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Humoral and Cell-Mediated Immune Status in Mice Exposed to Chloral Hydrate

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Chloral hydrate has been found in our drinking water supplies at levels up to 5 μg/1. The purpose of this study was to evaluate the functional status of the immune system in random-bred CD-1 mice exposed to chloral hydrate for 14 and 90 days. Male mice, following 14 or 90 days of exposure to 1/10 and 1/100 the actual oral LD_{50}, exhibited no alterations in either humoral or cell-mediated immunity. However, female mice exposed for 90 days to chloral hydrate in the drinking water demonstrated a significant depression in humoral immune function. This depression was observed when spleen cells from exposed mice were evaluated for their ability to produce antibody against sheep erythrocytes. These females did not demonstrate any changes in cell-mediated immune status.

Introduction

This study describes the effects of subchronic exposure to chloral hydrate on the immune system. There has been renewed interest in chloral hydrate for, in addition to its use as a hypnotic in man, it is utilized industrially in the production of polyurethanes. Thus it enters the drinking water supply and has been found in six of ten water supplies examined by the EPA (*1*). Additionally, trichloroethylene, a common industrial solvent, is transformed into chloral hydrate during its metabolism (*2*); this provides two potential sources for absorption of chloral hydrate via the drinking water.

Little is known about the long-term effects of subchronic oral exposure to chloral hydrate. Therefore, this immunological investigation was conducted, as well as a general toxicological study, which was conducted concurrently and is described in a companion report (*3*). The parameters evaluated here are humoral immunity, determined by measuring plasma hemagglutination titers and the number of spleen antibody producing cells of mice sensitized to sheep erythrocytes; cell-mediated immunity, measured as delayed type hypersensitivity to sheep erythrocytes; lymphocyte responsiveness to T- and B-cell mitogens; bone marrow status, determined by cell number and DNA synthesis and the functional activity of the reticuloendothelial system, as measured by the vascular clearance rate and tissue uptake of ^{51}Cr sheep erythrocytes.

Methods

Exposure of Animals

Complete details concerning housing of the randomized CD-1 mice and their exposure to chloral

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hydrate are given in the preceding paper (*3*). The actual doses of chloral hydrate consumed as time-weighted averages from the two concentrations in the drinking water are also given in the preceding paper. In the 14-day gavage study, animals were sacrificed the day after the final dose. For the drinking water study, animals were maintained on chloral hydrate until sacrificed.

Humoral Immunity

The primary IgM response to sheep erythrocytes was estimated by the hemolytic plaque assay of Jerne as modified by Cunningham and Szenberg (*4*). Mice were immunized by IP injection with 4×10^8 sheep erythrocytes (sRBC) 4 or 5 days prior to sacrifice. Spleen cell suspensions were prepared in RPMI 1640 culture medium and adjusted to a cell concentration of 5×10^5 cells/ml for assay of antibody forming cells (AFC).

As a complement to the plaque assay, the plasma antibody titer was measured by the hemagglutination technique. Mice were injected IP with 10^9 sRBC 7 days prior to sacrifice and assay (day 7 for the 14-day study; day 83 of the 90-day study). An equal volume of a 0.5% suspension of sRBC was added to each of the dilutions in a microtiter dish. 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 type hypersensitivity response to sRBC. Sensitization was done 4 days before the end of the exposure period by injecting 10^8 sRBC in a volume of 20 μl into the left hind footpad.

Four days following sensitization (the last day of exposure), the mice were challenged in the same footpad with 4×10^8 sRBC in a volume of 40 μl. Seventeen hours following the challenge, the mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (Mallinckrodt) (80,000 cpm/0.1 ml). After 2 hr, the mice were sacrificed by cervical dislocation, and both hind feet were removed and placed in tubes for gamma counting. Paranjpe and Boone (*5*) have shown that ^{125}I-albumin extravasates into the extracellular space during the delayed type hypersensitivity response. The right footpad served as an unsensitized and unchallenged control for background infiltration of ^{125}I-albumin. A group of mice challenged with sRBC to determine nonspecific swelling served as unsensitized controls. Results are expressed as a stimulation index.

Lymphocyte Responsiveness 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, Con A, which is the plant lectin concanavalin A (Sigma). For mitogenicity assays, spleens were prepared as single-cell suspensions (5×10^6 ml) and 0.1 ml was added to three replicate wells of a microtiter dish containing mitogens. LPS was used in amounts of 5, 20, and 40 μg per well; Con A was used in amounts of 1, 5 and 10 μg per well. Plates were incubated at 37°C in 10% CO_2, 95% humidity, for 48 hr. The cells were then pulsed with an ^{125}I-iododeoxyuridine (IUdR; New England Nuclear)/fluorodeoxyuridine (FUdR; Sigma) solution (0.2 μCi IUdR in 1 μM FUdR). After an 18-hr incubation the cells were collected on filter disks, using a Titertek cell harvester, and counted in a Beckman gamma counter.

