaluates the ability of spleen T-cells to recognize allogeneic cells as "non-self" and proliferate in response to the presence of the foreign "stimulator" cells. Female B6C3F1 mice were exposed in vivo to the TVN for 14 days. On day 15, the mice were sacrificed and spleen cells prepared under sterile conditions by mincing the spleens between two frosted microscope slides. Spleen cells were counted and adjusted to  $1 \times 10^6$ /ml. Spleen cells were added to each well of a U-bottom microtiter plate at a concentration of  $1 \times 10^5$  cells per well. DBA/2 spleen cells were used as the allogeneic cell (stimulator cell) for the B6C3F1 (responder) mice. (Stimulator cells were treated with Mitomycin C to render them unable to proliferate; the ratio of stimulators to responders has previously been optimized at 4:1.) The cells were cultured for 5 days, during the last 18 hours in the presence of  $2 \mu\text{Ci } ^3\text{H-thymidine}$ . The cells were collected with a cell harvester and counted in an LKB liquid scintillation counter; data were collected directly onto computer diskettes for analysis. The incorporation of  $^3\text{H-thymidine}$  into the proliferating cells is used as the endpoint of the assay and the data are expressed as cpm/culture. The data are also expressed as a stimulation index, which is the cpm of the responder cells in the presence of the stimulator cells, divided by the cpm of the responder cells in the absence of the stimulator cells.

The MLR has proven to be a more sensitive assay than the spleen cell proliferative response to mitogens. This may be due to the more complex nature of the MLR response, in which recognition of the allogeneic cells and proliferation are necessary. At the present time, the MLR has proven to be a sensitive in vitro indicator of effects on cell-mediated immunity. A significant change in the MLR, when appropriately compared to untreated controls,

indicates that the test agent is capable of modulating recognition and/or proliferation in response to stimulation with allogeneic cells. Agents which produce such effects have the potential for modifying the immune system.

Listed below are publications in which the above assay has been utilized:

Holsapple, M.P., Munson, A.E., McCay, J.A., and Bick, P.H.: Suppression of cell-mediated immunocompetence after subchronic exposure to diethylstilbestrol in female B6C3F1 mice. *J. Pharmacol. Exp. Ther.* 227:130-138, 1983.

Holsapple, M.P., McNerney, P.J., Tucker, A.N. and White, K.L., Jr.: Effect of N-Nitrosodimethylamine on humoral immunity. *J. Pharmacol. Exp. Ther.* 229:493-500, 1984.

d. Delayed hypersensitivity response to KLH (SOP/DHR/008). The ability of mice to produce a delayed hypersensitivity response (DHR) to keyhole limpet hemocyanin (KLH; a known sensitizer) provides an overall assessment of cell-mediated immunity in the whole animal. Mice were exposed *in vivo* to TVN for 14 days. On the first day of dosing, the mice were induced by KLH (100 µg) via s.c. injection in the central portion of the back between the shoulders. On day 8 of exposure, a second inducing injection was made, as described previously. On day 14 of exposure, mononuclear cells were labeled *in vivo* by administering 2 µCi <sup>125</sup>I-5-Iododeoxyuridine (IUdR) per mouse (10 µCi/ml) by i.v. injection. The specific activity of the IUdR obtained was 2.2 Ci/mole. On day 15, the day following the final exposure, animals were challenged in the central portion of the left ear with an intradermal injection of KLH (30 µg). Twenty four hours after challenge, animals were sacrificed and a central portion of each ear was removed with a punch borer. Each ear biopsy was counted in an LKB gamma counter and the data entered into the computer for analysis. The data are presented as a stimulation index, which represents the counts in the challenged ear divided by the counts in the unchallenged ear, after correcting for non-specific monocyte influx.

As background, this holistic assay requires numerous immunological components to function in concert to produce the delayed hypersensitivity response. These include antigen recognition, cell proliferation, production of lymphokines, and the recruitment of final inflammatory cells capable of releasing substances that produce immunologic mediated inflammation. If the test article affects any or all of these immunological functions, a modified DHR can result. A significant increase or decrease in the DHR, when appropriately compared to untreated controls, indicates that the test article is capable of modulating cell mediated immunity in the whole animal and thus has potential for modifying the immune response.

