Mechanistic Considerations for Carcinogenic Risk Estimation: Chloroform
by R. H. Reitz,* T. R. Fox* and J. F. Quast*

Chloroform has been reported to induce cancer in rodents after chronic administration of high doses by gavage. However, the interpretation of these findings is hampered by a lack of knowledge concerning the relative roles of genetic and nongenetic mechanisms in these bioassays. The present studies were carried out in male B6C3F1 mice in order to investigate the potential of chloroform to induce genetic damage and/or organ toxicity at the sites where tumors have been observed in the various bioassays. These studies revealed that carcinogenic doses of chloroform produced severe necrosis at the sites where tumors later developed. This was demonstrated by light microscopy as well as by determination of the cellular regeneration index following administration of 3H-thymidine. Noncarcinogenic doses of chloroform failed to induce these responses. In contrast, studies of DNA alkylation and DNA repair in vivo failed to give any indication that chloroform had produced the type of genetic alterations associated with known genotoxic chemicals. These data suggest that the primary mechanism of chloroform-induced carcinogenesis is nongenetic in nature. If the same mechanism predominates in man, there should be little to no carcinogenic risk associated with exposure to noncytotoxic levels of chloroform.

Introduction

Some chemical carcinogens apparently act through direct alteration of DNA (by the induction of somatic mutations). This has led to the speculation that a single molecular event might influence the rate of cancer formation. Such a concept does not allow for the existence of an absolute threshold in carcinogens acting by this mechanism. However, considerations such as the multistage nature of chemical carcinogenesis, the existence of DNA repair systems and immune surveillance mechanisms, and the observation of threshold doses for other pathological responses support a possible threshold for at least some carcinogenic agents (1). There has been, and continues to be, considerable debate on the subject of thresholds in carcinogenesis.

Consequently, there has been some concern whenever large numbers of people are exposed to a material which has been shown to be an animal carcinogen, even if the levels of exposure are very much lower than those employed for the animal test. Chloroform is one example of such a chemical. It was tested for possible carcinogenicity following chronic administration by gavage (2) and found to induce liver tumors in B6C3F1 mice, as well as kidney and thyroid tumors in Osborne-Mendel rats.

Human exposures to chloroform are very much lower than those employed in the gavage bioassay. The primary source of exposure is to small amounts of trihalomethanes (including chloroform) formed during the chlorination of drinking water supplies. The Environmental Protection Agency has recently established a maximum contaminant level (MCL) for chloroform in finished drinking water of 100 ppb (0.1 mg.). The level chosen by EPA is based on a risk estimation which suggests that the excess cancer risk in populations exposed to this level of chloroform may be as high as 100 cases per million exposed population (3,4).

The risk estimation cited by the EPA is based upon the techniques outlined by the National Academy of Sciences (NAS) (5) in their book, “Drinking Water and Health.” However, there are at least two reasons why these techniques may not be appropriate for risk extrapolations with chloroform: (1) The NAS indicated that they felt it was “prudent” to assume, when there was no evidence

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*Toxicology Research Laboratory, Dow Chemical U.S.A., 1803 Building, Midland, Michigan 48640.
to indicate otherwise, that animal carcinogens were acting through genetic mechanisms and hence could not be assumed to have any "threshold" in their activity. This assumption led them to recommend that mathematical models such as the "one-hit" model be considered for risk extrapolation. (2) The NAS also noted that, as a general rule, processes such as metabolism and excretion occur less rapidly in man than in laboratory rodents (6). Since these processes are often involved in detoxification of foreign chemicals, the NAS also suggested that, when there was no evidence to indicate otherwise, man should be considered to be more sensitive to the toxicity (including carcinogenicity) of foreign chemicals than rodents.

However, there is reason to believe that neither of these assumptions are justified when the carcinogenic risk associated with exposure to low levels of chloroform is estimated. Pertinent data for a more realistic estimation of carcinogenic risk will be discussed below.

Methods

Animals

Male mice (CD-1 or B6C3F1 strains) and male rats (Sprague-Dawley strain) were obtained from Charles River Laboratories, Wilmington, Mass. All animals were acclimatized for at least one week before use.