Bone Marrow Assay

To assess bone marrow DNA synthesis, bone marrow cells were collected from one femur, counted, and cells per femur determined. The cell count was adjusted to 3×10^6 cells/ml, and 200 μl of cells and 20 μl of ^{125}I-IUdR/FUdR solution were added to each microtiter well. At 60, 120 and 180 min, six replicate wells were harvested per animal, and the cpm's of IUdR incorporated into the acid-precipitable fraction were determined.

Functional Ability of the Reticuloendothelial System

Freshly drawn sRBC were radiolabeled with ^{51}sodium chromate (New England Nuclear). After chromation, the sRBC were washed with Alsever's solution until the supernatant fluid was virtually radioactivity 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.

Mice used for evaluation of RES activity were weighed and placed in shoebox cages maintained at 39°C. At 0 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. At the end of 60 min, the mice were sacrificed by decapitation, drained of blood, and organs were removed for radioassay. Blood clearance is expressed as the phagocytic index, which is determined by the slope of the clearance curve. Organ distribution is expressed as percent

Table 1. Humoral immune response to sheep erythrocytes in CD-1 male mice exposed for 14 days to chloral hydrate.[a,b]

Exposure group	Body weight, g	Speen weight		AFC/10^6 spleen cells	10^5 × AFC/spleen
		mg	% body weight		
Deionized water	29.8 ± 0.7	190 ± 13	0.64 ± 0.04	2286 ± 296	5.26 ± 0.7
14.4 mg/kg	31.4 ± 0.7	197 ± 14	0.62 ± 0.04	2324 ± 234	4.98 ± 0.6
144 mg/kg	31.2 ± 0.7	181 ± 10	0.58 ± 0.03	2210 ± 255	4.62 ± 0.6

[a]Mice were immunized on day 11 of gavage, and AFC's were determined 24 hr after day 14 of gavage.
[b]Values represent the mean ± SE derived from 11-12 mice/group.

organ uptake and cpm's/mg tissue (specific activity).

Statistical Evaluation

If a one-way analysis of the variance of the means showed treatment effects, a Dunnett's T-test was performed (*6*). Values which differ from vehicle control at $p < 0.05$ are noted with an asterisk in the tables. Each of the values in the tables is given as the mean ± standard error (SE) of the mean.

Results

Fourteen-Day Exposure to Chloral Hydrate

Chloral hydrate was administered to male CD-1 mice daily for 14 days by stomach tube at doses of 14.4 and 144 mg/kg (1/100 and 1/10 the LD_{50}). There were no significant changes ($p < 0.05$) in spleen weight, spleen antibody-forming cells, or delayed type hypersensitivity response to sRBC (Tables 1 and 2).

Ninety-Day Exposure to Chloral Hydrate

Male and female CD-1 mice received chloral hydrate in their drinking water for 90 days. Based on fluid consumption, chemical concentration, and body weight, time-weighted averages of chloral hydrate consumed were 16 and 160 mg/kg for males and 18 and 173 mg/kg for females (*3*).

Humoral and Cell-Mediated Immunity

The number of splenic antibody forming cells produced against sRBC in mice exposed to chloral hydrate for 90 days is summarized in Table 3. The peak day of response was day 4 after immunization for both sexes. On day 4, female mice produced 36 and 40% fewer AFC/spleen compared to control, at dose levels of 0.07 and 0.7 mg/ml, respectively. When expressed as AFC/10^6 spleen cells, the only significant decrease occurred at the high dose level of 0.7 mg/ml (173 mg/kg), where there was a 32% suppression. No changes were observed in male mice.

Serum hemagglutination titers were also measured. As seen in Table 3, no changes from control antibody titers were detected.

Delayed type hypersensitivity to sRBC was measured as an indicator of cell-mediated immunity (Table 2). Male mice exposed to the high dose level of 0.7 mg/ml demonstrated a 74% increase in footpad swelling compared to vehicle control, as measured by ^{125}I-albumin extravasation. On the other hand,

Table 2. Cell mediated immune response to sheep erythrocytes in CD-1 mice exposed to chloral hydrate in the drinking water for 14 days or 3 months.

