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Immunotoxicologic Assessment of Subacute Exposure of Rats to Carbon Tetrachloride with Comparison to Hepatotoxicity and Nephrotoxicity[1]

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Immunotoxicologic Assessment of Subacute Exposure of Rats to Carbon Tetrachloride with Comparison to Hepatotoxicity and Nephrotoxicity. SMIALOWICZ, R. J., SIMMONS, J. E., LUEBKE, R. W., AND ALLIS, J. W. (1991). *Fundam. Appl. Toxicol* **17**, 186–196. The immunotoxicity, hepatotoxicity, and nephrotoxicity of subacute exposure to carbon tetrachloride (CCl_4) were evaluated in young adult (8–9 weeks old) male Fischer 344 rats dosed by gavage with CCl_4 for 10 consecutive days at 0, 5, 10, 20 or 40 mg/kg/day. Two days following the last treatment rats were evaluated for alterations in immune function by monitoring the following: body and lymphoid organ weights; mitogen and mixed leukocyte reaction lymphoproliferative responses; natural killer cell activity; and cytotoxic T lymphocyte responses. A separate group of similarly dosed rats was immunized with sheep red blood cells (SRBC) on Day 9 of dosing, and the primary antibody response was assessed 4 days later. Hepatic and renal toxicity were assessed 2 days after the last treatment by monitoring organ weights, serum indicators of hepatic and renal damage, and hepatic cytochrome P450 levels, as well as by histological evaluation. Significant increases in relative liver weights were observed in rats dosed at 40 mg/kg/day. Histologically, these livers displayed mild to moderate vacuolar degeneration and minimal to mild hepatocellular necrosis. In addition, serum levels of aspartate aminotransferase and alanine aminotransferase were elevated at this dosage, as well as at 20 mg/kg/day. There were no renal effects observed at these dosages of CCl_4. In addition, no consistent alterations were observed in the immune parameters examined in these same animals nor in the rats immunized with SRBC. Furthermore, there was no difference in the antibody response to SRBC in another set of rats dosed at 40, 80, or 160 mg/kg/day CCl_4. These results indicate that CCl_4 is not immunotoxic in the rat at dosages that produce overt hepatotoxicity.

For many years, carbon tetrachloride (CCl_4) was widely used as a solvent, cleaning agent, grain fumigant, and intermediate in the production of chlorofluorocarbons. However, because of its toxicity, production of CCl_4 steadily declined. Nevertheless, despite reductions in the production of CCl_4 (ATSDR, 1989) over 750 million pounds were manufactured in the United States in 1988 (CEN, 1989). While human exposure to CCl_4 has decreased over the years, there remains the potential for exposure from occupational and environmental sources. CCl_4 is very stable in the environment, with a half-life of 30 to 100 years (ATSDR, 1989). Sources of human exposure from the environment include CCl_4-contaminated air (Singh *et al.*, 1976; Lillian *et al.*, 1975), food (McConnell *et al.*, 1975; Jagielski *et al.*, 1978), and water (Marx, 1977).

Exposure to CCl_4 can lead to damage in a number of tissues; however, the primary target

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organ for CCl_4-induced toxicity in most animal species is the liver (Bruckner *et al.*, 1986). In fact, CCl_4-induced liver damage has been used as a model for studying the pathogenesis of hepatic necrosis and the consequences of induced alterations in hepatic function (Zimmerman, 1978). CCl_4-induced hepatotoxicity is believed to result from the metabolism of the parent compound to a highly reactive radical intermediate by the cytochrome P450 mixed-function oxidase system (Recknagel, 1983); however, the sequence of events between cytochrome P450-mediated metabolism of CCl_4 and hepatocellular damage and death remains to be elucidated. At low levels, CCl_4 exposure of experimental animals results in characteristic fatty livers. As the levels of CCl_4 exposure increase, centrilobular necrosis ensues and following prolonged exposure to CCl_4, cirrhosis and hepatic carcinoma may develop. In addition to its hepatotoxicity, CCl_4 is also recognized for its nephrotoxicity (Striker *et al.*, 1968; Kluwe, 1981).

Animal studies have also been conducted to evaluate the carcinogenic potential of CCl_4. In B6C3F1 mice exposed to time-weighted average doses of 1250 and 2500 mg/kg/day CCl_4, 5 days/week for 78 weeks, the incidence of hepatocellular carcinoma was reported to be almost 100% in both sexes (NCI, 1976). The incidence of hepatocellular carcinoma was substantially lower (about 5%) in male and female Osborne–Mendel rats exposed to 47 or 94 and 80 or 159 mg/kg/day CCl_4, respectively, under the same conditions (NCI, 1976). In more recent studies, it has been suggested that chronic exposure to a variety of chlorinated hydrocarbons, including CCl_4, may be carcinogenic in humans (Blair *et al.*, 1979; Capurro, 1979). Since the immune system plays a significant role in surveillance against neoplasia, recent studies by Kaminski *et al.* (1989, 1990) addressed the question of whether the immune system might also be affected by CCl_4 exposure. Female B6C3F1 mice exposed to CCl_4, by ip injection on 7 consecutive days at 500, 1000, or 1500 mg/kg, were found to have marked suppression of both humoral and cell-mediated immune function. Humoral immunity, as measured by the T-dependent antibody response to sheep erythrocytes, was found to be the most sensitive indicator of CCl_4 immunotoxicity (Kaminski *et al.*, 1989, 1990).

In the present study, the immunotoxic potential of CCl_4 was evaluated in the rat using a battery of immune function tests that have been developed and evaluated in this lab (Smialowicz *et al.*, 1985, 1989). Results from the immune function tests were compared with results of tests used to monitor liver and kidney effects, in order to determine which of these target organ systems was most sensitive to CCl_4-induced toxicity.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (Charles River Laboratories, Raleigh, NC) were used for CCl_4 treatment and male Wistar/Furth rats (Harlan Sprague–Dawley, Inc., Indianapolis, IN) were used as a source of stimulator cells in the mixed lymphocyte reaction in these experiments. The rats were housed, three per cage, in polycarbonate cages containing heat-treated pine shavings (Beta Chips, North Eastern Products Inc., Warrensburg, NY) and given feed (Purina lab chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. An ambient temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and a 12-hr light–dark cycle were provided. Rats were acclimatized for at least 2 weeks prior to the start of the experiments.