Materials

$^{14}$C-Chloroform was obtained from New England Nuclear (Catalog No. NEC-351) with a reported specific activity of 5.4 mCi/mmol and a radiochemical purity of $>97\%$. Nonradioactive chloroform was Baker Analytical Reagent grade containing 0.7% ethanol as a preservative (and less than 0.1% of any other materials). All other materials were reagent grade from commercial supply houses.

Enzymes used in the purification of DNA $\alpha$-amylase, tpsin, chymotrypsin, ribonuclease-A, and deoxyribonuclease (DN-100) were obtained from Sigma Chemical Co., St. Louis, Mo.

DNA Alkylation

The potential of chloroform to cause DNA alkylation in vivo was estimated by measuring the specific radioactivity of DNA isolated from animals sacrificed 4 hr after exposure to $^{14}$C-chloroform (240 mg/kg, PO). DNA was isolated from the livers and kidneys of these animals by the method of Marmur (7) as modified by Reitz et al. (8).

The modified procedure involved the precipitation of isolated DNA from solution with 5% trichloroacetic acid (TCA), hydrolysis of the precipitate in a buffered solution of purified deoxynucleosine acid, and reprecipitation of insoluble impurities by addition of more TCA (final concentration 5%). Following the second precipitation, the incubation is filtered through a Millipore filter (5 μm pore size) and the filtrate is collected. This last step removes many contaminating macromolecules such as protein, RNA, and glycogen.

These conditions are similar to those employed by others (9-11). The mild acid and enzyme treatments would not be expected to degrade covalently bonded DNA adducts, and levels of DNA alkylation obtained using these methods are consistent with those reported by other investigators (12).

DNA Repair in Vivo

DNA repair was estimated by administering non-radioactive chloroform to animals and subsequently determining the rate of incorporation of $^3$H-thymidine into DNA in animals receiving doses of hydroxyurea sufficient to depress normal DNA synthesis. Details of this procedure have been described previously (8).

Other Analyses

Glycogen, RNA, and protein in the final DNA preparation were determined by the methods of Shields and Burnett (13), Brown (14), and Bradford (15), respectively. Radioactivity was determined by liquid scintillation counting with automatic quench correction (Beckmann LS-9000). DNA concentration was estimated by the method of Burton (16).

Cellular Regeneration

Male mice were gavaged with various doses of unlabeled chloroform, held for 4 hr, and then injected IP with $^3$H-thymidine. Four hours later, these animals were sacrificed, and DNA was isolated from liver and kidney samples as outlined by Reitz et al. (8). The cellular regeneration index was estimated by determining the relative specific radioactivity in the DNA from treated and control groups.

Histopathological Assessment

Samples of kidney and liver were removed at necropsy and fixed in buffered 10% formalin. The
tissues were processed by routine histologic procedures. Sections (5–6 μm) were stained with hematoxylin and eosin and examined by light microscopy.

Statistical Analyses

Data were analyzed by the procedure of Wilcoxon (17) as modified by Mann and Whitney (18) for unequal sample sizes. Outliers were removed as outlined by Grubb (19) before analysis. The level of significance was chosen to be \( p < 0.05 \).

Results

DNA Alkylation

The levels of radioactivity incorporated into DNA isolated from the liver and kidney of B6C3F1 mice exposed to \(^{14}\text{C}-\text{chloroform}\) are summarized in Table 1. These data are reported as micromole equivalents of chloroform bound per mole of DNA phosphorus, adjusted to an equivalent dose of 1 mmole/kg.

The levels of radioactivity in the DNA isolated from the organs of the chloroform-treated mice represented about 10 to 20 dpm over background counts in samples of DNA isolated from untreated mice. (All samples were counted for 100 min in order to accumulate sufficient counts for accurate estimation of the low amounts of radioactivity present.) It must be emphasized that the radioactivity observed in the DNA may also arise from biosynthetic incorporation of radioactive one carbon fragments during normal DNA synthesis. Hence the value reported must be considered an "upper limit" rather than an accurate estimate of the level of DNA alkylation.

DNA Repair

Repair of DNA (estimated as hydroxyurea-resistant incorporation of \(^3\text{H}-\text{thymidine}\) into DNA) also served as an indicator of the potential of chloroform to cause genetic effects. Intraperitoneal administration of dimethylnitrosamine (DMN) caused large increases in DNA repair in the liver of B6C3F1 mice (Fig. 1), but chloroform (240 mg/kg, PO) was inactive in this system. Thus these data also fail to indicate any significant genotoxic activity for orally administered chloroform in mice.

