Enhancement of the Hepatotoxicity of Chloroform in B6C3F1 Mice by Corn Oil: Implications for Chloroform Carcinogenesis

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A recent study of the ability of chloroform in drinking water to produce cancer reported that male Osborne-Mendel rats developed renal tumors, but that female B6C3F1 mice failed to develop hepatocellular carcinomas. The results obtained in the male Osborne-Mendel rats were comparable to those observed in an earlier study sponsored by the National Cancer Institute (NCI). On the other hand, the lack of an increased incidence of hepatocellular carcinomas in female B6C3F1 mice was in sharp contrast to previously reported results. The doses of chloroform used were comparable to that which produced an 85% incidence in the NCI study.

We have investigated the extent to which the vehicle might be responsible for the different results in these two studies by examining the differential effects of chloroform when it was administered by gavage using corn oil versus a 2% Emulphor suspension as the vehicle. Male and female B6C3F1 mice were administered chloroform at 60, 130, and 270 mg/kg per day for 90 days. At sacrifice, body and organ weights were measured, and blood was recovered to perform the following serum chemistry measurements (in order of priority): glutamate oxaloacetate transaminase (SGOT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and triglyceride (TG) levels. The liver was sectioned for histopathological examination. Chloroform increased SGOT levels significantly only when administered in corn oil at a dose of 270 mg/kg in both male and female mice. It had no effect on LDH activity. There was a small increase in BUN when chloroform was administered in corn oil, but not when administered in 2% Emulphor. When administered in corn oil, chloroform significantly decreased serum TG levels but was without effect on this parameter when administered in 2% Emulphor. Chloroform decreased body weight and increased liver weight with both vehicles, but the effects were significantly greater when it was administered in corn oil. Mice administered chloroform in corn oil displayed a significant degree of diffuse parenchymal degeneration (5 of 10 males and 1 of 10 females) and mild to moderate early cirrhosis (5 of 10 males and 9 of 10 females); significant pathological lesions were not observed in the animals administered corn oil without chloroform nor in mice receiving chloroform in 2% Emulphor.

These data indicate that administration of chloroform by corn oil gavage results in more marked hepatotoxic effects than observed when it is provided in an aqueous suspension. A major difference between two recent carcinogenesis bioassays of chloroform in this same mouse strain was the vehicle used; corn oil in the study that yielded an increased incidence of hepatocellular carcinoma (2) and drinking water in the negative bioassay reported by Jorgenson et al.

Introduction

Chloroform is one of a group of chemicals that is formed during the chlorination of drinking water (1,2) and from the treatment of experimental animals with aqueous solutions of chloride by oral gavage (3,4). These observations gave rise to considerable public concern when it was shown that chloroform was capable of increasing the incidence of renal tumors in male Osborne-Mendel rats and liver tumors in B6C3F1 mice (5).

From the time they were first observed, chloroform-induced liver tumors were associated with overt necrosis of the organ (6). This observation has led a number of people to suggest that tumors arising from chloroform treatments are secondary to tissue necrosis and repeated reparative hyperplasia (7). Adding to this argument has been the fact that chloroform and/or its metabolites do not interact extensively with DNA in vivo (7–10). These observations have been generally supported by the absence of genotoxic activity of chloroform in bacterial systems (11–14). Chloroform also
failed to induce chromosomal damage or sister-chromatid exchange in human lymphocytes treated in vitro (13) or mutations at the 8-azoguanine locus in Chinese hamster lung fibroblast cells (15). On the other hand, chloroform has been shown to induce mutation in yeast (16) and to enhance viral transformation of Syrian hamster ovary cells in vitro (17) and reported by another group to induce sister-chromatid exchange in human lymphocytes in vitro and mouse bone marrow cells in vivo (18). In vivo treatments with chloroform have been reported to increase mutagenicity in the urine of mice and to induce polychromatic erythrocytes (19). Chloroform treatment was reported to increase spermid abnormalities in mice in one study (20) and to be without such effects in another (21). In general, to the extent that genotoxic activities have been associated with chloroform, that activity has been weak. Nevertheless, it is difficult to rule out a role for such activity in the carcinogenic responses to chloroform without positive evidence for alternative mechanisms.