Dosing. Male Fischer 344 rats were assigned to one of several dosing groups. One group of 9-week-old rats was dosed by gavage with 0, 5, 10, 20, or 40 mg/kg/day carbon tetrachloride (analytical reagent grade, Mallinckrodt, St. Louis, MO) in corn oil (Sigma, St. Louis, MO) for 10 consecutive days, at a dosing volume of 2.5 ml/kg body wt. Two days later these rats were used to assess immune function parameters, excluding the primary antibody response to sheep red blood cells (SRBC), as well as liver and kidney parameters. Two separate groups of 8-week-old rats were dosed either with 0, 5, 10, 20, or 40 mg/kg/day or 0, 40, 80, or 160 mg/kg/day CCl_4 for 10 consecutive days and used to measure the primary antibody response to SRBC. Rats were weighed daily and dosing volumes adjusted accordingly. Fresh dosing solutions were used daily for each dosage group.

Immune parameters. After the last dosing with CCl_4, rats were fasted from feed but not from water for 16–18 hr prior to euthanasia. The rats were weighed, anesthetized with 50 mg/kg sodium pentobarbital (Nembutal, Abbott Lab., Chicago, IL) ip, and exsanguinated from the abdom-

inal aorta. The spleen, thymus, and mesenteric lymph nodes were aseptically removed. Body, spleen, and thymus weights were recorded.

The proliferative response of splenic lymphocytes was determined for the T cell mitogens phytohemagglutinin (PHA-P; Burroughs–Wellcome, Research Triangle Park, NC) and concanavalin A (Con A; Difco Laboratories, Detroit, MI), the B cell mitogen *Salmonella typhimurium* mitogen (STM; Ribi Immunochem, Hamilton, MT), and the T and B cell mitogen pokeweed mitogen (PWM; GIBCO, Grand Island, NY). Spleen cell cultures (i.e., erythrocyte-lysed, unfractionated splenocytes) were prepared in complete medium, which consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 mM Hepes, gentamycin at 50 μg/ml (GIBCO), and 5×10^{-5} M 2-mercaptoethanol (2-ME). Cells were cultured at 5×10^5 splenocytes/well in 96-well flat-bottom culture plates (Costar, Cambridge, MA) for 72 hr. Cultures were labeled with tritiated thymidine ([^{3}H]TdR, 0.5 μCi/well, sp act 6.7 Ci/mM, New England Nuclear, Boston, MA) 4 hr prior to harvest on a MASH II automated harvester (Microbiological Associates, Bethesda, MD). Lymphoproliferative responses were determined in triplicate at several concentrations of each mitogen. The optimal responses for each mitogen are reported (i.e., 1 μg/culture of Con A and PHA, 20 μl/culture of PWM, and 16 μg/culture of STM).

Natural killer (NK) cell activity of splenocytes from F344 rats dosed with CCl_4, cultured in supplemented RPMI medium without 2-ME, was determined in a 4-hr ^{51}Cr release assay. W/Fu-G1 rat lymphoma cells and YAC-1 mouse lymphoma cells, free of mycoplasma, were used as target cells in this assay. ^{51}Cr-labeled target cells (2×10^4/well) were cocultured in triplicate with effector cells (splenocytes) in a volume of 200 μl in round-bottom microtiter plates (Linbro) at effector:target cell (E:T) ratios of 25:1, 50:1, and 100:1. After a 4-hr incubation at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, the plates were centrifuged for 5 min at 250*g*, and the supernatant was collected using a supernatant collection system (SCS, Skatron, Inc., Sterling, VA). The percentage of specific ^{51}Cr release was calculated using the formula: $[(E - S)/(T - S)] \times 100$, where E is the ^{51}Cr released from target cells in the presence of spleen cells, S is the spontaneous release of ^{51}Cr from target cells alone, and T is the maximum release of ^{51}Cr from target cells in the presence of 0.25% Triton X-100. Spontaneous release was typically less than 10% of the maximum releasable ^{51}Cr activity.

The one-way mixed lymphocyte reaction (MLR) was performed using responder (CCl_4-dosed F344 rat) and stimulator (Wistar/Furth rat) lymph node lymphocytes. Stimulator cells were treated with mitomycin C (Sigma) and resuspended in supplemented medium containing 2-ME at 5×10^6 cells/ml. Responder cells were resuspended in the same medium at 2×10^6 cells/ml. To quadruplicate wells of round-bottom microtiter plates containing 2×10^5 responder cells, complete medium and 5×10^5 stimulator cells or Con A were added in a total volume of 200 μl. The responder plus Con A cultures served as a positive control for the proliferative capacity of the responder cells. In addition, wells containing stimulator cells plus Con A were set up as an internal control for the efficacy of the mitomycin C treatment of stimulator cells. MLR cultures were incubated for 96 hr at 37°C and 5% CO_2. Four hours prior to harvest, cultures were pulsed with 0.5 μCi/well [^{3}H]TdR. The results are expressed as the net CPM, subtracting the CPM of responder only cultures from the CPM of responder plus stimulator cultures.

Cytotoxic T lymphocytes (CTL) were generated *in vitro* with lymph node cells from CCl_4-dosed F344 (responder) and Wistar/Furth (stimulator) rats as in the MLR assay. Equal numbers (5×10^5) of responder and stimulator cells were added to quadruplicate round-bottom microtiter wells in a total volume of 200 μl. To another set of wells 100 μl of responder cells only was added. Duplicate wells of responder plus stimulator cells, responder cells only, and stimulator cells only were prepared on a separate microtiter plate. This second set of cultures was used for the determination of the final E:T ratios at the time of the CTL assay based on the number of viable cells remaining. All cultures were incubated for 96 hr at 37°C and 5% CO_2. At the end of the incubation period the CTL cultures were centrifuged at 250*g* for 5 min and 100 μl of culture supernatant was removed. The cell pellets were resuspended and 100 μl of ^{51}Cr-labeled W/Fu-G1 cells (4×10^5 cells/ml) was added to each well. These cultures were incubated for 4 hr, after which they were centrifuged (250*g*), and the supernatants collected. The percentage of ^{51}Cr release, as a measure of CTL activity, was determined as described for the NK assay.