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Listed below are publications in which the above assay has been utilized:

Holsapple, M.P., Munson, A.E., McCay, J.A., and Bick, P.H.: Suppression of cell-mediated immunocompetence after subchronic exposure to diethylstilbestrol in female B6C3F1 mice. *J. Pharmacol. Exp. Ther.* **227**:130-138, 1983.

Holsapple, M.P., Page, D.G., Bick, P.H. and Shopp, G.M.: Characterization of the delayed hypersensitivity response to a protein antigen in the mouse. I. Kinetics of reactivity and sensitivity to classical immunosuppressants. *Int. J. Immunopharmacol.* **6**(5): 399-405, 1984.

Holsapple, M.P., Bick, P.H. and Duke, S.S.: Effects of N-Nitrosodimethylamine on cell-mediated immunity. *J. Leuk. Biol.* **37**:367-381, 1985.

e. Multiple variables can be assessed in the same animals. Table 1 shows the six basic studies with the primary and concomitant assays listed.

#### 4. Data Handling and Statistical Analysis

The data obtained in these studies were either entered directly onto computer forms or entered from instruments, i.e., liquid scintillation counter, electronic balances, etc. The data were verified and outliers removed and documented prior to modification. Outliers were identified by two methods: (1) if there is an identifiable experimental reason for the data point to be deleted, such as questionable injection (for these studies most deletions were made in the DHR; this assay requires intradermal injections in the pinna of the ear where increased variability is known to occur); or (2) if the data point is 2 standard deviations from the mean when the mean is calculated without the questionable data point. Data modification was performed based on the type of data. The modified data obtained in this study were first tested for homogeneity of variances using the Bartlett's Chi Square Test. Homogeneous data were evaluated by a parametric one-way analysis of variance. When significant differences occurred, treatment groups were compared to the vehicle control using the multiple range Dunnett's t Test. Non-homogeneous data were evaluated using a non-parametric analysis of variance. When significant differences occurred, treatment groups were compared to the vehicle control using the Wilcoxon Rank Test. The Jonckheere's Test performs a trend analysis and provides insight into dose dependency.

#### 5. Data and Record Retention

All data and records, including tissues, pertaining to this study will be retained in the MCV/VCU Archives in the Strauss Building for 7 years.

#### 6. Personnel

Curriculum vitae for the personnel involved in this study have become part of the permanent records for this study.

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**7. Safety**

**The appropriate safety precautions for the use of chemicals, as described in separate guidelines established by the MCV/VCU Cancer Center, were followed.**

## RESULTS

### A. General

Mice were administered TVN, solubilized in acetone:olive oil (4:1), daily by the dermal route to either an abraded or non-abraded site. Exposure occurred without significant trauma or stress to the animal. The animals continued to preen, including the area of exposure, therefore, exposure by the oral route with absorption via the gastrointestinal tract must be considered likely. Mice tolerated TVN without major incident. There were no overt adverse effects. The fur was maintained in a healthy state, there were no discharges from orifices, and the feces appeared normal with respect to consistency and amount. There was no evidence of diuresis, as judged by the status of the bedding. The mice with abraded skin showed minor local blushing of the skin at the time of dermabrasion. One mouse was lost to the study because it escaped and was outside its cage for 24 hours.

Cyclophosphamide was administered as a positive control for the immunological studies and to demonstrate changes in the spleen and thymus, and in the leukocyte counts. The cyclophosphamide group is presented in all tables and for all parameters. For many of the parameters, cyclophosphamide is not a positive control. It should be emphasized here that cyclophosphamide was administered on the last 5 days of the 14-day TVN exposure period and thus body weights for the first week in these groups are without treatment.

The studies were designed to use the experimental animals for multiple parameters. The outline shown in the Methods of Procedure (Table 1) indicates the study number, primary assay and concomitant assays. The results are divided into Standard Toxicology and Immunology.

