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In Vivo Assessment of Immunotoxicity

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The organs, tissues, and cells of the lymphoreticular system have received considerable attention as targets for chemicals causing adverse effects. A basic toxicological approach is described for assessing the risk of a chemical perturbing the immune system. CD-1 mice were exposed for 14 or 90 days to one of several chlorinated hydrocarbons: 1,2-dichloroethane, 1,2-dichloroethylene or 1,1,2-trichloroethylene. Other mice were exposed to dexamethasone, a known immunosuppressive agent. The immune system is evaluated against a background of the more standard toxicological parameters such as fluid consumption, body and organ weights, hematology, clinical chemistries, and blood coagulation. Reported here are the results for the male mice after 14-day exposure to three chlorinated hydrocarbons and after 90-day exposure to 1,2-dichloroethane and dexamethasone.

Acute toxicity studies were performed to provide a basis for doses used in the subchronic studies. The LD_{50} values are reported.

The status of the humoral immune system was determined by measuring the number of IgM spleen antibody-forming cells to sRBC, the serum antibody level to sRBC, and the lymphocyte response to the B-cell mitogen, LPS. Of the three chlorinated hydrocarbons, only dichloroethane produced a significant ($p < 0.05$) reduction in antibody-forming cells. The other two chemicals produced trends towards suppression. Mice exposed to dichloroethane in the drinking water for 90 days showed no alteration in AFC, serum antibody titers or response to the B-lymphocyte mitogen, LPS. Subchronic 90-day exposure to dexamethasone produced a dose-dependent inhibition of AFC/spleen but not AFC/10^6 spleen cells when measured on the peak day of response. Response to LPS was not altered, and spleen weight and spleen cell number were reduced as much as 42%. These data suggest that dexamethasone administered in the drinking water is nonspecifically cytotoxic to the spleen cells.

Cell-mediated immunity was assessed by measuring the DTH response to sRBC and the response to the T-lymphocyte mitogen, concanavalin A. After 14 days of exposure, trichloroethylene produced a 15 and 60% suppression at 24 and 240 mg/kg, respectively. Dichloroethylene produced a non-dose-dependent inhibition at 4.9 and 49 mg/kg, which was slight, but significant ($p < 0.05$). Subchronic 90-day exposure to dichloroethane did not alter the DTH response or spleen lymphocyte response to concanavalin A. In contrast, dexamethasone produced a dose-dependent inhibition of the DTH response and a hyperresponsiveness to concanavalin A.

Dichloroethane did not alter the functional activity of the reticuloendothelial system, as measured by the vascular clearance rate and tissue uptake of ^{51}Cr sRBC. In the case of dexamethasone exposure, only the spleen and thymus showed decreased uptake of ^{51}Cr sRBC, which was directly related to decrease in size.

The approaches and results from these types of studies provide a basis for judging a chemical's potential risk to the immune system.

Introduction

The organs, tissues and cells of the lymphoreticular system have received considerable attention as targets for chemicals which cause adverse effects. The immune system, like all other systems in the body, is complex, with several types of cells working independently and in concert to carry out a role in homeostasis. Immunotoxicology is the subject of increased activity and awareness because the physiology and biochemistry of the immune

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system and the basic processes involved in host defense mechanisms are now better understood. Another reason for this interest is that the immune system's cells, having been exposed to a chemical, can be readily removed and their function(s) examined *in vitro*. This is in keeping with the direction that toxicology is taking—complementing morphological changes with functional alterations.

We are currently using a basic toxicological approach to assess a chemical's risk to the immune system. This approach includes using male and female random-bred mice in subchronic 14-day exposures as range-finding studies, followed by a more definitive subchronic 90-day study. The route of administration for these studies mimics human exposure. Except for carcinogenic, mutagenic and reproductive effects, the subchronic 90-day exposure is believed to produce an adequate manifestation of a chemical's toxicity. This may not prove true for the immune system because of long-lived T-lymphocytes. The immune response is evaluated by using assays that measure complex cell interactions, interactions of intermediate complexity and certain direct cellular assays. The analysis of the immune system is determined against a background of more conventional toxicological procedures such as body and organ weights, hematology, clinical chemistries, urinalysis and microsomal enzymes.

The data presented in this paper are selected from results obtained from acute, subchronic 14-day studies on dichloroethane, dichloroethylene and trichloroethylene and subchronic 90-day studies on dichloroethane and dexamethasone. Presented are results only from male mice. The results of the female mice were not remarkably different from the males. Dichloroethane was selected because of its environmental importance and because it was devoid of effects on the immune system. Dexamethasone was used because of its known immunosuppressive effects and solubility in water. The chemicals were administered orally: by gavage for the 14-day exposure and in the drinking water for the 90-day exposure. Historical controls are shown to provide indicators of variance for these immunologic parameters in random-bred mice.

Materials and Methods

Animals and Housing

All CD-1 mice were obtained from Charles River Breeding Laboratories, Wilmington, Mass. and housed four per shoebox plastic cage with sawdust bedding and maintained on Agway Lab Chow ad libitum. The animal rooms were maintained at 70-75°F and relative humidity at 40-60%. The light-dark cycle was maintained on 12-hr intervals. Upon arrival, all mice were quarantined for 1 week prior to use.

For acute toxicity studies, 6-week-old male and female mice were used, and for the subchronic 14- and 90-day studies, 4-week-old mice were obtained, with exposures beginning at 5 weeks of age.

Chemicals

1,2-Dichloroethane, Lot #120487, was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin; trans-1,2-dichloroethylene, Lot #083187, was also obtained from Aldrich Chemical Co. 1,1,2-Trichloroethylene, Lot #713839, was obtained from Baker Chemical Co., Phillipsburg, N.J. Dexamethasone phosphate, Lot #86837, was a gift of Merck, Sharpe, and Dohme, Inc., West Point, Pa. Flumethasone pivalate, Lot #R-4088, was supplied through the courtesy of Ciba-Geigy, Summit, N.J.

Chemical Administration

Solutions of dichloroethane, dichloroethylene, trichloroethylene, and dexamethasone were prepared fresh daily for acute and subchronic 14-day studies and the appropriate concentrations were administered by gavage in a volume of 0.01 ml/g body weight to achieve the desired dose. For the subchronic 90-day studies, dichloroethane was administered in the drinking water, diluted in deionized water to concentrations of 0.02, 0.2 and 2.0 mg/ml. For dexamethasone, the concentrations for the 90-day exposure were 0.1, 1.0 and 2.0 μg/ml. Drinking water solutions were maintained at room temperature in amber-colored bottles with stainless steel spouts fitted through the corks. The solutions were changed twice weekly, and less than 10% of dichloroethane and dexamethasone was lost during the three to four days between water bottle changes, as measured by gas chromatography for dichloroethane and high pressure liquid chromatography for dexamethasone.