CTL viable cell counts were determined using a modified pronase method (Stewart *et al.*, 1975). Suspensions were prepared from wells of responder plus stimulator, responder only, and stimulator only cultures. One full well of each culture was placed in a tube containing 100 μl of 2% (w/v) EDTA. To replicate tubes either 100 μl of pronase (Calbiochemical, sp act of 70,000 PUK/g, at 225 PUK/ml) or 100 μl of saline was added. The tubes were placed in a 37°C water bath for 10 min, after which they were mixed vigorously and diluted with 9.6 ml of Isoton followed by three drops of Zapoglobin (Coulter Electronics Inc., Hialeah, FL). Cell suspensions were counted on a Coulter Model ZBI particle counter. The viable cell counts of stimulated responder (i.e., effector) cells were determined by subtracting the count of stimulator cells (nonproliferative but viable cells) from the responder plus stimulator counts.

Separate groups of rats were immunized with a single intravenous injection of 0.5 ml of three times washed 10% SRBC in sterile saline prior to CCl_4 dosing on Day 9 of the 10-day dosing period. Four days later the primary antibody response to SRBC was determined using the direct plaque-forming cell (PFC) assay (Smialowicz *et al.*, 1988).

Liver and kidney parameters. Blood from the abdominal aorta was collected in sterile tubes (SST serum-separation

tubes, Becton–Dickinson, Lincoln Park, NJ). The blood was held on ice for a minimum of 30 min and then centrifuged at 1000*g* for 30 min. Serum was collected and frozen at −40°C until analyzed. Serum chemistry profiles were obtained by automated procedures (CentrifiChem System 500 centrifugal analyzer, Baker Instruments Corp., Allentown, PA) and included determination of the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKPH), lactate dehydrogenase (LDH), as well as the concentrations of total bilirubin (BILI), total cholesterol (CHOL), triglycerides (TRIGLYC), creatinine (CREAT), and urea nitrogen (BUN). Baker Instrument Corp. reagents and standards were used for all assays.

The liver and kidneys of each rat were excised, rinsed in physiological saline, blotted, and weighed. Relative organ weights (the ratio of organ weight to body weight) were calculated for each rat. Aliquots of liver from the median lobe were weighed (approximately 2 g) and used for determination of hepatic cytochrome P450 by the method of Omura and Sato (1964). The concentration of microsomal protein was determined by the method of Lowry *et al.* (1951).

Longitudinal and horizontal sections of the left and right kidney, respectively, and samples from the left hepatic lobe were taken for open histopathological examination (STP, 1986; Prasse *et al.*, 1986). Lesions were evaluated on hematoxylin and eosin stained sections of tissues fixed in 10% phosphate-buffered formalin. Hepatocellular vacuolar degeneration and necrosis were graded for lobular location (centrilobular, periportal, midzonal) and for severity as previously described (Simmons *et al.*, 1988), according to the following criteria: none; minimal (one to several hepatocytes affected); mild (no more than one-fourth of the hepatocytes in the affected zone involved); moderate (expansion of the number of damaged hepatocytes up to one-half of the affected zone); and marked (over one-half of the lobular architecture involved). Kidney nephropathy was graded on a scale of increasing severity as none; minimal (scattered foci of tubular cell regeneration with affected tubules having an increased number of cells with intense cytoplasmic and nuclear staining with up to 25% of cortex affected); mild (increased extent and number of regenerative foci, basement membranes surrounding regenerative tubules and within glomeruli thickened, tubular protein casts with 25–50% of the cortex affected); moderate (further increases in number and severity of regenerative foci, increased number of tubular protein casts, increased glomerular damage with adhesions between the glomerular tuft and the parietal epithelium, hemosiderin and lipochrome pigments possibly present in tubular epithelial cytoplasm with 50–75% of the cortex affected); and marked (diffuse involvement of cortex, "end-stage" kidney with sufficient impairment to result in either secondary hyperparathyroidism or contribute to death).

Statistical analysis. The immune function data were analyzed by one-way analysis of variance (ANOVA). Differences between control and treated groups were determined using Dunnett's *t* test (Dunnett, 1955) for multiple comparisons with a $p < 0.05$ considered significant.

The liver and kidney numerical data were subjected to Bartlett's test for homogeneity of variances (Sokal and Rohlf, 1981). The criterion for significance for Bartlett's test was $p < 0.001$ (Anderson and McLean, 1974). When necessary, the data were transformed to corresponding logarithms for analysis. Data were analyzed by ANOVA (SAS, 1985). When the significance criterion was met, differences between control and treated groups were determined by Sidak's method (SAS, 1985) for multiple comparison of means with a $p < 0.05$ considered significant.

Rat weight gain during the course of dosing was analyzed by obtaining the slope and intercept for each rat by linear regression analysis (SAS, 1985). These slopes and intercepts were analyzed as described for the liver and kidney data.