Table A-1 (Appendix) lists the individual mice and the assays performed.

### B. Standard Toxicology

#### 1. Body Weights

Female B6C3F1 mice were exposed to TVN on non-abraded skin daily for 14 days. One day after the last exposure, the mice were anesthetized with Metaphane, blood was collected by cardiac puncture, the mice were necropsied and selected organs were removed, cleaned of connective tissue and weighed. No gross pathology was seen in any of the animals. For each study, body weights and body weight changes were recorded or calculated. A table of body weights and body weight changes is provided for each of the six studies. Table 2 shows the body weights and body weight changes derived from the study in which lymphocyte proliferation to mitogens, the MLR and serum chemistries were performed (study 3). The skin was not abraded. There were no TVN-related changes in body weights over the exposure period. Mice receiving the vehicle gained 2.2 grams over the two week exposure period, as compared to 2.6, 1.6, and 1.9 grams for mice exposed to 0.7, 7.0 and 70 mg/kg TVN, respectively.

Female B6C3F1 mice were also exposed to TVN on abraded skin daily for 14 days. One day after the last exposure, the mice were anesthetized with Metaphane, blood was collected by cardiac puncture, the mice necropsied and selected organs were removed, cleaned of connective tissue and weighed. No gross pathology was seen in any of the animals. Table 3 shows the body weight and body weight changes of mice exposed to TVN on abraded skin in study 4. There were no TVN-associated changes in body weights over the exposure period. The dose-dependent increase in body weight seen here was not significant at any particular level and was not reflected in the body weight changes.

### 2. Organ Weights

Body and selected organ weights for the non-abraded study are shown in Table 4. There were no TVN-associated changes in wet tissue weight of the brain, liver, lung or kidneys. There was an increase in spleen weight and a dose-dependent decrease in thymus weight. The 36% increase in spleen weight of mice receiving 70 mg/kg TVN was statistically significant ( $p < 0.01$ ). This significant increase was reflected in absolute spleen weight, as a percent of spleen to total body weight, and as a ratio of spleen to brain weight. Thymus weight was decreased dose-dependently with respect to TVN; the highest dose of TVN produced a 23.5% decrease ( $p < 0.01$ ). Cyclophosphamide, the positive control for the immunological studies, produced the expected decrease in spleen weight (39.4%) and thymus weight (67.2%). The data on the spleen and thymus weights of non-abraded-skin animals are shown in Figure 1.

Body and selected organ weights for the abraded group are shown in Table 5. There were no TVN-associated changes in wet tissue weight of the brain, lung or kidneys. There was a dose-dependent increase in liver weight. Liver weights of mice receiving 70 mg/kg TVN were increased by 16% over vehicle control. The increase in liver weight was not seen in the non-abraded mice. As seen in the non-abraded-skin animals, there was a dose-dependent increase in spleen weight and a dose-dependent decrease in thymus weight. The 52% increase in spleen weight of mice receiving TVN was statistically significant ( $p < 0.01$ ). Again, the significant increase was reflected in absolute spleen weight, as a percent of spleen to total body weight, and as a ratio of spleen to brain weight. Thymus weight was decreased dose-dependently with respect to TVN; the highest dose of TVN produced a 31% decrease ( $p < 0.01$ ). Cyclophosphamide, the positive control for the immunological studies, produced the expected decrease in spleen weight (42.3%) and thymus weight (67.3%). The data on the spleen and thymus weights of abraded-skin animals are shown in Figure 2.

### 3. Hematology

Selected hematological values for mice receiving TVN on non-abraded skin are shown in Tables 6 and 7. There were no TVN-associated changes in the erythroid elements, i.e., erythrocyte number, hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin concentration (Table 6). There was a dose-dependent increase in the leukocyte number (Table 6), which was a result of an increase in both polymorphonuclear leukocytes (polys) and lymphocytes (Table 7). The most pronounced

effect was in the polys, where a 4-fold increase was seen. It should be noted that the percentage of lymphocytes decreased, but the total increased, whereas the percentage of polys increased along with an increase in the total number. Cyclophosphamide produced the expected decrease in leukocyte number. The data on leukocytes are presented graphically in Figure 3. Because the erythroid elements in this study were out of the historical control range, the study was repeated; these repeat data are shown in Table 6R. The results show the same trends, i.e., an increase in leukocytes, but no significant or biologically-relevant change in erythroid elements.