For the subchronic 90-day study, fluid consumption was estimated by the change in fluid weight over the three- or four-day period when solutions were changed. Twelve cages of control mice and eight cages of each treatment group for each sex were used to estimate fluid consumption. Chemical consumption was calculated from fluid consumption and is reported as ml/mouse/day and mg/kg/day.

Acute Toxicity

The chemicals were administered to male and female mice by an 18-gauge stainless steel stomach tube 18 hr after fasting from food. Following gavage, the mice were observed for overt toxicological effects continuously for 4 hr and then twice daily for 14 days. Mice dying during the experimental procedure were necropsied, and gross pathological changes were described. Mice surviving the 14-day observation period were sacrificed and gross pathology described. Log probit analysis (*1*) was used to determine the LD_{50} and 95% confidence limits.

Animal Necropsy

Animals were anesthetized with chloroform, and blood was collected immediately by cardiac puncture. Gross pathological examinations were performed on all mice. The organs (brain, liver, spleen, lungs, thymus, kidneys and testes) were then removed, trimmed and weighed. Certain organs were used in further studies.

The plasma from the blood samples was assayed for extrinsic activity by measuring the prothrombin time. Reagents for this assay were obtained from General Diagnostics. Fibrinogen levels were determined by the kinetic method, using reagents from Dade Diagnostics, Inc.

Hematology

Blood samples were taken in 3.2% sodium citrate. Leukocyte, erythrocyte and platelet counts were performed on a Coulter Counter, Model ZBI. Hematocrits were performed with microhematocrit equipment, and hemoglobins determined as cyanomethemoglobin.

Humoral Immunity: Spleen Cell Antibody Response

The primary IgM response to sheep erythrocytes was estimated by the hemolytic plaque assay of Jerne (*2*) as modified by Cunningham and Szenberg (*3*). Mice were immunized four or five days prior to sacrifice with 4×10^8 sheep erythrocytes by IP injection. Spleen cell suspensions were prepared in RPMI 1640 culture medium by using stainless steel mesh screens and adjusted to a cell concentration of 10^6/ml for assay of antibody-forming cells (AFC).

As a compliment to the plaque assay, the plasma antibody titer was measured by the hemagglutination technique. Seven days after IP injection of 10^9 sheep erythrocytes, blood was collected by cardiac puncture from chloroform-anesthetized animals into 3.2% sodium citrate. After centrifugation, the plasma was heat inactivated, and serial (1:1) dilutions were made in phosphate buffered saline. To each of the dilutions in a microtiter dish, an equal volume of 0.5% suspension of sheep erythrocytes was added. After incubation for 2 hr at 37°C, the plates were observed on a magnifying mirror for agglutination of the erythrocytes. The antibody titers were expressed as $\log_2$ of the reciprocal of the first dilution where there was no visible agglutination.

Cell-Mediated Immunity

Cell-mediated immunity was evaluated by measuring the delayed hypersensitivity (DTH) response to sheep erythrocytes. This is a modification of the methods of Legrange et al. and Paranjpe et al. (*4*, *5*). Sensitization was accomplished by injecting 10^8 sRBC in a volume of 20 µl into the left footpad (LFP). Four days following sensitization, the mice were challenged in the LFP with 4×10^8 sRBC in a volume of 40 µl. At 17 hr following challenge, the mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (HSA) (~80,000 cpm/0.1 ml). Two hours later, the mice were sacrificed by cervical dislocation and both hind feet were removed at the ankle joint and radioassayed in a gamma counter. The RFP served as an unchallenged control for background ^{125}I-HSA. A group of mice which were not sensitized but were challenged as above acted as unsensitized controls to determine nonspecific swelling. Results are expressed as a stimulation index (SI), which is calculated as follows:

$$SI = \left[\frac{\text{LFP sensitized}}{\text{RFP sensitized}}\right] - \left[\bar{X}\left(\frac{\text{LFP unsensitized}}{\text{RFP unsensitized}}\right)\right]$$

Spleen Cell Response to Mitogens

Lymphocyte responsiveness was assessed using the B-cell mitogen LPS, which is lipopolysaccharide from *Salmonella typhosa* 0901 (Difco), and the T-cell mitogen concanavalin A. For mitogenicity assays, spleens were removed aseptically and pushed through a stainless steel 80-mesh screen to provide a single cell suspension. Cells were diluted to 5×10^6/ml in RPMI 1640 medium supplemented with 2 *mM* L-glutamine and 10% heat-inactivated

fetal calf serum. A 100 μl portion of the cell suspension was added to each of three replicate wells into previously prepared microtiter plates containing 50 μl of the mitogen, which had been stored at -70°C. LPS was used in amounts of 1, 5 and 20 μg per well; concanavalin A was used in amounts of 1, 5 and 10 μg per well. Plates were incubated at 37°C in 10% CO_2 and 95% humidity for 48 hr. The cells were then pulsed with an I-iododeoxyuridine/fluorodeoxyuridine solution (0.2 μCi IUdR in 1μ*M* FUdR). After an 18-hr incubation, the cells were collected on filter discs using a Titertek cell harvester and counted in a Beckman gamma counter.

Functional Ability of the Reticuloendothelial System

We have previously compared several particles for measuring the phagocytic activity of the fixed macrophage system (*6*). For these studies we used sRBC.

Freshly drawn sRBC (5×10^9 cells/ml) were radiolabeled with ^{51}Na chromate (NEN) in a 37°C shaker bath with 1 mCi/5 ml of cells for 30 min. After chromation, the sRBC were washed with Alsever's solution until the supernatant was virtually radioactive free. Unlabeled sRBC (5×10^9 ml) were added to the labeled cells until the hematocrit was approximately 10% The resulting CPM's were approximately 200,000/0.1 ml. The sRBC were refrigerated and used the following day. Before use, the cells were washed to remove any free chromium released overnight.

Mice used for evaluation of RES activity were weighed and placed in shoebox cages maintained at 29°C. At zero time, 0.1 ml of labeled particle/10 g body weight was injected intravenously. Blood samples (10 μl) were taken from the tip of the tail at 2, 4, 6, 8, 10 and 15 min. The blood samples were put into 1 ml of distilled water and radioassayed. At the end of 60 min, the mice were sacrificed by decapitation and drained of blood. The liver, spleen, lungs, thymus and kidneys were removed, weighed and placed in tubes for counting in a gamma counter. Blood clearance was expressed as the phagocytic index, which is determined by the slope of the clearance curve. Organ distribution was expressed as percent organ uptake and cpm/mg tissue (specific activity).

Statistical Evaluation

If a one-way analysis of variance of the means showed treatment effects, a Dunnett's *T*-test was performed (*7*). Values which differ from vehicle control at $p < 0.05$ are noted in tables. Each of the values is given as the mean ± standard error (S.E.) of the mean, except the historical controls, which are shown with both standard deviation and standard error.

Results

Acute Toxicity

The acute toxicity of each of the chemicals was determined to provide range-finding doses to be used in the subchronic studies and possibly implicate target organs for toxicity.