The present work was part of a large-scale followup of the original National Cancer Institute (NCI) bioassay of chloroform (5). Tumor pathology from this study has been previously reported (22) and is only reviewed in the present paper. In this paper we report selected clinical chemistry data obtained in interim sacrifices of rats subjected to treatments that paralleled those in the carcinogenesis study and the results of shorter-term (90-day) studies in mice that were designed to determine the extent to which vehicles used in carcinogenesis bioassays of chloroform influence the hepatic responses to this agent.

**Experimental Methods**

**Treatment of Animals**

The treatment of male Osborne-Mendel rats in the carcinogenicity bioassay was previously described by Jorgenson et al. (22). In short, 960 rats were distributed among six treatment groups that received 0, 200, 400, 900, or 1800 mg of chloroform per liter of drinking water. The sixth group received no chloroform but was matched to the high-dose group for drinking water consumption. The groups contained 330, 330, 150, 50, 50, and 50 animals, respectively, at the start of the experiment. Toxicity information presented in this paper was gathered from separate groups of animals that received parallel treatments. In this case, 10 to 20 animals were treated per dose, per sacrifice time.

Female B6C3F1 mice used in the carcinogenicity study were assigned in much the same manner (22). In this case, the groups receiving the above identified concentrations of chloroform in their drinking water had 430, 430, 150, 50, 50, and 50 animals assigned to the respective groups.

The study of the influence of vehicle on the hepatocellular adenoma and adenocarcinoma in male Osborne-Mendel rats treated with chloroform.

| Dose, mg/kg | Animals with tumors, % | Dose, mg/kg | Animals with tumors, % |
|------------|------------------------|------------|------------------------|
| 0          | 20                     | 0          | 415                    |
| Matched 0  | 47                     | Matched 0  | 47                     |
|            | 0                      | 34         | 410                    |
|            | 0                      | 65         | 142                    |
| 238        | 45                     | 130        | 47                     |
| 477        | 41                     | 263        | 44                     |

*Dosage administered in corn oil, by gavage, 5 days/week for 78 weeks.

**Table 2. Hepatocellular adenoma and carcinomas in female B6C3F1 mice treated with chloroform.**

| Dose, mg/kg | Animals with tumors, % | Dose, mg/kg | Animals with tumors, % |
|------------|------------------------|------------|------------------------|
| 0          | 45                     | 263        | 44                     |

*Dosage administered in corn oil, by gavage, 5 days/week for 78 weeks.

**Figure 1.** Serum triglyceride levels in male Osborne-Mendel rats at different periods of exposure to chloroform in their drinking water at the indicated concentrations. Each experimental group contained 10 or 20 animals at each time of sacrifice.
Table 3. Effects of vehicle on general parameters of the subchronic toxicity of chloroform in B6C3F1 mice.*