Selected hematological values for mice receiving TVN on abraded skin are shown in Tables 8 and 9. There was a dose-dependent decrease in erythroid elements as a result of the high vehicle control value (Table 8). Erythrocyte number decreased from a control value of  $9.60 \times 10^6/\text{mm}^3$  to  $8.49 \times 10^6/\text{mm}^3$  in mice receiving 7.0 mg/kg TVN (an 11.6% decrease). The historical control range for erythrocyte number in this laboratory is 7.0 to  $8.5 \times 10^6/\text{mm}^3$ . The decrease in erythrocytes was manifested also in the hemoglobin levels, which decreased from 15.2 g% to 13.4 g% (an 11.8% decrease). The hematocrit decreased dose-dependently, but did not reach statistical significance. The erythrocyte size (MCV) and total hemoglobin per erythrocyte were not altered. The mean corpuscular hemoglobin concentration showed a statistically-significant decrease in mice receiving 70 mg/kg TVN. The statistically-significant changes in erythroid elements are not considered biologically significant because the values for the control group erythroid elements are at the high end of the historical control range. Although the increase in total leukocyte number was not statistically significant, as in the study on non-abraded skin (Table 6), there was a 3.3-fold increase in polys (Table 9). Also, as previously seen in the non-abraded-skin animals, the percentage of lymphocytes decreased, but with abraded-skin animals the total number of lymphocytes did not change. The data on leukocytes are presented graphically in Figure 4.

#### 4. Serum Chemistries

Selected serum chemistries of mice exposed to TVN on non-abraded skin are shown in Table 10. The chemistries selected were performed to determine effects on the liver or kidney. SGPT and BUN levels of mice exposed to TVN were not different from the vehicle control group. There was a dose-dependent decrease in albumin and total protein which reached statistical significance ( $p < 0.05$ ) for mice exposed to 70 mg/kg TVN. The decrease in the 70 mg/kg TVN group was 16.1% for albumin and 14.8% for total protein. Although there was a TVN dose-dependent decrease in blood glucose, significance was not reached for a specific experimental group.

Selected serum chemistries of mice exposed to TVN on abraded skin are shown in Table 11. In contrast to the study on non-abraded skin, there were no TVN-associated changes in the selected serum chemistries. The levels seen in the control groups for both the abraded and non-abraded studies are within the historical control range for the MCV/VCU Immunotoxicology Program.

## C. Immunology

### 1. IgM Spleen Antibody Forming Cell Response to T-Dependent Antigen

Female B6C3F1 mice were exposed to TVN on non-abraded skin for 14 days. On day 11 of exposure, the mice were immunized with sheep erythrocytes by the intravenous route. On day 15, one day after the last exposure and 4 days after immunization, the mice were sacrificed and the number of spleen antibody forming cells enumerated. Table 12 shows the body weights and body weight changes of mice in this study and Table 13 shows the immunological data. Although there was a dose-dependent trend for a decrease in body weight gain over the two week exposure period, no statistically-significant changes in body weights or body weight gains occurred for a specific TVN-exposed group.

Table 13 shows the data on the ability of mice to respond to the T-dependent antigen, sheep erythrocytes. Spleen weights in the mice exposed to TVN were not increased, as was seen in a standard toxicology study (see Table 4) due to the immunization with sheep erythrocytes. Spleen cells proliferate as a result of immunization which may have masked the effect of TVN on spleen weight. The response of mice to the T-dependent antigen was increased dose-dependently. At 70 mg/kg TVN, mice showed a 58% increase over the vehicle control group when the data are expressed as specific activity, i.e., AFC/10<sup>6</sup> spleen cells, or 56%, when expressed on a per spleen basis. Cyclophosphamide almost completely ablated this response and reduced the spleen size. The data are graphically presented in Figure 5.