The LD_{50} of dichloroethane administered as a single gavage was 489 (424-552) and 413 (337-499) mg/kg for male and female CD-1 mice, respectively. (The numbers in parentheses represent the 95% confidence limits.) The mice died over a 48-hr period. Those surviving 48 hr recovered and appeared normal at the end of the 14-day observation period. Upon gross inspection, the target organs for the acute effects appeared to be the lungs and liver.

In contrast to dichloroethane, the LD_{50} for dichloroethylene was 2221 (1987-2469) and 2391 (2056-2788) mg/kg in male and female CD-1 mice, respectively. The mice died between 2 and 24 hr, apparently by central nervous system suppression. The LD_{50} of trichloroethylene was 2402 (2065–2771) mg/kg for male mice and 2454 (1040–3062) mg/kg for female mice, with the mice dying over a 9-day period. At the high doses, the deaths occurred within 24 hr, most probably of central nervous system suppression; the lower lethal doses caused deaths later, presumably with the liver and kidney being the target organs.

Dexamethasone was administered at doses up to 1000 mg/kg without consistent lethal effects. Doses higher than this were not used because subchronic toxic doses were known to be less than 10 mg/kg.

Subchronic 14-Day Studies

The subchronic 14-day exposures served to provide range-finding doses that were used to establish the doses for subchronic 90-day studies. These data from the 14-day exposures can also be used to supplement other information in establishing acceptable exposure levels in acute environmental spills.

CD-1 male mice were administered the organic water contaminants dichloroethane, dichloroethylene and trichloroethylene daily for 14 days by gavage at doses 0.01 and 0.1 times the LD_{50}. This exposure did not alter body weight over the 14-day

Table 1. Hematological and coagulation values of CD-1 mice gavaged for 14 days with three organic water contaminants.[a]

Treatment	Parameter	1,2-Dichloroethane	1,2-Dichloroethylene	1,1,2-Trichloroethylene
Control	Leukocytes × 10^3/mm^3	8.24 ± 0.94	10.06 ± 1.02	5.93 ± 0.46
	Hematocrit, %	39 ± 0.9	42 ± 0.7	42 ± 0.5
	Hemoglobin, g-%	13 ± 0.4	14.4 ± 0.4	12.2 ± 0.3
	Fibrinogen, mg/dl	280 ± 8.0	371 ± 11.0	283 ± 5.0
	Prothrombin time, sec	9.1 ± 0.2	6.9 ± 0.1	8.5 ± 0.1
0.01 × LD_{50}	Leukocytes × 10^{-3}/mm^3	7.60 ± 0.52	8.66 ± 1.17	5.15 ± 0.44
	Hematocrit, %	40 ± 0.6	42 ± 0.7	42 ± 0.4
	Hemoglobin, g-%	12.8 ± 0.3	14.4 ± 0.4	12.7 ± 0.3
	Fibrinogen, mg/dl	262 ± 6.0	373 ± 15	294 ± 7
	Prothrombin time, sec	9.1 ± 0.1	6.8 ± 0.1	8.5 ± 0.1
0.1 × LD_{50}	Leukocytes × 10^{-3}/mm^3	5.76 ± 0.49[b]	7.44 ± 0.53	6.15 ± 0.38
	Hematocrit, %	41 ± 0.5	42 ± 0.1	39 ± 0.7[b]
	Hemoglobin, g-%	12.9 ± 0.1	14.2 ± 0.2	11.4 ± 0.5
	Fibrinogen, mg/dl	278 ± 8	327 ± 13	272 ± 8
	Prothrombin time, sec	9.0 ± 0.1	6.4 ± 0.1	8.5 ± 0.1

[a]CD-1 male mice were exposed to daily gavage of the organic water contaminant for 14 days. Twenty-four hours after the last gavage, blood was collected by cardiac puncture into 3.2% sodium citrate. The values represent the mean ± S.E. derived from 10-12 mice per group.

[b]$p < 0.05$ compared to control.

period. The average initial weights were approximately 30 g. During a necropsy performed 24 hr after the last chemical administration, the liver, spleen, lungs, thymus, kidney, and brain were weighed. There were no changes seen in the organ weights for dichloroethane and dichloroethylene. Trichloroethylene produced a 33% increase in liver weight at the high dose.

Blood obtained by cardiac puncture was analyzed for selected hematological and coagulation parameters (Table 1). Dichloroethane caused a 30% decrease in leukocyte number. Trichloroethylene produced

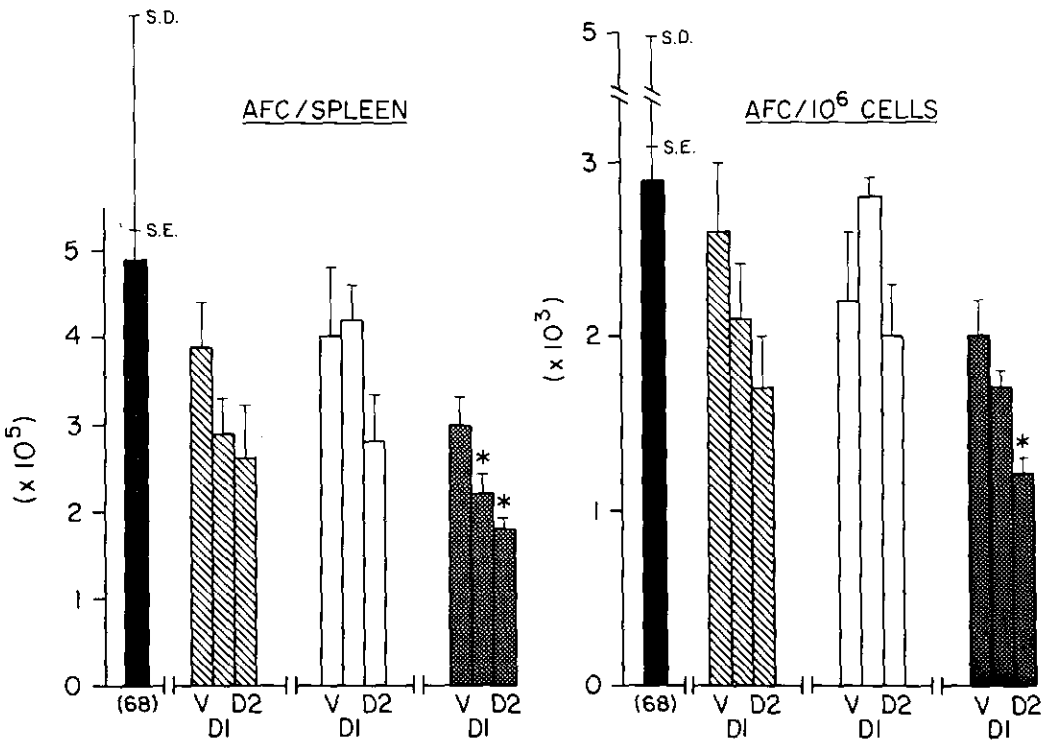

FIGURE 1. Humoral immune response to sheep erythrocytes. CD-1 male mice were exposed by gavage to (■) dichloroethane, (□) dichlorethylene and (▨) trichlorethylene at 0.01 (D1) and 0.1 (D2) × LD_{50} dose, daily for 14 days. Antigenic sensitization was performed on day 11 and antibody response measured 24 hr after the last gavage. The bar represents the mean ± S.E. derived from 10-12 mice per group, except the historical controls (■), which show the mean ± S.D. ± S.E. derived from 68 mice. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental controls.