Exposure time	Exposure group	Stimulation index	
		Males	Females
14 days[a]	Deionized Water	4.47 ± 0.50	–
	14.4 mg/kg	3.66 ± 0.48	–
	144 mg/kg	3.27 ± 0.49	–
3 months[b]	Deionized water	3.14 ± 0.45	4.57 ± 0.31
	0.07 mg/ml	2.77 ± 0.52	3.27 ± 0.35[c]
	0.7 mg/ml	5.48 ± 0.51	4.00 ± 0.42

[a]Mice were sensitized on day 10 of gavage, challenged on day 14, and measured for DTH on day 15. Values represent the mean ± SE derived from 12 mice/group.
[b]Values represent the mean ± SE derived from 17-20 mice in the deionized water group and 15-16 mice in the other groups.
[c]Significantly different from control at $p < 0.05$ as determined by Dunnett's T-test.

Table 3. Peak day humoral immune response to sheep eythrocytes and hemagglutination liters in CD-1 mice exposed to chloral hydrate in the drinking water for 3 months.

Sex	Exposure group	Humoral immune response[a]		Hemagglutination titers ($\log_2$ titer)[b]
		10^5 × AFC/spleen	AFC/10^6 cells	
M	Deionized water	4.31 ± 0.52	2273 ± 132	9.42 ± 0.10
M	0.07 mg/ml	3.01 ± 0.55	2042 ± 125	9.71 ± 0.14
M	0.7 mg/ml	2.98 ± 0.28	1984 ± 167	9.70 ± 0.16
F	Deionized water	4.09 ± 0.30	2089 ± 82	9.37 ± 0.19
F	0.07 mg/ml	2.61 ± 0.39[c]	1695 ± 159	9.40 ± 0.31
F	0.7 mg/ml	2.47 ± 0.51[c]	1411 ± 199[c]	9.17 ± 0.19

[a]Values represent the mean ± SE derived from 12 mice in the deionized water group and 8 mice in the other groups.
[b]Values represent the mean ± SE derived from 20-21 mice in the deionized water group and 13-16 mice in the other groups.
[c]Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

female mice exposed to the low dose level (0.07 mg/ml or 18 mg/kg) demonstrated a 28% inhibition in response.

Table 4 shows the responsiveness of spleen lymphocytes to the optimal concentrations of a T-cell mitogen (Con A) and a B cell mitogen (LPS). There were no exposure-related changes in lymphocyte response. Baseline response in the absence of mitogen is also shown in Table 4.

The functional activity of the reticuloendothelial system was not altered in male or female mice (data not shown). Even though there was a significant increase in kidney uptake of ^{51}Cr-labeled sRBC in female mice at the low dose, it was not considered biologically significant.

The number of bone marrow cells/femur was unchanged. There was a slight increase in bone marrow DNA synthesis in males as measured by incorporation of ^{125}I-IUdR (Table 5). This increase occurred at both exposure levels. It should be noted that DNA synthesis was higher in the control females, as compared to the males.

Table 4. Lymphocyte responsiveness in the absence and presence of mitogen using spleens from CD-1 mice exposed to chloral hydrate in the drinking water for 3 months.

Sex	Exposure group	No mitogen[a]	Con A (5 μg)[b]	LPS (20 μg)[b]
M	Deionized water	7419 ± 1294	258960 ± 6387	91291 ± 8977
M	0.07 mg/ml	7391 ± 731	253154 ± 8876	105541 ± 7851
M	0.7 mg/ml	8305 ± 1057	277594 ± 4954	88723 ± 8996
F	Deionized water	10697 ± 1439	260582 ± 9652	140246 ± 4903
F	0.07 mg/ml	7742 ± 1264	274527 ± 5593	138401 ± 4616
F	0.7 mg/ml	7569 ± 934	275839 ± 6699	129907 ± 6538

[a]Values represent the mean cpm's ± SE derived from 17-22 mice in the deionized water group and 13-16 mice in the other groups.
[b]The optimum concentration of concanavalin A (Con A) and lipopolysaccharide (LPS) were employed. Values represent the mean cpm's ± SE derived from 17-22 mice in the deionized water group and 13-16 mice in the other groups.

Table 5. Bone marrow synthesis in CD-1 mice exposed to chloral hydrate in the drinking water for 3 months.[a]

Exposure group	Males				Females			
	10^7 × cells/femur	60 min.	120 min.	180 min.	10^7 × cells/femur	60 min.	120 min.	180 min.
Deionized water	2.46 ± 0.17	2838 ± 246	5096 ± 385	6379 ± 429	1.99 ± 0.37	4475 ± 269	7084 ± 434	8523 ± 360
0.07 mg/ml	2.36 ± 0.17	3692 ± 190[b]	6496 ± 409[b]	8142 ± 429	1.58 ± 0.11	4289 ± 259	7378 ± 418	9164 ± 502
0.7 mg/ml	2.42 ± 0.20	4632 ± 283[b]	7526 ± 334[b]	8571 ± 403[b]	1.76 ± 0.17	4056 ± 294	6158 ± 274	8269 ± 393