Tables 14 and 15 provide the data on the body weights and IgM AFC response to sheep erythrocytes of mice exposed to TVN on abraded skin. No significant changes in body weight or body weight gain were seen for a given TVN exposure group, but a trend towards a decrease was noted for the day 15 body weights ( $p < 0.05$ ; Table 14). Although spleen weights in the immunized mice receiving TVN were not statistically increased for a given TVN exposure group (see above), there was a trend towards increased spleen weight ( $p < 0.05$ ; Table 15). The IgM AFC response to sheep erythrocytes of mice exposed on abraded skin to TVN was not changed from the vehicle control group. The decrease in the 0.7 mg/kg TVN group (1596 AFC/10<sup>6</sup> spleen cells, as compared to 2102 AFC/10<sup>6</sup> spleen cells for the vehicle) is indicative of a high vehicle control group. Although 2101 AFC/10<sup>6</sup> spleen cells is within the historical control range of the MCV/VCU Immunotoxicology Program, it may be slightly out of line in this study. If this were the case, there would be a trend towards an increased AFC response, as was seen in the study with non-abraded skin (Table 13). The data from this study are graphically represented in Figure 6.

### 2. Spleen Cell Response to Mitogens

Female B6C3F1 mice were exposed to TVN on non-abraded skin daily for 14 days. One day after the last exposure, the mice were sacrificed, spleen cells were prepared, and responsiveness to T- and B-cell mitogens was measured. Body weights and body weight changes were presented in Table 4. There were no TVN-associated changes in body

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weight or body weight gain. The data on spleen cell response to mitogens are presented in Tables 16 and 17. Table 16 shows the data as uptake of  $^3\text{H}$ -thymidine in counts per minute (cpm) and Table 17 shows the data as a stimulation index, which is the cpm of the culture with mitogen divided by the cpm of the culture without mitogen. The stimulation index normalizes the data for any change in baseline uptake of  $^3\text{H}$ -thymidine in spleen cells due to xenobiotic exposure. In this study, the baseline uptake of  $^3\text{H}$ -thymidine was not altered by exposure to TVN (see Medium, Table 16). Four concentrations of the T-cell mitogens, phytohemagglutinin (PHA) and concanavalin A (CON A), and two concentrations of the B-cell mitogen, lipopolysaccharide (LPS), were used to assure the selection of an optimal concentration, based on the responses in the vehicle control groups (Table 16). The optimal concentration of PHA was  $0.5 \mu\text{g/culture}$ , while the optimum for LPS was  $10 \mu\text{g/culture}$ . In this study both 1 and  $5 \mu\text{g Con A/culture}$  produced the same optimal level of response. Exposure of mice to TVN produced a dose-dependent decrease in the response to CON A at the  $1 \mu\text{g}$  level, but not at the  $5 \mu\text{g}$  level. There was no alteration of the PHA response at its optimal concentration. The response to the B-cell mitogen, LPS, was decreased dose-dependently in TVN-exposed mice (Table 16); this response was significant ( $p < 0.05$ ) at  $7.0 \text{ mg/kg TVN}$ . The decreases were 3.5%, 44%, and 77% for 0.7, 7.0 and  $70 \text{ mg/kg}$ , respectively; these compare to 82.5% for the positive control, cyclophosphamide. The data shown in Table 17 are the mitogen response data expressed as the stimulation index. A similar decrease in response to CON A at the  $1 \mu\text{g}$  level was seen, with no effects on the PHA response. The dose-dependent decrease in the LPS response was seen.