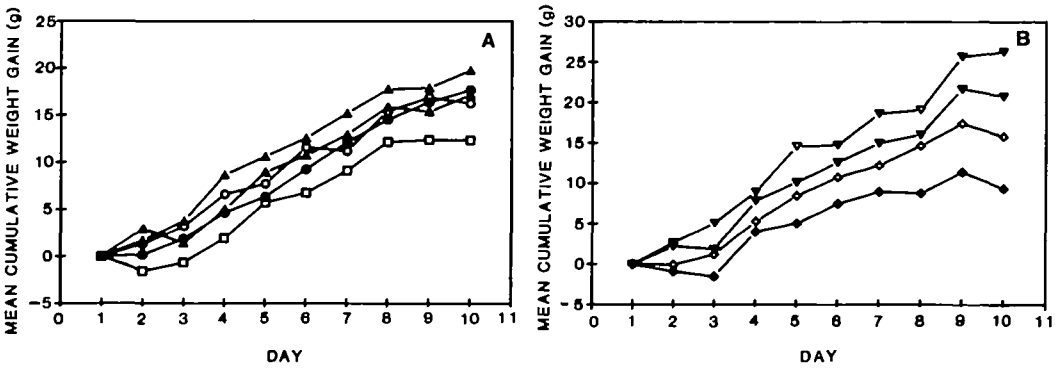

FIG. 1. Weight gain of rats dosed by oral gavage on 10 consecutive days with carbon tetrachloride. Data presented are mean body weights of six (A) or eight (B) rats per dosage group. Key: ○, corn oil; ●, 5 mg/kg; △, 10 mg/kg; ▲, 20 mg/kg; □, 40 mg/kg (A) or ▽, corn oil; ▼, 40 mg/kg; ◇, 80 mg/kg; ◆, 160 mg/kg (B).

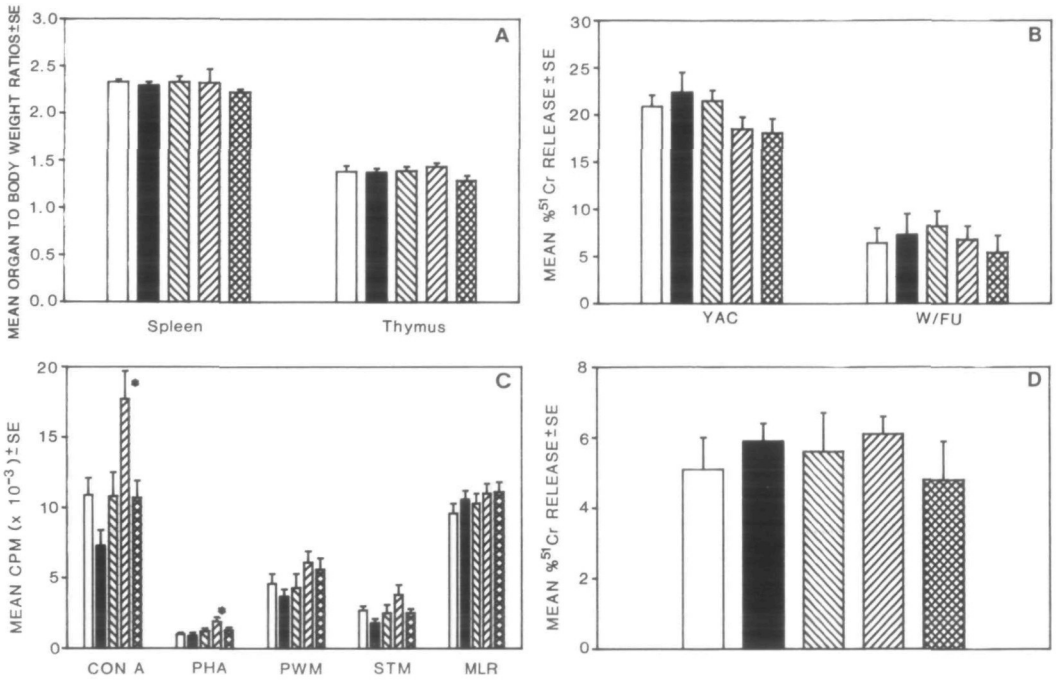

FIG. 2. Effect of carbon tetrachloride on immune parameters in rats dosed by oral gavage on 10 consecutive days. Relative spleen and thymus weights (A); natural killer cell activity against YAC-1 and W/Fu-G1 target cells at an effector to target cell ratio of 100:1 (B); lymphoproliferative responses to concanavalin A (Con A), phytohemagglutinin (PHA), pokeweed mitogen (PWM), *Salmonella typhimurium* mitogen (STM), and the one-way mixed lymphocyte reaction (MLR) (C); and allogeneic cytotoxic T lymphocyte reaction (D). Key: □, corn oil; ■, 5 mg/kg; ▨, 10 mg/kg; ▧, 20 mg/kg; ▩, 40 mg/kg. Data are expressed as the means ± SE. $*p < 0.05$ compared with corn oil control ($N = 6$).

RESULTS

Body weight gains of rats exposed to 10 daily doses of CCl_4 are shown in Fig. 1. There were no differences in weight gain by rats dosed at 5 to 40 mg/kg/day CCl_4 during the dosing period (Fig. 1A). However, when the three separate groups of rats that received 40 mg/kg/day (see Dosing under Materials and Methods) and their respective control groups were analyzed by two-way ANOVA, with CCl_4 and replicates as factors, a significant decrease in weight gain attributable to this dosage of CCl_4 was detected. Significant decreases in weight gain, as determined by comparison of the slopes of the weight gains over the 10-day dosing period, were observed for rats dosed at 80 and 160 mg/kg/day CCl_4 (Fig. 1B).

The results of the immune function tests that were examined in rats dosed at 5 to 40 mg/kg/day CCl_4 are shown in Fig. 2. Exposure of rats to these dosages of CCl_4 had no apparent effect on spleen or thymus weights (Fig. 2A), natural killer cell activity (Fig. 2B), or the *in vitro*-generated allogeneic cytotoxic T lymphocyte response (Fig. 2D). Enhancement of the lymphoproliferative response to Con A and PHA, both T cell mitogens, was observed for splenocytes from rats dosed at 20 mg/kg/day CCl_4 (Fig. 2C). However, these enhanced responses appear to be anomalous since there were no indications of an association between the CCl_4 dose and these changes. Furthermore, there were no effects of CCl_4, at this or any other dosage, on the T cell-dependent PWM and MLR responses (Fig. 2C).

Figure 3 shows the results of two separate experiments in which the primary antibody response to SRBC was determined in rats dosed with CCl_4. There were no effects of CCl_4 exposure on the PFC response to SRBC, as expressed as PFC/10^6 spleen cells, at either dosages of 5 to 40 mg/kg/day (Fig. 3A) or 40 to 160 mg/kg/day (Fig. 3B). Furthermore, there were no significant differences in the primary antibody responses of rats dosed with CCl_4 expressed as PFC/total spleen or as measured by serum hemagglutination titer (data not shown).