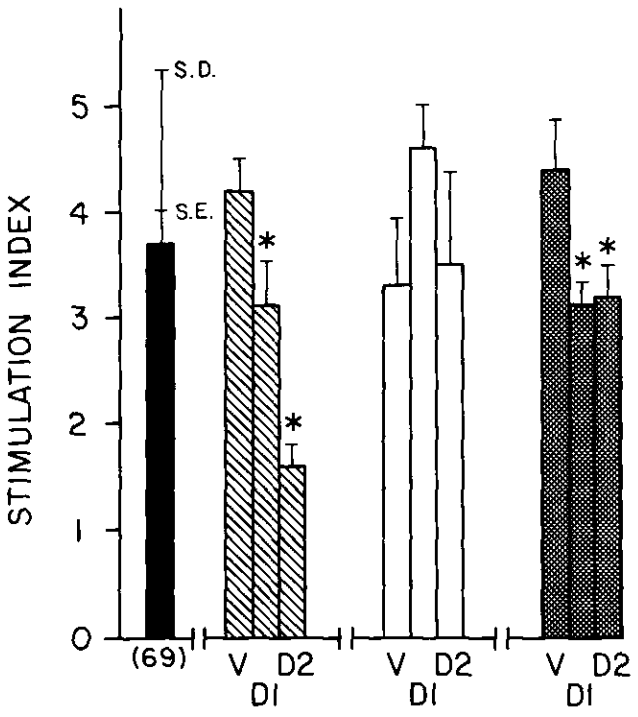

FIGURE 2. *Cell-mediated immune response to sheep erythrocytes.* CD-1 male mice were exposed by gavage to (▩) *dichloroethane,* (□) *dichloroethylene and* (▨) *trichloroethylene* at 0.01 (D1) and 0.1 (D2) × LD_{50} dose, daily for 14 days. *Antigenic sensitization was performed on day 11,* challenge was carried out on day 15, and delayed hypersensitivity quantified 19 hours after challenge. The bar graph represents the mean ± S.E. derived from 12 experimental controls (V) and 10 chemically exposed mice. The historical controls (■) are the mean ± S.E. ± S.D. derived from 69 mice. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental controls.

a 7% ($p < 0.05$) decrease in hematocrit and a concomitant decrease ($p < 0.1$) in hemoglobin.

Serum obtained by cardiac puncture was analyzed for lactic dehydrogenase, serum glutamic pyruvate transaminase, and blood urea nitrogen levels. There were no elevations in any of these parameters after 14 days exposure to the three organic compounds (data not shown).

The humoral immune response was assessed by measuring the number of IgM antibody forming cells (AFC) to sRBC. On the AFC/spleen basis, all three chemicals showed a trend towards producing immunosuppression (Fig. 1). However, the only chemical which showed an effect at the $p < 0.05$ level was dichloroethane, which produced a 25 and 40% suppression at 4.9 and 49 mg/kg, respectively. The suppression of AFC/spleen produced by trichloroethylene and dichloroethylene was significant at $p < 0.1$.

Cell-mediated immune response was measured by quantifying the extravasation of human serum albumin during the delayed hypersensitivity response to sRBC (Fig. 2). Trichloroethylene produced 25 and 60% suppression in the DTH response at 24 and 240 mg/kg, respectively. Dichloroethylene showed no effect, while dichloroethane produced a slight effect which was not dose-dependent.

Subchronic 90-Day Study

Based upon data derived from the subchronic 14-day study, the concentrations of the chemicals in the drinking water solutions were set so as to deliver daily doses equivalent to those administered by gavage. The calculations were based on our data showing that male and female mice drink about 9 or 7 ml/day, respectively. For dichloroethane, the desired daily doses were 4.9, 49 and 490 mg/kg. The 490 mg/kg dose was added because the data from the subchronic 14-day exposure showed that doses higher than 49 mg/kg could be tolerated.

The concentrations for dichloroethane in the drinking water were 0.02, 0.20 and 2.0 mg/ml. The calculated time weighted average doses delivered based on actual fluid consumed were 3, 24 and 189 mg/kg. The differences between the expected doses and actual doses delivered were caused by decreased fluid consumption in the middle and high dose groups.

In the case of dexamethasone, the concentrations in the drinking water were set at 0.1, 1.0 and 2.0 μg/ml, with the expected doses being 0.024, 0.24 and 0.49 mg/kg. The doses delivered based on fluid consumption were 0.02, 0.2 and 0.4 mg/kg.

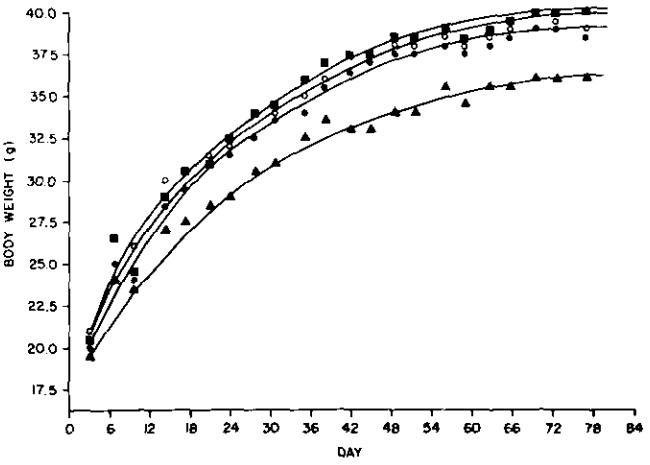

FIGURE 3. Growth chart of male CD-1 mice exposed to 1,2-dichloroethane in the drinking water: (■) deionized water; (●) 3 mg/kg delivered, 0.1 mg/ml in water; (O) 24 mg/kg delivered, 1.0 mg/ml in water; (Δ) 189 mg/kg delivered, 2.0 mg/ml in water. There were 48 mice in the deionized water group, and 32 mice in each of the dexamethasone-exposed groups.