The mitogen response data on the TVN exposure on abraded skin are shown in Tables 18 and 19. The baseline uptake of  $^3\text{H}$ -thymidine was unaltered by exposure to TVN. The optimal CON A and PHA concentrations were  $5.0 \mu\text{g/culture}$  and  $1.0 \mu\text{g/culture}$ , respectively. At the optimal concentration of the T-cell mitogens, mice exposed to TVN showed no differences from vehicle control mice. The lack of an effect was also seen when the data are expressed as a stimulation index (Table 19). Mice injected with the positive control, cyclophosphamide, showed a 67.3% and 65.1% reduction in response to PHA and CON A, respectively. The vehicle control response to LPS was not within the historical control range of the MCV/VCU Immunotoxicology Program. The test article response, although low, showed that mice exposed to TVN had a decreased response to LPS using the  $10 \mu\text{g/culture}$  stimulus. A repeat study using CON A and LPS was performed and the data are shown in Tables 18R and 19R. The repeat data were in the historical control range and a dose-dependent suppression was seen for both LPS and CON A. For CON A, the optimal level was again  $5 \mu\text{g}$ . In contrast to the study shown in Tables 18 and 19, TVN exposed mice showed a decreased responsiveness to the T-cell mitogen CON A. There was a 42% reduction in the spleen cell response to the T-cell mitogen CON A in the  $70 \text{ mg/kg}$  exposed group. A significant ( $p < 0.01$ ) reduction (41%) in LPS response was measured in the  $7 \text{ mg/kg}$  exposed group. Table 19R shows the mitogen responsiveness data in the form of a stimulation index. TVN-exposed mice showed a dose-dependent decrease in responsiveness to both CON A and LPS.

In studies reported in Tables 16 and 18, i.e., mitogen response in non-abraded- and abraded-skin animals, the spleen cell number was increased dose-dependently in the TVN-exposed mice; statistical significance ( $p < 0.05$ ) was reached at  $70 \text{ mg/kg TVN}$ . These

were the mice used in the organ weight studies (Tables 4 and 5) which showed increased spleen weight.

The immunological data from this study are shown graphically in Figures 7 and 8.

### 3. Mixed Leukocyte Response (MLR)

Female B6C3F1 mice were exposed to TVN on non-abraded skin daily for 14 days. One day after the last exposure, the mice were sacrificed, spleen cells were prepared and responsiveness to an allogeneic cell (DBA/2 spleen cell) was measured. Body weights and body weight changes were presented in Table 2. There were no TVN-associated changes in body weight or body weight gain. The data on the mixed leukocyte response (MLR), presented in Table 20, are expressed as uptake of  $^3\text{H}$ -thymidine in counts per minute (cpm) and as a stimulation index, which is the cpm of the culture with stimulator cells divided by the cpm of the culture without stimulator cells. The stimulation index normalizes the data for any change in baseline uptake of  $^3\text{H}$ -thymidine in spleen cells due to xenobiotic exposure. In this study, the baseline uptake of  $^3\text{H}$ -thymidine was not altered by exposure to TVN (see "Responders Alone", Table 20). The response in cpm for vehicle-exposed mice was 28611. A TVN dose-dependent decrease in the MLR occurred, with statistical significance ( $p < 0.01$ ) reached in mice exposed to 70 mg/kg TVN (69% decrease, as compared to vehicle control). The stimulation index confirmed that TVN exposure reduced the MLR response, but that there was no effect on basal uptake of  $^3\text{H}$ -thymidine in responder cells alone.

No response was measured in the MLR for mice in the abraded study (Table 21). At present we have no explanation for the lack of a response. In the past when the response did not occur, the problem has been with the stimulator cells (spleen cells from DBA/2 mice). A repeat study was conducted using stimulator cells from a new lot of DBA/2 mice; the response was robust and within the historical control range. The data presented in Table 21R show an 87% reduction in the MLR response in the mice exposed to 70 mg/kg TVN. Mice exposed to lower doses showed a normal MLR response.

The data from these studies are graphically presented in Figure 9.