Cellular Regeneration

Cellular regeneration was increased 14-fold in the liver of male mice treated with 240 mg/kg (Table 2). Much smaller effects on cellular regeneration in

![Figure 1. DNA repair in the liver of mice treated with dimethylnitrosamine (DMN) or chloroform (CHCl₃) relative to control groups.](image)

Table 1. Chemical binding indexes (CBI) for binding of various chemicals to DNA in \textit{vivo} at a standard dose of 1 mmole/kg according to Lutz (26).

| Potency rating | Chemical | Chemical bound to DNA (μmole/mole DNA) |
|----------------|----------|--------------------------------------|
| Strong         | Aflatoxin | 17,000* |
|                | Dimethylnitrosamine | 6,000* |
|                | Dimethylnitrosamine | 7,430b |
| Moderate       | 2-Acetylaminofluorene | 560a |
|                | 0-Aminoazotoluene | 220a |
| Weak           | Urethane | 29–90a |
|                | 4-Dimethylaminoazobenzene | 6a |
| Experimental results for selected chemicals | Chloroform | 1.5 (Det. Limit = 1)b |
|                | Perchloroethylene | 0.0 (Det. Limit = 10)b |

*Data from Lutz (26).

bData gathered in Dow Laboratories.
Table 2. Cellular regeneration (estimated by determination of relative incorporation of \(^3\)H-thymidine into DNA) in tissues of male B6C3F1 mice or male Osborne-Mendel rats 48 hr after a single gavage dose of chloroform.

| Tissue/dose   | DPM/g DNA ± SD | Ratio  |
|---------------|----------------|--------|
| Liver (mice)  |                |        |
| Control       | 3.6 ± 1.5 (n = 5) | —      |
| 15 mg/kg      | 3.4 ± 2.2 (n = 5) | 0.94   |
| 60 mg/kg      | 8.0 ± 3.3 (n = 6) | 2.2    |
| 240 mg/kg     | 50.6 ± 13.0 (n = 6) | 14.0* |
| Kidney (mice) |                |        |
| Control       | 2.4 ± 0.21 (n = 5) | —      |
| 15 mg/kg      | 1.9 ± 0.32 (n = 5) | 0.79   |
| 60 mg/kg      | 19.6 ± 16.2 (n = 6) | 8.2*  |
| 240 mg/kg     | 59.4 ± 10.6 (n = 6) | 24.8* |
| Liver (rats)  |                |        |
| 180 mg/kg     | 2.6*           |        |
| Kidney (rats) |                |        |
| 180 mg/kg     | 1.4*           |        |

*Significantly different from control (p < 0.05, Mann Whitney U test).

Data from Reitz et al. (20).

The results were significant in the groups receiving either 60 mg/kg (2.2-fold increase) or 15 mg/kg.

The kidney tissue of mice was more sensitive than the liver tissue to the effects of chloroform. Cellular regeneration was increased 25-fold in the kidneys of mice receiving 240 mg/kg, and 8-fold in mice receiving 60 mg/kg. No effects on cellular regeneration were seen in the kidneys of mice receiving 15 mg/kg of chloroform by gavage (Table 2).

In order to further characterize the effects of chloroform on liver and kidney tissue, small groups of mice (two/dose) were gavaged with various doses of chloroform and then sacrificed 48 hr later for examination by histopathological techniques. Tissue damage was readily observable in the tissues where cellular regeneration had been stimulated (Table 3). Damage was severe enough to be judged necrotic following 240 mg/kg in both liver and kidney, while tubular epithelial regeneration was noted in the corticomedulary region of kidneys from mice gavaged with 60 mg/kg. No microscopic changes were observed in liver or kidney tissue obtained from mice gavaged with 15 mg/kg of chloroform (Table 3).

Previous experiments indicated that rats were less sensitive than mice to the toxic effects of chloroform, including the production of increased cellular regeneration. Male Osborne-Mendel rats dosed with 180 mg/kg of chloroform showed a 160% increase in cellular regeneration in the liver, and a 40% increase in the kidney (20) (Table 2).