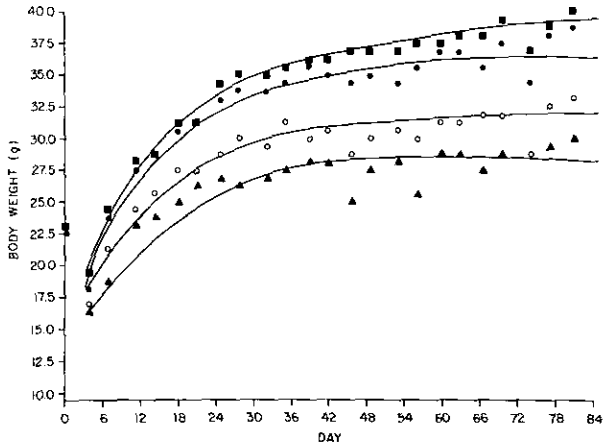

FIGURE 4. Growth chart of male CD-1 mice exposed to dexamethasone in the drinking water: (■) deionized water; (●) 0.04 mg/kg delivered, 0.1 μg/ml in drinking water; (O) 0.2 mg/kg delivered, 1.0 μg/ml in drinking water; (▲) 0.4 mg/kg delivered, 2.0 μg/ml in drinking water. There were 48 mice in the deionized water group and 32 mice in each of the dexamethasone-exposed groups.

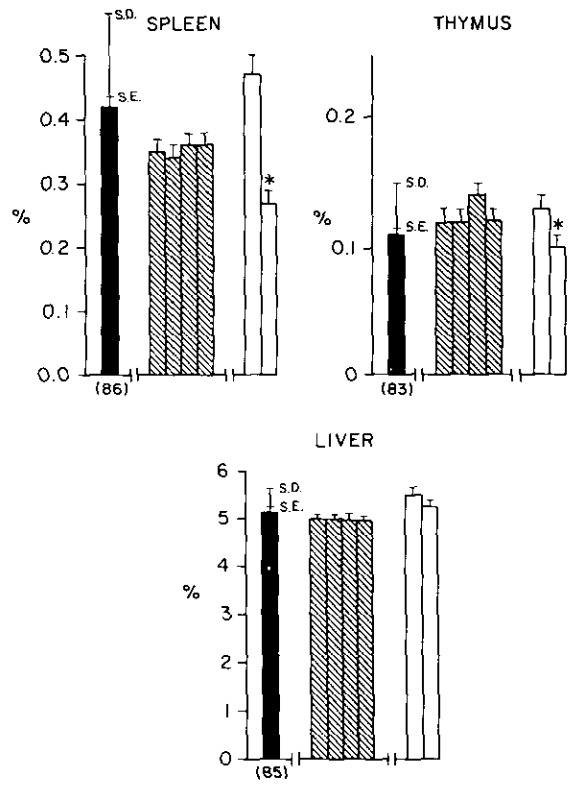

FIGURE 5. Organ weights (as % of body weight) of CD-1 mice exposed to dichloroethane or dexamethasone: (■) historical controls (numbers in parentheses represent number of mice); (▨) dichloroethane at 0, 3, 24, and 189 mg/kg; (□) dexamethasone at 0 and 0.4 mg/kg. The bars represent the mean ± S.E., except for the historical control, where the S.D. is also presented. There were 24 mice in the deionized water group and 16 mice in each of the dichloroethane- and dexamethasone-exposed groups. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental control.

The growth curves for mice exposed to dichloroethane and dexamethasone in the drinking water are shown in Figures 3 and 4. Both compounds caused a concentration-dependent decrease in growth rate. However, fluid consumption was unaltered by the presence of dexamethasone, as seen in 5.0, 4.5, 4.5 and 4.5 ml/mouse/day consumed for the 0, 0.01, 1.0 and 2.0 μg/ml dose groups, respectively. Fluid consumption in mice exposed to dichloroethane was decreased in relationship to the concentration, as seen in 5.0, 5.5, 4.2 and 2.8 ml/mouse/day consumed for the 0, 0.02, 0.2 and 2.0 mg/ml dose groups, respectively. As will be seen, decreased growth rate alone, or decreased fluid consumption alone does not predispose the immune system to altered reactivity.

Organ Weights. Dichloroethane did not alter the weights of the liver, spleen, lungs, thymus or kidneys at the levels of exposure. The weights, expressed as percent of body weight for the liver, spleen and thymus, are shown in Figure 5. The absence of effects on organ weights was also seen when the data were expressed on an organ to brain ratio or in milligrams of tissue. The positive control, dexamethasone, administered at a dose of 0.4 mg/kg, showed a 42% decrease in spleen weight and a 30% decrease in thymus weight, with no changes detected in liver weight (Fig. 5).

Hematology. Hemoglobin, hematocrit, erythrocytes, leukocytes, and platelets were not altered by exposure of mice to dichloroethane. The leukocyte data are shown in Figure 6. In contrast, mice receiving dexamethasone showed a 24% decrease in total leukocytes and 43% decrease in the percent of peripheral blood lymphocytes. This lymphopenia was accompanied by a corresponding increase in polymorphonuclear leukocytes. There were no exposure-related changes in the number of blood monocytes.

Humoral Immunity. The number of IgM spleen antibody forming cells (AFC) was quantitated on days 4 and 5 after sRBC immunization. In separate groups of mice, serum hemagglutination antibody levels were determined seven days after sRBC immunization. The AFC responses of mice exposed to dichloroethane and dexamethasone are shown for the peak day of response, which was day 4 (Fig. 7). Dichloroethane exposure caused no significant change in the number of AFC/spleen or per 10^6 nucleated spleen cells. A 55% reduction in AFC/spleen was seen in dexamethasone exposed mice. However, the specific activity, i.e., AFC/10^6

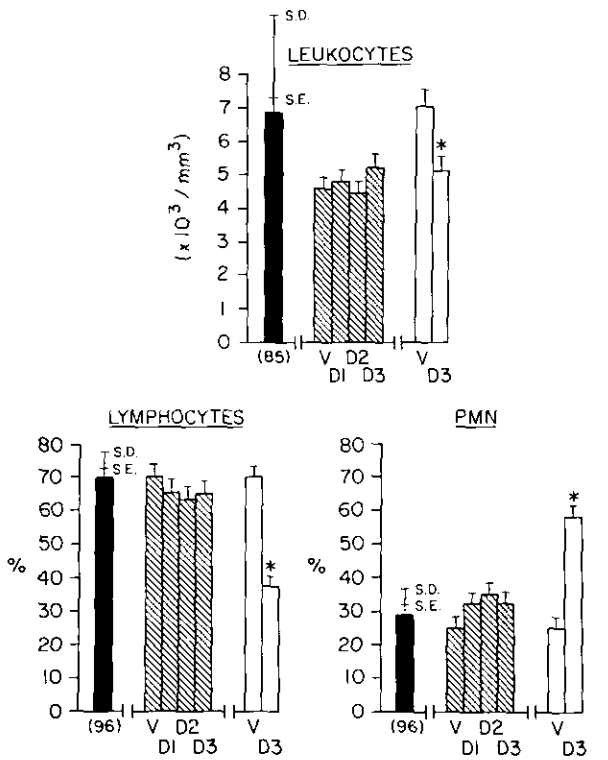

FIGURE 6. Leukocyte and differential counts of mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = 0 mg/kg, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = 0 mg/kg and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in the deionized water group and 16 mice in each of the dichloroethane- and dexamethasone-exposed groups. PMN = polymorphonuclear leukocytes. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental controls.