### 4. Delayed Hypersensitivity Response (DHR) to Keyhole Limpet Hemocyanin (KLH)

Female B6C3F1 mice were exposed to TVN on non-abraded skin for 14 days. On days 1 and 8 of exposure, the mice were immunized with KLH subcutaneously in the nape of the neck. On day 14 of exposure, the mice were injected intravenously with  $^{125}\text{I}$ UdR, which labels the bone marrow cells. On day 15, one day after the last exposure to TVN, the mice were challenged in the pinna of the left ear with KLH. On day 16, the left and right ears were biopsied and radioassayed. An influx of labeled bone marrow cells is indicative of a cell-mediated immune response to KLH. Mice in this study showed no changes in body weight or body weight gain over the experimental period (Table 22). The DHR of vehicle control mice was 4.66, which is within the historical control range for the MCV/VCU Immunotoxicology Program. Two mice were excluded from the vehicle controls because

they were considered outliers. One animal from each of the TVN-exposed groups was removed because they, too, were considered outliers. The DHR to KLH was not significantly altered in mice exposed to TVN (Table 23). The high TVN-exposed group showed the greatest variability. Cyclophosphamide completely inhibited the DHR to KLH.

The results of the DHR to KLH studies of mice exposed to TVN on abraded skin are shown in Tables 24 and 25. Body weights and body weight gains for mice in this study were not changed by exposure to TVN (Table 24). The response of mice to KLH was increased dose-dependently, with statistical significance ( $p < 0.05$ ) reached in the group receiving 70 mg/kg TVN; enhancement was 2.82-fold (see Table 25). The vehicle control stimulation index in this group (abraded) was 2.32, as compared to 4.66 for the vehicle group in the non-abraded study. The vehicle responses are within the limits of the historical range of the MCV/VCU Immunotoxicology Program. However, the 2.32 is on the lower end of the acceptable range.

The data for the DHR studies are graphically presented in Figure 10.

## DISCUSSION

The purpose of this study was to determine the potential effects of TVN on the immune system when applied to the non-abraded and abraded skin. If effects were seen, the lowest effect level was to be determined. Based on previous reports, TVN, administered by gavage at doses between 60 and 1000 mg/kg, produced changes in spleen weight, thymus weight and leukocyte number; a no-effect level was not established. The current studies were designed to administer TVN by the intended route of exposure, to determine the effects on the same parameters, and to measure selected immune functions which may provide more sensitive indicators of adverse effects on the lymphoreticular system. The studies were designed so that multiple parameters could be obtained from the same group of exposed animals.

Because of the preening which was observed to occur at the site of application, the possibility that the effects observed were at least in part due to oral absorption must be seriously considered. Exposure of the chemical was accomplished by daily application to the abdominal area where there is easy access to oral licking. The chemical was also administered in an acetone:olive oil vehicle. The acetone evaporates quickly and the animals may have been attracted to the oil which contained the xenobiotic.

Doses were initially selected based on the previous studies, but due to the route of administration and solubility of the compound, had to be reduced to 0.7, 7, and 70 mg/kg. Mice were exposed on the shaved abdomen in a manner similar to that used for evaluation of contact hypersensitivity. The vehicle was acetone:olive oil in a ratio of 4:1, which was selected because of the solubility of TVN and to provide rapid evaporation from the skin.

Table 26 summarizes the results and gives the lowest effect level (LOWEL). The target organs or cellular elements identified as being affected by TVN were the thymus, spleen, and polymorphonuclear leukocytes (polys). These results indicate that the action may be at the level of the bone marrow. The bone marrow is the source of the immunocompetent cells which take up residence in the thymus and spleen and is also the source of the polys in the blood. A decrease in thymus weight was noted at doses of TVN producing no changes in body weight or body weight gain. Histopathology of the thymus was not performed and, therefore, the affected cell type is not known.

Several immune function tests were performed which require T-lymphocytes, derived from the thymus, to participate. The ability of lymphocytes derived from mice exposed to TVN to respond to the T-cell mitogen CON A and allogeneic cells (MLR) were decreased. The effect on responsiveness to CON A is not as convincing as that for the MLR. The holistic, cell-mediated response of the animal to KLH was increased in abraded mice. This response also requires T-cell participation. It appears that TVN is acting in a manner similar to an adjuvant, such as Freund's Complete Adjuvant. An adjuvant is a substance which increases an endline immune response. Adjuvants are used to increase the amount of an antibody (a B-cell function) or products of cell-mediated (T-cell) immunity, such as cytotoxic T-lymphocytes, in response to a specific antigen. In the studies conducted here, the endline response such as the DHR to KLH, was enhanced. With the process of