In contrast to the lack of any consistent alterations in immune parameters following exposure to CCl_4, dose-dependent hepatotoxicity was observed in these rats. Table 1 summarizes the liver function parameters that were examined in rats dosed at 5 to 40 mg/kg/day CCl_4. An increase in relative liver weight was observed in rats dosed at 40 mg/kg/day CCl_4. CCl_4 exposure also increased serum levels of AST and ALT at 20 and 40 mg/kg/day. Although a significant ANOVA result was obtained for hepatic cytochrome P450, indicating significant differences among the various groups, no treatment group was significantly different from control. Histopathological examination of livers revealed an increase in vacuolar degeneration in the centrilobular region of the hepatic lobule and centrilobular hepatocellular necrosis with increasing dose of CCl_4 (Table 2).

Examination of a number of renal parameters, in rats exposed to CCl_4 at dosages of 5 to 40 mg/kg/day, failed to reveal any effects (Table 3). Furthermore, histopathological examination of kidneys obtained from these rats revealed no nephropathy (Table 2).

DISCUSSION

Evaluation of the potential immunotoxic effects of CCl_4 exposure in rats revealed no consistent alterations in humoral or cell-mediated immune function at dosages which clearly resulted in body weight decreases and hepatotoxicity. These results are in contrast to those reported by Kaminski *et al.* (1989, 1990) in which immune alterations were observed in B6C3F1 mice exposed to CCl_4, albeit at much higher doses.

The liver is considered the principal target organ following exposure to CCl_4 in most species (Bruckner *et al.*, 1986). The kidney is also a target organ in humans and animals, although in animals it appears to be less sensitive to CCl_4-induced injury than the liver following oral administration (ATSDR, 1989), as confirmed in the present study. The potential public health significance of CCl_4-induced immune system deficits depends, at least in part, on the sensitivity of this target organ relative to that of the liver or the kidney. That is, when the lowest observed effect level for

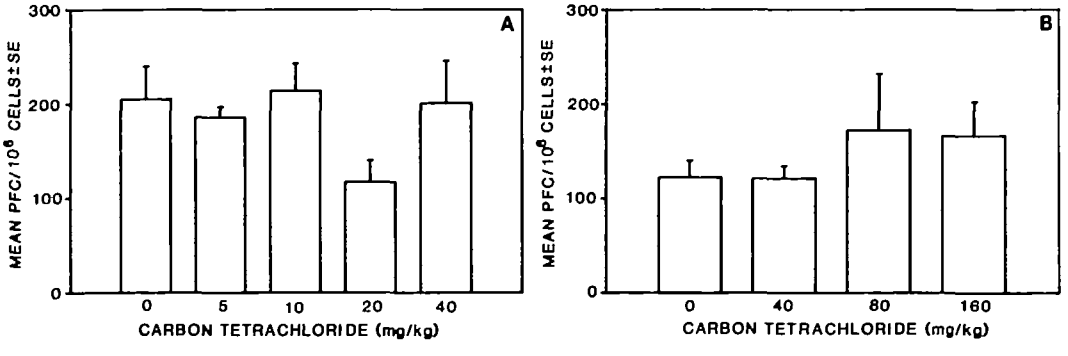

FIG. 3. Effect of carbon tetrachloride on the plaque-forming cell (PFC) response to sheep red blood cells of rats dosed for 10 consecutive days. Data presented are means ± SE of six (A) and eight (B) rats per dosage group.

TABLE 1

THE EFFECT OF 10 DAILY DOSES OF CARBON TETRACHLORIDE ON LIVER PARAMETERS

Parameter (units)	Carbon tetrachloride (mg/kg/day)[a]				
	0	5	10	20	40
Body weight (g)	202.17 ± 4.18	203.17 ± 5.66	202.92 ± 3.76	198.53 ± 4.01	194.45 ± 2.34
Liver weight (g)	6.92 ± 0.33	7.19[b] ± 0.54	7.15 ± 0.19	7.44 ± 0.27	7.77 ± 0.15
Relative liver weight (%)	3.41 ± 0.11	3.58[b] ± 0.17	3.52 ± 0.05	3.74 ± 0.06	4.00** ± 0.06
P450 (nmol/mg microsomal protein)	1.12 ± 0.05	1.06 ± 0.03	1.20 ± 0.03	1.25 ± 0.03	1.14 ± 0.04
Microsomal protein (mg/g liver)	17.56 ± 0.84	16.71 ± 1.37	18.22 ± 0.54	16.97 ± 0.86	16.16 ± 0.72
ALKPH (U/liter)	215.83 ± 24.19	238.33 ± 16.82	198.67 ± 10.45	223.00 ± 5.32	251.17 ± 5.28
BILI (mg/dl)	0.25 ± 0.02	0.30 ± 0.03	0.29 ± 0.03	0.27 ± 0.02	0.26 ± 0.03
LDH (U/liter)	93.50 ± 13.65	123.00 ± 8.01	107.83 ± 12.06	130.67 ± 15.69	145.83 ± 14.33
AST (U/liter)	49.33 ± 2.36	61.33 ± 1.28	56.50 ± 4.92	72.00* ± 3.71	132.00** ± 18.78
ALT (U/liter)	31.67 ± 0.99	39.00 ± 1.44	46.00 ± 4.98	64.33** ± 3.48	172.00** ± 28.96
TRIGLYC (mg/dl)	13.93 ± 2.34	25.02 ± 3.82	26.77 ± 8.04	18.98 ± 2.82	16.58 ± 3.78
CHOL (mg/dl)	41.33 ± 4.76	35.87 ± 3.61	31.63 ± 1.76	29.90 ± 1.79	30.55 ± 1.58

[a] Data are presented as the mean ± SE; $N = 6$.
[b] $N = 5$.
* $p < 0.05$ vs control.
** $p < 0.01$ vs control.

the immune system is larger than that for the liver or the kidney, exposure guidelines designed to prevent hepatic or renal effects should adequately guard against immune effects. Thus, the relative sensitivity and the relationship of the liver and the immune system to CCl_4-induced damage are important. The present study indicates that in the male rat, immune system effects do not occur at CCl_4 dosages that produce significant hepatotoxicity.