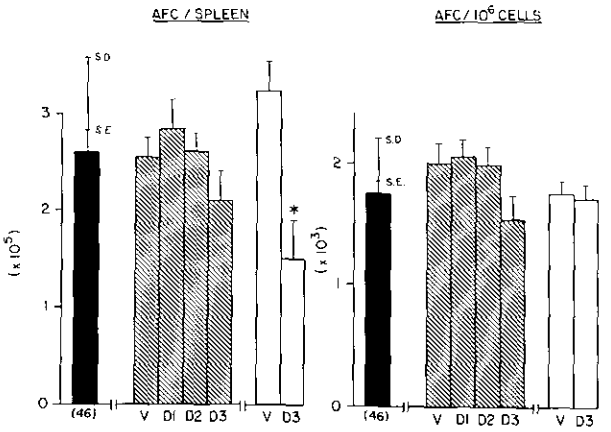

FIGURE 7. Humoral immune response to sRBC of CD-1 mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = 0 mg/kg, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = 0 and D1 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in the deionized water group and 16 mice in each of the dichloroethane- and dexamethasone-exposed groups. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental control group.

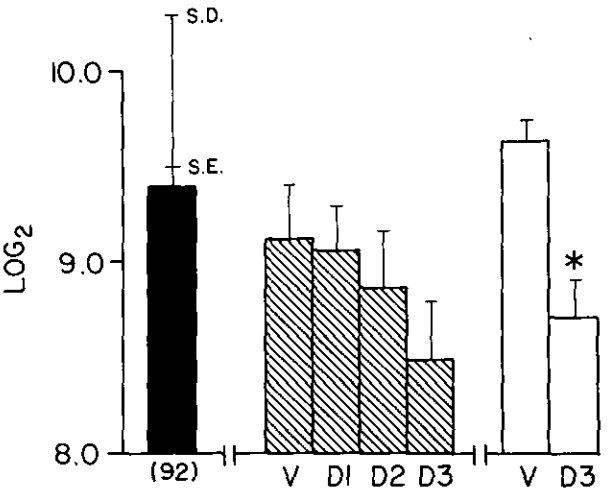

FIGURE 8. Serum hemagglutination titers in CD-1 male mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = deionized water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = deionized water, D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in the deionized water group and 16 mice in each exposure group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to experimental control group.

spleen cells, was not altered, indicating that the reduction in spleen size (Fig. 5) and spleen cell number (data not shown) was not specific for antibody-producing cells. The serum antibody titer in the dexamethasone-exposed mice was reduced from 1/787 to 1/415, a 48% suppression, confirming the results seen in AFC response (Fig. 8).

There appeared to be a dichloroethane dose-dependent reduction in hemagglutination titer. Although this was not significant at the $p < 0.05$ level, it is important to note that AFC/spleen and AFC/10^6 spleen cells were also reduced, albeit not significant at the $p < 0.05$ level.

A more complete presentation of the dexamethasone suppression of humoral immunity is seen in Figure 9. The peak day of response was day 4. Dose-dependent suppression was seen on both days 4 and 5 on an AFC/spleen basis. On day 4, there was no effect when presented as AFC/10^6 spleen cells, but there was a slight suppression on day 5.

Bacterial lipopolysaccharide (LPS) is a known B-cell mitogen. The spleen cells of dichloroethane

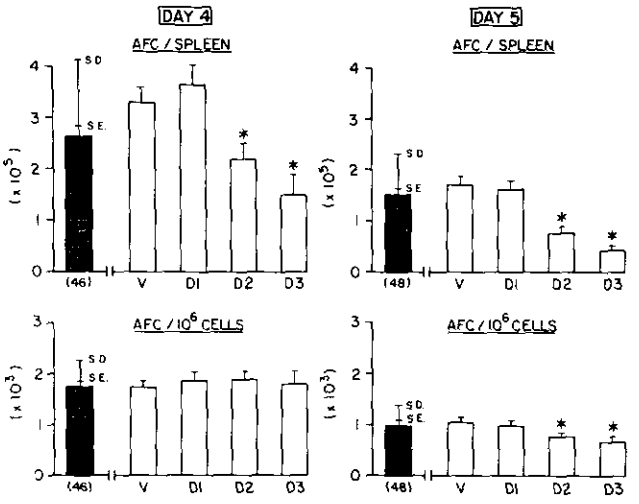

FIGURE 9. Humoral immune response to sRBC in CD-1 mice exposed to dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (□) dexamethasone at V = distilled water, D1 = 0.02 mg/kg, D2 = 0.2 mg/kg, and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in the deionized water group and 16 in each dexamethasone-exposed group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to distilled water (control) group.

and dexamethasone were evaluated for their ability to respond to this mitogen. The data seen in Figure 10 show that neither dichloroethane or dexamethasone alters the spleen cell response to three concentrations of LPS. The spleen cell response of control mice in the dexamethasone experiment was at the upper limits of the historical controls and was about 1.8 times that of the control mice in the dichloroethane experiment.

Cell-Mediated Immunity. The DTH response to sRBC was used as an *in vivo* indicator of the status of cell-mediated immunity. Mice exposed to dichloroethane showed no significant change in the DTH response. Mice exposed to dexamethasone showed a 65% reduction in the DTH response. Flumethasone injected parenterally also produced a marked suppression of 64% (Fig. 11). These data do not provide insight into which arm of the DTH response (afferent or efferent) was the site of action.

Spleen cell responsiveness to concanavalin A, a specific T-lymphocyte mitogen, was evaluated in mice exposed to dichloroethane and dexamethasone. The results are shown in Figure 12. Three concentrations of mitogen were used, producing the expected bell-shaped curve. This is seen by examin-