| Parameter                        | Chloroform dose, mg/kg per day | Male | Female |
|----------------------------------|---------------------------------|------|--------|
|                                  | 2% Emulphor                     | Corn oil | 2% Emulphor | Corn oil |
| Final body weight, g ± SEM       | 33.6 ± 0.5                      | 35.5 ± 0.4d | 26.5 ± 0.6 | 25.4 ± 0.4 |
|                                  | 60                              | 31.0 ± 0.8 | 31.7 ± 0.4 | 25.4 ± 0.6 | 25.5 ± 0.7 |
|                                  | 130                             | 31.6 ± 0.8 | 30.6 ± 0.5 | 26.4 ± 0.6 | 26.0 ± 0.3 |
|                                  | 270                             | 29.3 ± 0.6d | 26.6 ± 0.5d | 25.4 ± 0.3 | 24.0 ± 0.7 |
| Liver weight, g ± SEM            | 1.29 ± 0.03                      | 1.17 ± 0.03d | 0.98 ± 0.03 | 1.00 ± 0.04 |
|                                  | 60                              | 1.18 ± 0.03 | 1.32 ± 0.03d | 1.09 ± 0.04d | 1.19 ± 0.05d |
|                                  | 130                             | 1.33 ± 0.05 | 1.36 ± 0.03d | 1.13 ± 0.04d | 1.22 ± 0.03d |
|                                  | 270                             | 1.36 ± 0.03 | 1.50 ± 0.02d | 1.22 ± 0.03d | 1.39 ± 0.03d |
| Liver/body weight ratio, g/100 g ± SEM | 4.02 ± 0.49                      | 3.58 ± 0.10d | 4.32 ± 0.10 | 4.38 ± 0.15 |
|                                  | 60                              | 4.28 ± 0.06 | 4.67 ± 0.06d | 4.84 ± 0.10d | 5.25 ± 0.16d |
|                                  | 130                             | 4.84 ± 0.11d | 5.09 ± 0.09d | 4.94 ± 0.12d | 5.47 ± 0.08d |
|                                  | 270                             | 5.34 ± 0.07d | 6.64 ± 0.09d | 5.58 ± 0.12d | 6.86 ± 0.16d |
| Liver/brain weight ratio, g/g ± SEM | 2.31 ± 0.30                      | 2.33 ± 0.05d | 1.90 ± 0.05 | 1.96 ± 0.07 |
|                                  | 60                              | 2.47 ± 0.04 | 2.72 ± 0.07d | 2.14 ± 0.08d | 2.31 ± 0.11d |
|                                  | 130                             | 2.72 ± 0.10 | 2.74 ± 0.05d | 2.15 ± 0.08d | 2.39 ± 0.08b,d |
|                                  | 270                             | 2.87 ± 0.06d | 3.10 ± 0.07d | 2.42 ± 0.05d | 2.83 ± 0.05d |

*For each sex and dose level, significant differences between Emulphor and corn oil groups of p ≤ 0.05 or p ≤ 0.01, respectively, based on Student’s t-test. Number of animals per group = 9 or 10.

*b p ≤ 0.05.

*c p ≤ 0.01.

*d Significantly different from corresponding control of p ≤ 0.05 by one-way analysis of variance and pairwise Student’s t-test.

Table 4. Comparison of clinical chemistry parameters of mice treated with chloroform in corn oil vs. Emulphor for 90 days.

| Parameter          | Chloroform dose, mg/kg/day | 2% Emulphor | Corn oil | 2% Emulphor | Corn oil |
|--------------------|-----------------------------|-------------|----------|-------------|----------|
| SGOT, mU/mL        | Control                     | 284 ± 53    | 181 ± 36 | 151 ± 27    | 193 ± 22 |
|                    | (7)                          | (10)        | (9)      | (8)         |          |
|                    | 60                           | 142 ± 32ab  | 158 ± 31 | 113 ± 26    | 126 ± 16 |
|                    | (4)                          | (9)         | (7)      | (8)         |          |
|                    | 130                          | 176 ± 43    | 116 ± 14 | 169 ± 25    | 127 ± 14 |
|                    | (7)                          | (8)         | (8)      | (9)         |          |
|                    | 270                          | 167 ± 31    | 298 ± 31bd | 117 ± 17    | 335 ± 60bd |
|                    | (6)                          | (8)         | (7)      | (7)         |          |
| Triglycerides, mg-%| Control                     | 58 ± 8      | 80 ± 10  | 63 ± 10     | 64 ± 5   |
|                    | (9)                          | (10)        | (10)     | (10)        |          |
|                    | 60                           | 75 ± 12     | 75 ± 4   | 62 ± 9      | 65 ± 5   |
|                    | (10)                         | (9)         | (10)     | (10)        |          |
|                    | 130                          | 54 ± 6      | 60 ± 5b  | 50 ± 4      | 55 ± 5   |
|                    | (9)                          | (10)        | (10)     | (9)         |          |
|                    | 270                          | 60 ± 4      | 43 ± 3bd | 55 ± 3      | 41 ± 3bd |
|                    | (10)                         | (10)        | (10)     | (10)        |          |

*Mean ± SEM, number of animals in parentheses.

b Significantly different from corresponding control at p ≤ 0.05.

c For each sex and dose level, significant differences of p ≤ 0.05 between Emulphor and corn oil groups were based on Student’s t-test.

d Significant differences of p ≤ 0.01, as in footnote c.
Table 5. Effect of vehicle on chloroform-induced accumulation of lipid in the liver of B6C3F1 mice.