Oral exposure of rats, which is the most relevant route, to CCl_4 dosages as high as 160 mg/kg/day failed to alter the primary antibody response to SRBC. This differs from the results obtained for mice in which a dose-dependent decrease in the T-dependent antibody response to SRBC was observed following 7 consecutive

TABLE 2

INCIDENCE OF HISTOPATHOLOGY OF LIVERS AND KIDNEYS FROM RATS DOSED BY ORAL GAVAGE FOR 10 DAYS WITH CARBON TETRACHLORIDE

Organ[a]	Carbon tetrachloride (mg/kg/day) 0	5	10	20	40
Liver[b]					
Vacuolar degeneration					
None	6	0	0	0	0
Minimal	0	6	0	0	0
Mild	0	0	6	6	5
Moderate	0	0	0	0	1
Marked	0	0	0	0	0
Hepatocellular necrosis					
None	6	6	3	1	0
Minimal	0	0	3	5	5
Mild	0	0	0	0	1
Moderate	0	0	0	0	0
Marked	0	0	0	0	0
Kidney					
Renal nephropathy					
None	3	1	0	2	1
Minimal	3	5	6	4	5
Mild	0	0	0	0	0
Moderate	0	0	0	0	0
Marked	0	0	0	0	0

[a] Lesions were graded according to the criteria described under Materials and Methods, $N = 6$.

[b] All observed hepatic changes occurred in the centrilobular region of the hepatic lobule.

days of ip injections with 500, 1000, and 1500 mg/kg CCl_4 (Kaminski *et al.*, 1989). This difference in results is probably not due to differing routes of administration because in a subsequent study Kaminski *et al.* (1990) found that there was no difference in the ability of CCl_4 to inhibit the PFC response in mice when dosing was by either the ip or the oral route.

Except for the anomalous enhancement in the responses by splenocytes to Con A and PHA from rats dosed at 20 mg/kg/day CCl_4, no alterations in lymphoproliferative responses to these or other mitogens or in the MLR were observed. Furthermore, the *in vitro*-generated allogeneic CTL response and NK cell activity were unaffected by CCl_4 exposure. This is in contrast to results reported for mice exposed to CCl_4, in which the MLR response and the mitogen-stimulated responses to Con A and lipopolysaccharide were suppressed (Kaminski *et al.*, 1989). Mice exposed to CCl_4 also displayed significantly decreased thymus weights; however, these decreases were not associated with any alterations in body, liver, or kidney weights (Kaminski *et al.*, 1989). In contrast, liver weights were increased in rats dosed at 40 mg/kg/day CCl_4, in the absence of any change in thymus weights, in the present study. Furthermore, body weights were reduced in rats dosed at 80 and 160 mg/kg/day CCl_4. There were no indications of nephrotoxicity in rats dosed at 5 to 40 mg/kg/day CCl_4.

Kaminski *et al.* (1989) demonstrated both immunotoxicity and hepatotoxicity in female B6C3F1 mice following exposure to 500 mg/kg/day CCl_4 for 7 days. Hepatotoxicity, assessed the day following cessation of exposure, was very severe at this dosage level as indicated by a 34-fold elevation, relative to control, in serum ALT level. In a subsequent investigation, hepatic and immune effects were assessed in female B6C3F1 mice following 7 or 30 days of exposure to lower dosages of CCl_4 (Kaminski *et al.*, 1990). Exposure to 250 mg/kg/day CCl_4 for 7 days had no apparent effect on either the immune system (T-dependent antibody response) or the liver (ALT levels). However, serum ALT levels were not measured until 5 days after cessation of exposure, which is a sufficient time interval for significant recovery to have occurred. This conclusion is based on results from studies in which ALT levels returned to control levels within a few days following CCl_4 exposure. For example, ALT values apparently returned to control levels within 4 days following ip administration of 1.8 ml/kg CCl_4 or approximately 2869 mg/kg in male Swiss Webster mice (Hurwitz, 1972). In rats, ALT levels returned to control levels within 5 days of ip exposure to 1.0 ml/kg CCl_4 or approximately 1594 mg/kg (Charbonneau *et al.*, 1985), and apparently within 3 days of oral exposure to

TABLE 3

THE EFFECT OF 10 DAILY DOSES OF CARBON TETRACHLORIDE ON KIDNEY PARAMETERS

	Carbon tetrachloride (mg/kg/day)[a]				
Parameter (units)	0	5	10	20	40
Kidney weight (g)	1.49 ± 0.07	1.47[b] ± 0.05	1.48 ± 0.04	1.47 ± 0.05	1.49 ± 0.03
Relative kidney weight (%)	0.73 ± 0.02	0.74[b] ± 0.01	0.73 ± 0.01	0.74 ± 0.02	0.77 ± 0.01
BUN (mg/dl)	20.32 ± 1.00	21.67 ± 1.18	22.33 ± 0.83	22.90 ± 0.96	22.78 ± 1.14
CREAT (mg/dl)	0.62 ± 0.03	0.63 ± 0.04	0.56 ± 0.02	0.64 ± 0.03	0.65 ± 0.04
BUN/CREAT	33.22 ± 2.35	34.63 ± 1.68	39.76 ± 1.47	36.48 ± 3.03	36.26 ± 3.68

[a] Data are presented as the mean ± SE; $N = 6$ except where indicated.
[b] $N = 5$.

1.5 ml/kg CCl_4 or approximately 2391 mg/kg (Teschke *et al.*, 1983).