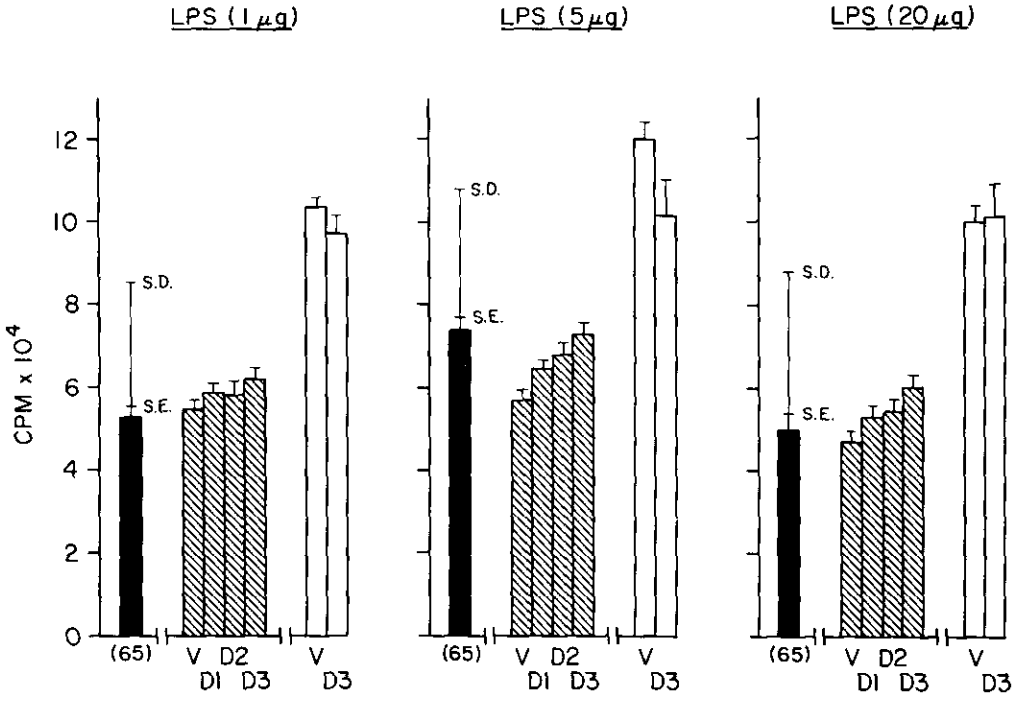

FIGURE 10. Spleen cell responsiveness to lipopolysaccharide (*S. typhose* 0901): (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = distilled water, and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in the deionized water group and 16 in each of the dichloroethane- and dexamethasone-exposed groups.

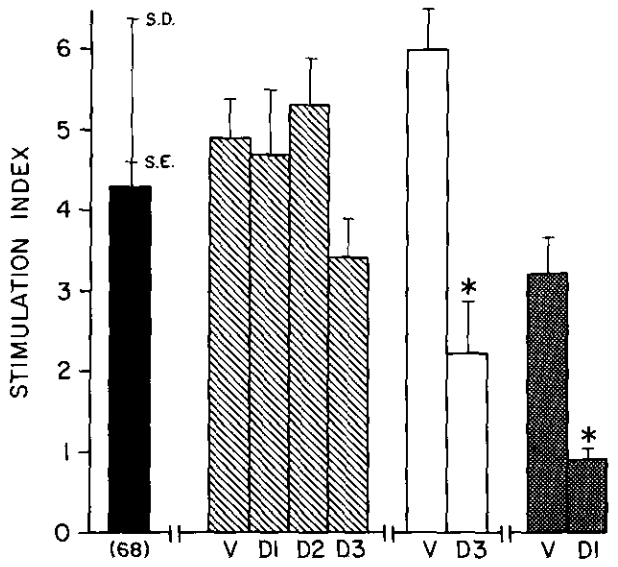

FIGURE 11. Cell-mediated immune response to sheep erythrocytes in CD-1 male mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = distilled water, D3 = 0.4 mg/kg; (▩) flumethasone at V = 0.15*M* sodium chloride IP daily for 3 days, D1 = 5 mg/kg IP daily for 3 days. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in each of the experimental control groups and 16 mice in each chemically exposed group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control group.

ing the historical and experimental controls. Dichloroethane exposure caused no alteration in spleen cell response to concanavalin A, except for the low dose group at the low (1 μg) concentration of mitogen. The results for dexamethasone were different than expected: at every concentration of mitogen, the response was significantly enhanced. In contrast, *in vitro* exposure of dexamethasone always produced a suppression of lymphocyte response to the mitogen, with an ED_{50} of about 5 μg/5 × 10^6 spleen cells (data not shown).

Functional Activity of the Reticuloendothelial System. There are many assays for macrophage function. For these studies, the vascular clearance rate and organ distribution of ^{51}Cr sRBC were used. Mice exposed to dichloroethane and dexamethasone showed no significant change in the rate of clearance, which is primarily a function of liver and spleen phagocytosis (Fig. 13). Flumethasone, administered parenterally for three days, caused a 67% reduction in clearance rate.

Liver, spleen, lung, thymus, and kidney uptake of ^{51}Cr sRBC were determined 1 hr after IV administration. This was the time previously determined to be the plateau phase of uptake. The results for the spleen, thymus, and liver of mice exposed to dichloroethane and dexamethasone are shown in Figure 14. Dichloroethane produced no changes in phagocytic uptake in the organs analyzed. Mice exposed to dexamethasone showed a reduced uptake in the spleen and thymus, but not the liver. The size of the spleen and thymus was reduced by this exposure (Fig. 5). Calculated as specific activity, i.e., cpm/mg, there was no suppression of phagocytosis in the organs examined.

Discussion

Successful interfacing of immunological assays into the toxicological evaluation of chemicals is in its early stages. We present here an approach to immunotoxicity assessment from the viewpoint of the toxicologist. After acute toxicity studies, which provide a basis for dose levels and potential target organs, subchronic 14- and 90-day studies are conducted. Immunotoxicity studies are not conducted after acute administration because of the difficulties associated with the time of antigen presentation in relationship to the time of chemical administration. In the subchronic studies, the antigens are given during the exposure period, which allows for the target cells to be at some equilibrium with the chemical during the pre- and post-antigen period. The route relevant to human exposure is used in random-bred male and female mice and the sensitivity of the immune system is evaluated against a background of the more standard toxicological methods.

Of the ten chemicals studied over the last five years, we selected dichloroethane, dichloroethylene and trichloroethylene to represent subchronic 14-day exposure, and dichloroethane and dexamethasone for subchronic 90-day exposure. Dichloroethane is used to follow a single agent through the immunotoxicological evaluation and also to show that reduction in body weight gain alone does not *a priori* predispose the immune system to toxicity. Dexamethasone is used as a positive control for many of the immunological assays.

The historical control data are presented along with the experimental control data to provide a picture of the variability in the assays. It is important to reiterate that these assays are performed in random-bred mice and the relationship of immune responses to the immune response genes are well known. Refinement of immunotoxicological assays benefits by maintaining and referring to historical controls.