differentiation which must occur in the endline response there is a feedback inhibition of proliferation. The reduction in proliferation is seen in the MLR response, which is T-cell mediated, and in the response to the T-cell mitogen, CON A. This same rationale can be seen for the B-cell side of the system. There was an increase in the end-line response, i.e., IgM AFC response to a specific antigen, sheep erythrocytes. A possible reason for the fact that increases in end-line assays, such as the IgM AFC response to sheep erythrocytes and DHR response to KLH, did not occur in both the abraded and non-abraded mice is the fact that the adjuvant effect is not profound.

The meaning of an adjuvant action of a chemical in terms of risk assessment can only be speculated. There is a balance in the immune system as it responds to non-self. Allergy is often an imbalance in recognition of non-self antigens and the inability to suppress immunological responses to the antigens. Certain individuals are more prone to developing immune responses to antigens and are considered atopic. An adjuvant could increase the chances of an individual inappropriately responding to environmental antigens and developing allergies to the antigens. Another potential problem in the enhancement of the immune response is in the area of autoimmunity. The presence of an adjuvant over an extended period of time may increase the chances of autoimmunity. A number of infectious processes; particularly viral infections, cause the formation of neo-antigens, which can elicit immunological responses that, in the presence of an augmented immune state, could increase the potential for an immune response to be directed towards self.

Spleen size increased in TVN-exposed mice as compared to vehicle control mice. The increase in spleen size was masked in mice immunized with sheep erythrocytes. Spleen size increases ranged from 15-30% during the period of immunization. A xenobiotic causing a small increase in spleen size may not be detected in mice undergoing spleen cell proliferation in response to an antigen. The increase in spleen size and the increase in the IgM AFC response to sheep erythrocytes are compatible with an adjuvant effect.

The increase in polys is also compatible with an adjuvant effect. Polys are expected to be called out from the mobile pool of the bone marrow when an adjuvant is administered. This effect occurred at the 70 mg/kg level. Although there was a decrease in the percentage of lymphocytes in the blood, this relative decrease was more a function of the increase in polys. Total lymphocyte counts were either not changed, as in the abraded-skin TVN exposure, or increased, as in the non-abraded-skin TVN exposure. This effect was also seen in the previous report.

Overall, the lowest effect level was not seen in the endline immune assays, such as the IgM AFC response and DHR to KLH, but was seen in the lymphocyte response to the B-cell mitogen LPS. The lowest effect level is 7 mg/kg TVN administered via the dermal route.

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## CONCLUSION

TVN administered to the abraded or non-abraded skin of female B6C3F1 mice produced changes in the lymphoreticular system, as manifested in a decrease in thymus weight, an increase in spleen weight, an increase in polymorphonuclear leukocytes, an increase in humoral- and cell-mediated immune responses (AFC and DHR), and a decrease in lymphocyte proliferative responses (MLR and response to mitogenic stimulation); these changes are consistent with an adjuvant effect. The lowest effect level was 7 mg/kg, as seen in the decrease in lymphocyte proliferation to the B-cell mitogen LPS. Most of the effects were observed at 70 mg/kg.

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Table 1  
Overall Experimental Design

Study Number	Primary Assay	Abraded Skin	Concomittant Assay
1.	IgM AFC	No	Hematology Leukocyte Differentials Body Weights
2.	IgM AFC Abraded	Yes	Hematology Leukocyte Differentials Body Weights
3.	Lymphocyte Proliferation (Mitogens)	No	Mixed Leukocyte Response Serum Chemistries Organ Weights Body Weights
4.	Lymphocyte Proliferation (Mitogens)	Yes	Mixed Leukocyte Response Serum Chemistries Organ Weights Body Weights
5.	Delayed Hypersensitivity Response	No	Body Weights
6.	Delayed Hypersensitivity Response	Yes	Body Weights
7.	Hematology - Repeat	No	Hematology
8.	Lymphocyte Proliferation - Repeat (Mitogens)	Yes	Mixed Leukocyte Response