Suppression of T-dependent antibody response occurred in female B6C3F1 mice following ip exposure to 25 mg/kg/day CCl_4 for 30 consecutive days; hepatotoxic effects were not monitored in these mice (Kaminski *et al.*, 1990). It is reasonable to expect that significant hepatotoxicity occurred in the mice exposed to 25 mg/kg/day based on the following observations. First, the ED50 for ALT elevation (data were transformed to an all or none response) in mice following a single ip injection was 0.0098 ml/kg CCl_4 or approximately 15.6 mg/kg (Klaassen and Plaa, 1966) determined by one method and 0.008 ml/kg CCl_4 or approximately 12.8 mg/kg determined by an alternate method (Klaasen and Plaa, 1967). Second, increasing the duration of exposure to CCl_4 from 1 to 11 days resulted in an increase in the severity of hepatic injury in rats (Bruckner *et al.*, 1986). Furthermore, mice are reported to be more sensitive than rats to the acute hepatotoxic effects of CCl_4 (Diaz Gomez *et al.*, 1975), with significant hepatotoxicity observed in rats following oral exposure to 33 mg/kg/day, 5 days/week for 4 or 6 weeks (Bruckner *et al.*, 1986).

Kaminski *et al.* (1990) have suggested that metabolic activation of CCl_4 in the liver is necessary to produce immunosuppression in the mouse and have presented circumstantial evidence for this view. The results of the present study show evidence for CCl_4 hepatotoxicity in the rat and therefore the presence of metabolic activation. However, there is no evidence of immunosuppression in the rat, albeit at doses around 25-fold lower than those to which mice were exposed (Kaminski *et al.*, 1989). If the suggestion of Kaminski *et al.* (1990) is valid, then the rat must not possess the necessary mechanism(s) linking hepatotoxicity to immunosuppression. This result may provide a clue to the putative linking mechanism in the mouse. Other possibilities also exist and species differences in immunotoxicity of other chemical agents are known. For example, rats but not mice, guinea pigs, chickens, or Japanese quail display thymic atrophy and suppressed immune function when exposed to the organotin compounds di-*n*-octyltin dichloride or di-*n*-butyltin dichlo-

ride (Seinen *et al.*, 1977; Seinen and Penninks, 1979). Species (Vos *et al.*, 1973; Sharma, 1981) and strain (Clark *et al.*, 1983; Nagarkatti *et al.*, 1984) sensitivity to the immunotoxic effects of the halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin have also been reported.

At present, other than the obvious difference in the doses of CCl_4 administered to mice (Kaminski *et al.*, 1989, 1990) versus rats, the reason(s) for our failure to observe immune alterations in the rat is (are) not known. It may be that CCl_4 doses higher than those employed in this study are required to produce immune alterations in the rat. However, exposure of rats to doses in excess of those which we observed to produce hepatotoxicity and reduced weight gain would be overtly toxic, and as such trigger the adrenal axis which would profoundly influence the immune system. Clearly, further work is necessary to determine if a species difference in CCl_4-induced immunotoxicity exists and if so to elucidate the underlying reasons for a species difference. This is of particular importance because of the role that interspecies extrapolation plays in the risk assessment process.

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REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR) (1989). *Toxicological Profile for Carbon Tetrachloride.*

ANDERSON, V. L., AND MCLEAN, R. A. (1974). *Design of Experiments: A Realistic Approach*, pp. 16–22. Dekker, New York.

BLAIR, A., DECOUFLE, P., AND GRAUMAN, D. (1979). Cause of death among laundry and dry cleaning workers. *Am. J. Pub. Health* **69**, 508–511.

BRUCKNER, J. V., MACKENZIE, W. F., MURALIDHARA, S., LUTHRA, R., KYLE, G. M., AND ACOSTA, D. (1986). Oral Toxicity of carbon tetrachloride: Acute, subacute and subchronic studies in rats. *Fundam. Appl. Toxicol.* **6**, 16–34.

CAPURRO, P. U. (1979). Cancer in a community subject to air pollution by solvent vapors. *Clin. Toxicol.* **14**, 285–294.

CHARBONNEAU, M., IIJIMA, M., COTE, M. G., AND PLAA, G. L. (1985). Temporal analysis of rat liver injury following potentiation of carbon tetrachloride hepatotoxicity with ketonic or ketogenic compounds. *Toxicology* **35**, 95–112.

Chemical and Engineering News (CEN) (1989). *Organic Chemicals: 1987's Strong Rise Was Often Repeated in 1988.* June 19, p 41.

CLARK, D. A., SWEENEY, G., SAFE, S., HANCOCK, E., KILBURN, D. G., AND GAULDIE, J. (1983). Cellular and genetic basis for suppression of cytotoxic T cell generation by haloaromatic hydrocarbons. *Immunopharmacology* **6**, 143–153.

DIAZ GOMEZ, M. I., DE CASTRO, C. R., D'ACOSTA, N., DE FENOS, O. M., DE FERREYRA, E. C., AND CASTRO, J. A. (1975). Species differences in carbon tetrachloride-induced hepatotoxicity: The role of CCl_4 activation and of lipid peroxidation. *Toxicol. Appl. Pharmacol.* **34**, 102–114.

DUNNETT, C. W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am Stat Assoc.* **5**, 1096–1121.

HURWITZ, A. (1972). Effects of microsomal enzyme inducers on animals poisoned with hepatotoxins. *Toxicol. Appl. Pharmacol.* **22**, 339–346.

JAGIELSKI, J., SCUDAMORE, K. A., AND HEUSER, S. G. (1978). Residues of carbon tetrachloride and 1,2-dibromoethane in cereals and processed foods after liquid fumigant grain treatment for pest control. *Pest Sci.* **9**, 117–126.

KAMINSKI, N. E., BARNES, D. W., JORDAN, S. D., AND HOLSAPPLE, M. P. (1990). The role of metabolism in carbon tetrachloride-mediated immunosuppression, *in vitro* studies. *Toxicol. Appl. Pharmacol.* **102**, 9–20.

KAMINSKI, N. E., JORDAN, S. D., AND HOLSAPPLE, M. P. (1989). Suppression of humoral and cell-mediated immune responses by carbon tetrachloride. *Fundam. Appl. Toxicol.* **12**, 117–128.

KLAASSEN, C. D., AND PLAA, G. L. (1966). Relative effects of various chlorinated hydrocarbons on liver and kidney function in mice. *Toxicol. Appl Pharmacol.* **9**, 139–151.