| Chloroform dose, mg/kg/day | Male 2% Emulphor | Male 2% Corn | Female 2% Emulphor | Female 2% Corn |
|---------------------------|------------------|--------------|---------------------|---------------|
| 0                         | 6.6 ± 1.1a       | 7.9 ± 0.7    | 6.8 ± 0.4           | 7.5 ± 0.8     |
| 60                        | 6.8 ± 0.6        | 9.4 ± 0.8    | 7.5 ± 0.3           | 13.1 ± 1.1b   |
| 130                       | 7.6 ± 0.7        | 7.8 ± 0.4    | 6.9 ± 0.3           | 9.2 ± 0.8     |
| 270                       | 5.3 ± 0.6        | 8.0 ± 0.3    | 6.0 ± 0.4           | 8.4 ± 0.4     |

*a* Average lipid content in % net weight ± SD. Nine or ten animals per group.

**Histopathology**

The brain, liver, spleen, lungs, thymus, kidneys, heart, and gonads of each mouse were weighed at necropsy. Four histological slides were prepared from freshly CO₂-frozen liver sections of each animal (two each from nonadjacent sections of the central lobe). One slide from each section was stained with Oil Red O and the other with hematoxylin and eosin (H&E). The left lobe of the liver was processed for quantitative determination of lipid content by organic solvent partitioning, solvent evaporation, and residue weight determinations.

**Statistical Analyses**

The effects of chloroform on the corresponding vehicle control were compared by using a one-way analysis of
variance (ANOVA). Treatment groups were compared with control groups using pairwise Student's t-tests if the ANOVA proved to be significant at \( p < 0.05 \). The effects of the same dose of chloroform administered in different vehicles were compared using Student's t-test.

Results

Table 1 compares the yield of renal tubular-cell adenomas and adenocarcinomas obtained in the NCI bioassay of chloroform (5) with the more recent results reported by Jorgenson et al. (22). Because the dosage indicated was administered 7 days/week for 2 years in the drinking water in the latter study and by gavage for only 5 days/week for 78 weeks in the NCI study, the total dose of chloroform administered in the NCI study is actually somewhat lower relative to the Jorgenson study than would be surmised from the table. Nevertheless, the results of the two studies were in substantial agreement despite the differences in the mode of chloroform administration.

A substantially different picture emerges when the yield of hepatocellular carcinomas in the female B6C3F1 mice is compared between these two studies presented in Table 2. Projecting from the response observed in the NCI study, a greater than 80% incidence of hepatocellular carcinoma should have been observed in the Jorgenson et al. (22) study at the highest dose administered (283 mg/kg per day). Instead, a 2% incidence was observed relative to the 5% incidence seen in the control group, and a 0% incidence in the smaller control group that was matched to the high-dose group for water consumption was observed.

As noted previously (23), few indications of toxicity were observed in rats exposed to chloroform in their drinking water. The major clinical chemistry finding was that serum triglyceride levels were depressed by chloroform treatment (Fig. 1). This finding was partially, but not completely, explained by decreased water consumption (that is assumed to be attributed to an associated decrease in food consumption). It is difficult to judge precisely the degree to which intermediate doses of chloroform add to the serum triglyceride depression produced by decreased water consumption. In the high-dose group, this depression was 32% to 80% relative to the group matched to it for water consumption throughout the first 18 months of the study. In fact, chloroform at the high dose appears to prevent almost completely an age-related increase in serum triglycerides in the rat. A definite trend in the data indicates that this effect is seen to a somewhat lesser extent at concentrations of chloroform as low as 400 mg/L. Comparisons at the 24-month time point were not dependable because of the relatively poor survival encountered in certain groups.

Renal pathology was evident in rats from all test groups. However, there was no clear indication that renal pathology was either more frequent or more se-
vere in animals that received the high dose of chloroform relative to the appropriate control groups.

Some interactions occurred between the vehicle used, food consumed, and body weight in the experiments designed to determine the impact of the vehicle on the hepatotoxicity of chloroform in B6C3F1 mice (data not shown). Corn oil gavage substantially reduced food consumption by about 25% in male mice and 15% in female mice. In both sexes, food consumption was significantly increased by chloroform in a dose-related manner when corn oil was administered as the vehicle. This phenomenon was not observed in the experiment using 2% Emulphor as the vehicle.