[a]Values represent the mean ± SE derived from 21 mice in the deionized water group and 13-16 mice in the other groups.
[b]Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

Discussion

The previous report on chloral hydrate described the general toxicological effects of acute and subchronic exposure in random-bred CD-1 mice (*3*). Following exposure for 14 or 90 days, gross pathology, organ weights, clinical chemistries, hematology, urinalysis, and the effects on the hepatic microsomal enzymes were assessed. The most notable effects were in the livers of the male mice, where a dose-dependent hepatomegaly and an elevation in SGOT and LDH were seen. There was also increased hepatic microsomal aminopyrine *N*-demethylase and aniline hydroxylase activity, without an increase in P-450 content. In this report we describe the effects of chloral hydrate on the immune system when mice were exposed at the same time and under the same conditions as in the general toxicological studies.

The amount of chloral hydrate administered in the 90-day drinking water study was high in comparison to levels existent in the drinking water supplies surveyed by the EPA; that survey disclosed levels of chloral hydrate ranging from 2 to 5 µl/l. For the 90-day study, the highest dose administered to mice was 173 mg/kg. An equivalent human dose (based on body surface area) would be 14 mg/kg, or less than one gram. On a day-to-day basis, this could be considered a potentially harmful dose, especially in those individuals who are already at immunological risk.

The immune system in male mice showed no exposure-related depression or stimulation following 14 or 90 days of exposure. Humoral immune status, as assessed by measurement of the production of AFC, hemagglutination titers, and spleen cell response to LPS, was unchanged. Cell-mediated immunity, evaluated by a delayed type hypersensitivity response to sRBC, was enhanced in male mice exposed to the high concentration of chloral hydrate (160 mg/kg). The reason for this responsiveness may be related to the control stimulation index of 3.14 (Table 2), which was low in comparison to the mean for our accumulated historical control values for this strain of mouse. Spleen cell responsiveness to the T-cell mitogen concanavalin A was not altered, nor was there any effect on the functional activity of the reticuloendothelial system.

The immune status of female mice following 90 days of exposure differed from the males. The major difference was that humoral immunity, assessed by the number of AFC produced against sRBC, was depressed on the peak day of response (day 4). However, two other indicators of humoral immune status, hemagglutination titers and spleen cell response to the B-cell mitogen LPS, were not changed. The AFC response is the most sensitive indicator of humoral immunity because of the complex cellular cooperations required and because the number of cells that produce antibody can be quantitated. Although cell-mediated immunity was suppressed in mice exposed to the low dose of chloral hydrate, we do not believe this was compound related because the suppression was slight and it was not dose related. The effect on humoral immune status, concurrent with little alteration in the status of cell-mediated immunity, was consistent with results of other studies we have conducted upon water contaminants such as trichloroethylene and various chlorinated hydrocarbons (*7*). Why these compounds affect primarily the humoral immune functions is not clear.

From this study, it appears that the immune system, particularly the ability to produce IgM antibody to a T-dependent antigen in female mice, is the most sensitive indicator of the toxicity of chloral hydrate. The previous paper, which dealt with the toxicology of chloral hydrate, indicated that the liver is the most sensitive organ in male mice. The lowest concentration used, i.e., 0.07 mg/ml or about 16 mg/kg, was effective in eliciting both these responses.

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REFERENCES

1. U.S. E.P.A. Preliminary assessment of suspected carcinogens in drinking water. Report to Congress, Washington, D.C., 1975.
2. Waters, E. M., Gerstner, H. B., and Huff, H. E. Trichlorethylene. 1. An overview. J. Toxicol. Environ. Health 2: 671-707 (1977).
3. Sanders, V. M., Kauffmann, B. M., White, K. L., Jr., Douglas, K. A., Barnes, D. W., Sain, L. E., Bradshaw, T. J., Borzelleca, J. F., and Munson, A. E. Toxicology of chloral hydrate in the mouse. Environ. Health Perspect. 44: 137-146 (1982).
4. Cunningham, A. G. and Szenberg, A. Further improvements in the plaquing techniques for detecting single antibody forming cells. Immunology 14: 599-600 (1968).
5. Paranjpe, M. S., and Boone, C. W. Delayed hypersensitivity to Simian virus 40 tumor cells in Balb/c mice demonstrated by a radioisotopic footpad assay. J. Natl. Cancer Inst. 48: 563-566 (1972).
6. Dunnett, C. W. New tables for multiple comparisons with a control. Biometrics 20: 482-491 (1964).
7. Sanders, V. M., Tucker, A. M., White, K. L., Jr., Kauffmann, B. M., Hallett, P., Carchman, R. A., Borzelleca, J. F., and Munson, A. E. Humoral and cell-mediated immune status in mice exposed to trichloroethylene. Toxicol. Appl. Pharmacol, in press.