KLAASSEN, C. D., AND PLAA, G. L. (1967). Relative effect of various chlorinated hydrocarbons on liver and kidney function in dogs. *Toxicol. Appl. Pharmacol.* **10**, 119–131.

KLUWE, W. M. (1981). The nephrotoxicity of low molecular weight halogenated alkane solvents, pesticides, and

chemical intermediates. In *Toxicology of the Kidney* (J. B. Hook, Ed.), pp. 179–193, Raven Press, New York.

LILLIAN, D., SINGH, H. B., APPLEBY, A., LOBBAN, L., ARNTS, R., GUMPERT, R., HAGUE, R., TOMMEY, J., KAZAZIS, J., ANTELL, M., HANSEN, D., AND SCOTT, B. (1975). Atmospheric fates of halogenated compounds. *Environ. Sci. Technol.* **9**, 1042–1048.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951). Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193**, 265–275.

MARX, J. L. (1977). Drinking water: Getting rid of the carbon tetrachloride. *Science* **196**, 632–636.

MCCONNELL, G., FERGUSON, D. M., AND PEARSON, C. R. (1975). Chlorinated hydrocarbons and the environment. *Endeavour* **34**, 13–18.

NAGARKATTI, P. S., SWEENEY, G. D., GAULDIE, J., AND CLARK, D. A. (1984). Sensitivity to suppression of cytotoxic T cell generation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is dependent on the Ah genotype of the murine host. *Toxicol. Appl. Pharmacol.* **72**, 169–176.

National Cancer Institute (NCI) (1976). *Report on Carcinogenesis Bioassay of Chloroform.* Carcinogenesis Program, Division of Cancer Cause and Prevention, Bethesda, MD.

OMURA, T., AND SATO, R. (1964). The carbon monoxide-binding pigment of liver microsomes. *J. Biol Chem* **239**, 2370–2378.

PRASSE, K., HILDEBRANDT, P., DODD, D., AND GOODMAN, D., *et al.* (1986). Letter to the Editor. *Vet. Pathol.* **23**, 540–541.

RECKNAGEL, R. O. (1983). A new direction in the study of carbon tetrachloride hepatotoxicity. *Life Sci.* **33**, 401–408.

SAS Institute, Inc. (1985). *SAS User's Guide: Statistics.* Version 5 ed. SAS Institute, Inc., Cary, NC.

SEINEN, W., AND PENNINKS, A. (1979). Immune suppression as a consequence of a selective cytotoxic activity of certain organometallic compounds on thymus and thymus-dependent lymphocytes. *Ann. N.Y. Acad. Sci.* **320**, 499–517.

SEINEN, W., VOS, J. G., SPANJE, I. V., SNOEK, M., BRANDS, R., AND HOOYKAAS, H. (1977). Toxicity of organotin compounds. II. Comparative *in vivo* and *in vitro* studies with various organotin and organolead compounds in different animal species with special emphasis on lymphocyte cytotoxicity. *Toxicol. Appl. Pharmacol.* **42**, 197–212.

SHARMA, R. P. (1981). Effects of tetrachlorodibenzo-*p*-dioxin (TCDD) on immunologic systems. In *Immunological Considerations in Toxicology* (R. P. Sharma, Ed.), Vol. 1, pp. 89–102. CRC Press, Boca Raton, FL.

SIMMONS, J. E., DEMARINI, D. M., AND BERMAN, E. (1988). Lethality and hepatotoxicity of complex waste mixtures. *Environ. Res.* **46**, 74–85.

SINGH, H. B., FOWLER, D. P., AND PEYTON, T. O. (1976). Atmospheric carbon tetrachloride: Another man-made pollutant. *Science* **192**, 1231–1234.

SMIALOWICZ, R. J., ANDREWS, J. E., RIDDLE, M. M., ROGERS, R. R., LUEBKE, R. W., AND COPELAND, C. B. (1989). Evaluation of the immunotoxicity of low level PCB exposure in the rat. *Toxicology* **56**, 197–211.

SMIALOWICZ, R. J., LUEBKE, R. W., RIDDLE, M. M., ROGERS, R. R., AND ROWE, D. G. (1985). Evaluation of the immunotoxic potential of chlordecone with comparison to cyclophosphamide. *J. Toxicol. Environ. Health* **15**, 561–574.

SMIALOWICZ, R. J., RIDDLE, M. M., ROGERS, R. R., ROWE, D. G., LUEBKE, R. W., FOGELSON, L. D., AND COPELAND, C. B. (1988). Immunologic effects of perinatal exposure of rats to dioctyltin dichloride. *J. Toxicol. Environ. Health* **25**, 403–422.

Society of Toxicologic Pathologists (STP). (1986). Society of Toxicologic Pathologists' position paper on blinded slide reading. *Toxicol. Pathol.* **14**, 493–494.

SOKAL, R. R., AND ROHLF, F. J. (1981). *Biometry: The Principles and Practice of Statistics in Biological Research,* Second ed., pp. 189–190 and pp. 402–412. Freeman, New York.

STEWART, C. C., CRAMER, S. F., AND STEWARD, P. G. (1975). The response of human peripheral blood lymphocytes to phytohemagglutinin: Determination of cell numbers. *Cell. Immunol.* **16**, 237–250.

STRIKER, G. E., SMUCKLER, E. A., KOHNEN, P. W., AND NAGLE, R. B. (1968). Structural and functional changes in rat kidney during CCl_4 intoxication. *Am. J. Pathol.* **53**, 769–789.

TESCHKE, R., VIERKE, W., AND GOLDERMANN, L. (1983). Carbon tetrachloride (CCl_4) levels and serum activities of liver enzymes following acute CCl_4 intoxication. *Toxicol. Lett.* **17**, 175–180.

VOS, J. G., MOORE, J. A., AND ZINKL, J. G. (1973). Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals. *Environ. Health Perspect.* **5**, 149–162.

ZIMMERMAN, H. J. (1978). *"Hepatotoxicity: The Adverse Effects of Drugs and Other Chemicals on the Liver,"* p. 198. Appleton–Century–Crofts, New York.