Table 3 describes the effects of chloroform on liver weight, liver:body weight, and liver:brain weight ratios when it was administered in corn oil compared to those associated with the administration of chloroform in the 2% Emulphor vehicle. Liver weight in male mice was significantly depressed by corn oil despite the fact that it significantly increased body weight. As chloroform doses were increased, however, liver weights were significantly increased when corn oil was used as the vehicle, but not when 2% Emulphor was used as the vehicle. Chloroform in corn oil depressed body weight in male mice to a much greater extent than when administered in equivalent doses in Emulphor. On the other hand, it had little effect on body weight when administered to female mice in either vehicle. Liver weight was increased with chloroform treatment using both vehicles in female mice, but to a significantly greater degree when administered in corn oil. Normalization of the differential effects of chloroform on body weight by expressing the data as liver:body weight and liver:brain weight ratios provided substantially greater increases in these ratios when corn oil was used as the vehicle relative to the use of Emulphor. Consequently, chloroform-induced increases in liver size were quite evident in both sexes.

Lactic dehydrogenase (LDH) in serum provided no evidence of organ damage in B6C3F1 mice with either vehicle. However, serum glutamic oxalacetic transaminases (SGOT) increased in a dose-related manner when chloroform was administered to mice in a corn oil vehicle (Table 4). This effect was also observed in both sexes. No such trend was observed when chloroform was administered in the same doses in the 2% Emulphor vehicle. In the same manner, serum triglycerides of mice became depressed in mice administered chloroform in corn oil, but not when Emulphor was used as the vehicle.

The lowest dose of chloroform used (60 mg/kg) increased the levels of lipid in the liver of both male and female mice when administered in corn oil (Table 5). At higher doses, the liver lipid levels decreased in concert with the depression of serum triglycerides. Lipid accumulation in liver was not evident when chloroform was administered in 2% Emulphor.

Figures 2 through 7 are typical photomicrographs of

**FIGURE 4.** Photomicrograph of frozen liver section from a B6C3F1 mouse given 60 mg/kg chloroform in corn oil for 90 days. Note extensive vacuolation and dark stain product indicating the presence of fat. Compare with Fig. 4. Oil Red O. Original magnification ×63.
liver sections taken from B6C3F1 mice that were treated with corn oil (controls) or with chloroform dissolved in corn oil. H&E-stained liver sections from animals receiving corn oil alone appeared normal with hepatocytes of uniform size and shape (Fig. 2). Animals that were administered 60 mg/kg chloroform in corn oil for 90 days displayed extensive vacuolation with H&E stain (Fig. 3), and frozen sections provided evidence of lipid when stained with Oil Red O (Fig. 4), whereas frozen sections from control animals did not (Fig. 5). The accumulation of lipid in the dose group was quite marked in three of nine male and three of ten female animals, a degree of severity not observed in the control group (corn oil only) nor in animals treated with higher doses of chloroform. Animals that had received 270 mg/kg of chloroform per day in corn oil displayed an extensive disruption of the normal hepatic architecture that was accompanied by infiltration of inflammatory and spindle cells (Fig. 6). The hepatocytes assumed bizarre shapes, were often substantially enlarged in size, but were only mildly vacuolated in animals subjected to this dose of chloroform. These enlarged hepatocytes were also observed in frozen sections (Fig. 7), but staining with Oil Red O was substantially reduced in the high-dose group relative to that observed in the groups treated with 60 mg/kg of chloroform in corn oil. The pathologist judged that five of ten male mice and seven of ten female mice displayed evidence of mild to moderate early cirrhosis at this high dose.

No such pathology was noted in animals administered these same doses of chloroform in 2% Emulphor. In this case, pathology was limited to minimal focal necrosis in two of nine male mice and two of ten female mice at 130 mg/kg and in two of ten female animals at 270 mg/kg. One male mouse of ten at 270 mg/kg chloroform in Emulphor displayed mild focal necrosis in the liver. In addition, no significant trends in the lipid content of the liver were apparent in these animals when frozen sections were observed after staining with Oil Red O (data not shown).