Dichloroethane at concentrations as low as 1/100

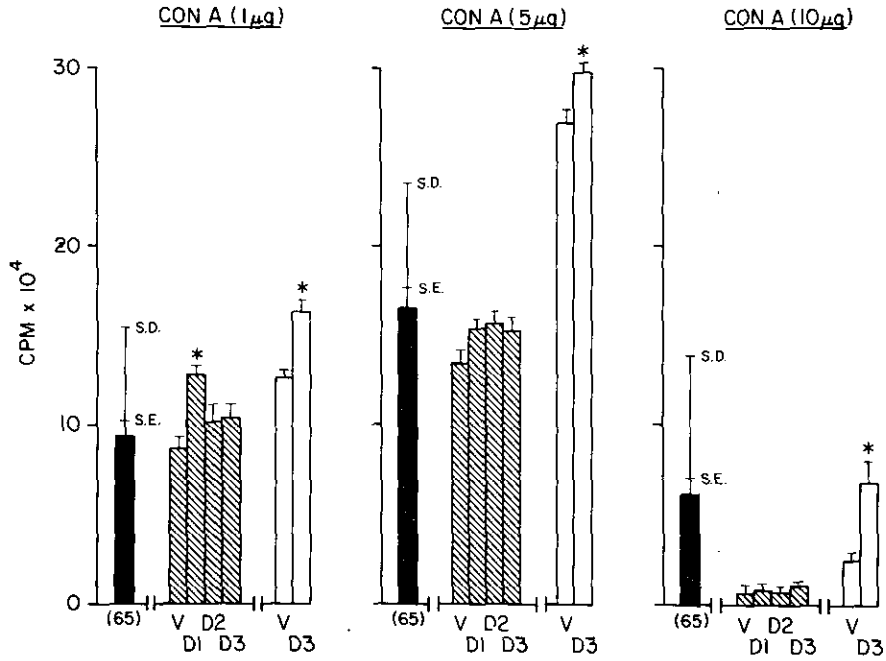

FIGURE 12. Spleen cell responsiveness to concanavalin A: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = distilled water and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in each of the distilled water groups and 16 in each of the dichloroethane- and dexamethasone-exposed groups. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control groups.

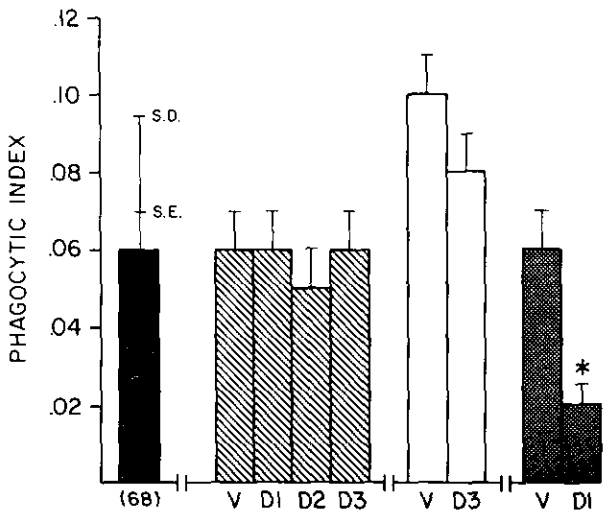

FIGURE 13. Vascular clearance rate of sRBC in CD-1 mice exposed to dichloroethane, dexamethasone and flumethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = distilled water, D3 = 0.4 mg/kg; (▩) flumethasone at V = distilled water, D1 = 0.5 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in each of the distilled water groups and 16 mice in each exposure group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control groups.

of the LD_{50} produced a suppression of both humoral and cell-mediated immunity when administered daily for 14 days by stomach tube. In the subchronic 90-day study, where doses as high as 189 mg/kg (0.4 of the LD_{50}) were consumed in the drinking water, there were no significant ($p < 0.05$) effects, although there were trends toward suppression at the high exposure level. There are at least two explanations for these results. First, the level at the biophase, i.e., immunocompetent cell, may be higher with the bolus presentation than with semi-continuous self-administration in the drinking water. Second, dichloroethane may induce its own metabolism over the longer exposure period, effectively reducing the amount of chemical reaching the immune cells.

Dexamethasone provides some interesting effects on the immune system. In these studies, it was administered in the drinking water, caused a dose-dependent decrease in body weight gain (with no change in fluid consumption), and unexpectedly did not markedly alter other toxicologic parameters such as hematology or clinical chemistries. In the case of humoral immunity, dexamethasone produced a dose-dependent reduction in AFC/spleen, but not an equivalent suppression of AFC/10^6 spleen cells, suggesting a nonspecific cytotoxic effect on the splenocytes. This is supported by the reduction in

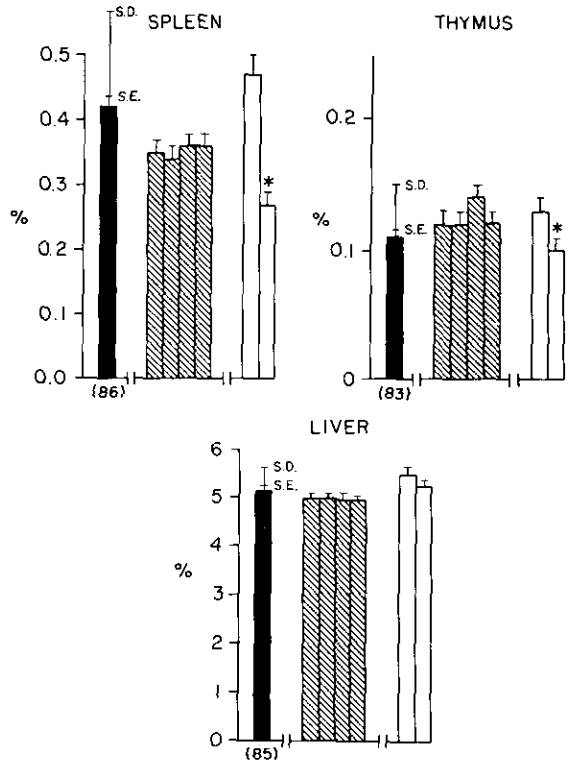

FIGURE 14. Organ uptake of sRBC in CD-1 mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = distilled water and D3 = 0.3 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in each of the distilled water groups and 16 mice in each of the exposure groups. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control groups.

serum antibody levels to sRBC and a normal B-lymphocyte mitogen response to LPS. In the mitogen assay, a constant number of spleen cells were added to the microtiter well, thus a reduction only in cell number would not alter mitogen response.

The effects on cell-mediated immunity present a more complex picture. There was a marked dose-dependent reduction in DTH response to sRBC in the presence of a hyper-responsiveness to the T-lymphocyte mitogen, concanavalin A. The increased response to concanavalin A could be caused by a change in T- and B-lymphocyte pool size. That is, a slightly greater cytotoxicity to the B-lymphocyte could result in more T-lymphocytes available in the spleen, thus more T-lymphocytes available to respond to the mitogen. This does not explain the suppression of the DTH response. Since steroids are excellent inhibitors of the efferent arm of the immune response (anti-inflammatory activity), it is quite likely that the reduced DTH response with this type of exposure is caused only by the effect on the expression of the DTH, but not on the development of the sensitized lymphocytes. This is supported by the data showing that the popliteal lymph node proliferation in these animals was not significantly affected (data not shown).

Approaches and results from these types of studies provide a good basis for evaluating a chemical's potential risk to the immune system.

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