Discussion

It is clear from the present results that the hepatotoxicity of chloroform is strongly dependent on the means of administration in B6C3F1 mice. The substantially different results obtained in the NCI (5) and the Jorgenson et al. (22) studies of chloroform-induced liver tumors in this same strain of mice may have also resulted from the dissimilarity in the means by which chloroform was administered. No such dependency on the means of chloroform administration was observed in the production of renal tumors in male Osborne-Mendel rats.

One effect of chloroform that appears to be common to both species is a substantial decrease in serum triglycerides concentrations. In the chronic studies where chloroform was administered in drinking water, a sig-
significant dose-related increase in liver fat occurred in B6C3F1 mice (23). A small, but significant, increase in liver lipid was observed only at the highest doses in male Osborne-Mendel rats. Despite these qualitative similarities, the two species appear to be divergent in sensitivity to chloroform-induced hepatotoxicity as well as to chloroform-induced hepatomas.

Accumulation of lipid in the liver of B6C3F1 mice was not as apparent in the present study when chloroform was administered in Emulphor. Although lipid accumulation was apparent in mice treated with 60 mg/kg chloroform in corn oil, the effect was not observed at higher doses. The biphasic nature of this dose-response relationship appeared to be related to the formation of atypical, enlarged hepatocytes and to the development of scarring typical of the initial stages of cirrhosis of the liver. Decreased levels of serum triglycerides may also have contributed to a lesser accumulation of lipid in the liver at higher doses of chloroform. However, the reasons for the differences in lipid accumulation in the liver when Emulphor was used as a vehicle compared to that associated with drinking water exposures are not readily apparent.

In the present study, the hepatotoxic effects of chloroform administered in two different vehicles in B6C3F1 mice were compared directly. A greater degree of hepatotoxicity was observed, using gross measurements of liver weight, clinical chemistry, and direct histopathological examination when corn oil was used as the vehicle. All three lines of evidence indicated that chloroform administered in corn oil is more hepatotoxic to the liver of B6C3F1 mice than when administered in a vehicle containing little or no unsaturated lipids. Withey et al. (24) demonstrated that the use of a corn oil vehicle actually slows the rate of chloroform absorption from the gastrointestinal tract. Consequently, it is unlikely that the difference in response can be explained on simple pharmacokinetic grounds.

The question of whether the carcinogenic responses to chloroform in the mouse liver were secondary to the hepatotoxic effects of this agent dates to the early observations of Eschenbrenner and Miller (6). These authors found that administration of chloroform to strain A mice induced hepatomas only at doses that produced frank liver necrosis. The supposition that chloroform induces liver tumors secondary to such tissue damage has been indirectly supported by the lack of a clear indication that it is capable of interacting with DNA (7-10). On the other hand, Reitz et al. (8) found that chloroform does increase the incorporation of $[^3]H$thymidine into liver DNA at doses of 60 mg/kg and above (unfortunately these authors did not specify the vehicle in which chloroform was administered). In a study that more directly addresses the influence of vehicle, Moore et al. (25) found that the incorporation of $[^3]H$thymidine into liver and kidney of CFLP outbred Swiss albino mice
was considerably enhanced when chloroform was administered in a corn oil vehicle compared to when it was administered using a toothpaste base as the vehicle. These data suggest that chloroform induced a regenerative hyperplasia that could stimulate the proliferation of spontaneously initiated neoplastic cells.

Newberne et al. (26) found that incorporation of corn oil into the diet increases the yield of aflatoxin B₁-induced tumors in rats. This latter observation would suggest that corn oil itself might promote the growth of initiated cells. However, the tumor yield observed in the control animals that received water as the vehicle in the Jorgenson et al. (22) study were quite consistent with the yields observed in untreated, historical controls (27) in this same strain of mice. Consequently, one is left with the hypothesis that the difference in carcinogenic response to chloroform observed with different vehicles may be attributable to interactions between the vehicle and chloroform. The present study has not provided direct evidence for such an effect. However, if the carcinogenic response in the mouse liver is secondary to the hepatotoxic effects of chloroform, such an interaction might well explain the divergent results obtained in two bioassays of this chemical.

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