### Discussion

Chloroform has been tested in several of the standard tests designed to detect mutagenic (and hence presumably carcinogenic) potential. The results have been generally negative. For instance, two groups reported that chloroform failed to induce mutations in Salmonella typhimurium (21,22), and chloroform was also negative in a sex-linked recessive lethal test conducted in Drosophila melanogaster (23). Most recently, chloroform was selected as one of several prototype chemicals to be evaluated in a comprehensive battery of short term tests for carcinogenicity. The results of these tests were summarized by Bridges et al. (24) and Brooks and Preston (25). Chloroform was consistently negative in tests for genotoxicity (24, 25).

The results of these studies of DNA alkylation and DNA repair are also inconsistent with the theory that chloroform can induce somatic mutations of the type that may lead to cancer. Doses of chloroform which exceeded those known to produce liver cancer in mice produced orders of magnitude less DNA alkylation than that observed with known genotoxic agents (Table 1). For comparative purposes, the degrees of alkylation of DNA produced in the target organs by known genotoxic chemicals (utilizing the same routes of administration known

Table 3. Histopathology in tissues of male B6C3F1 mice 48 hr after exposure to single oral doses of chloroform.

| Microscopic observation                                      | No. mice affected/no. mice examined after various doses of chloroform |
|--------------------------------------------------------------|-----------------------------------------------------------------------|
|                                                              | 0             | 15 mg/kg | 0 mg/kg | 240 mg/kg |
| Liver                                                        |               |          |         |           |
| Individual hepatocellular necrosis; inflammatory cell infiltration | 0/2     | 0/2      | 0/2     | 2/2       |
| Increased mitosis                                            | 0/2           | 0/2      | 0/2     | 2/2       |
| Centrilobular hepatocellular swelling (2/3 lobule involved)  | 0/2           | 0/2      | 0/2     | 2/2       |
| Kidney                                                       |               |          |         |           |
| No microscopic change                                        | 2/2           | 2/2      | 0/2     | 0/2       |
| Severe diffuse renal cortical necrosis                       | 0/2           | 0/2      | 0/2     | 2/2       |
| Focal tubular epithelial regeneration; corticomedullary junction | 0/2     | 0/2      | 2/2     | 2/2       |
to produce tumors) are summarized in Table 1. These data are from the publication of Lutz (26), and are all reported as micromoles of chemical bound per mole of DNA at a standard dose of 1 mmole/kg. Lutz observed a reasonable correlation between the in vivo potency for DNA alkylation and the potency as carcinogens (26) and proposed this method as a useful tool for estimating relative carcinogenic hazard.

Although it is not clear which sites of DNA are most sensitive to potentially mutagenic effects, or whether the various sites are equally susceptible to in vivo DNA repair mechanisms, the very low alklylation of DNA observed after chloroform administration suggests that the genotoxic potential of chloroform is minimal.

Furthermore, the failure of chloroform to stimulate any detectable activity in the DNA repair systems (Fig. 1) also suggests that no biologically significant alteration of DNA has occurred, even following administration of a dose which was carcinogenic in the NCI bioassay.

In contrast, severe tissue damage was produced in mice in the organs where tumors were reported to develop in two bioassays of chloroform (2,27). This damage was clearly visible at necropsy in the kidneys and livers of mice treated with high doses of chloroform.

This damage was also sufficient to stimulate cellular regeneration at these sites (Table 2). Both cellular regeneration and tissue damage were well correlated with the tumorigenicity in mice. For example, doses of 60 and 240 mg/kg stimulated cellular generation in the kidney, and Roe et al. (27) reported that 60 mg/kg induced kidney tumors in one of four strains of male mice. 15 mg/kg of chloroform failed to induce cellular regeneration and also was inactive (at any site) in the bioassay of Roe et al. The liver of male mice was less sensitive to the administration of chloroform. Cellular regeneration was not stimulated greatly at doses of chloroform below 240 mg/kg (Table 2), and liver tumors were only observed in mice after administration of doses above 100 mg/kg (2).

The presence of toxicity sufficient to produce necrosis (with attendant cellular regeneration) throughout the lifetime of the animal is a very significant observation, because it is well known that rapidly dividing cells are more sensitive to mutagenic stimuli than are quiescent cells (28,29). Increased cellular regeneration has been correlated with the tumorigenicity of other materials such as vinylidene chloride (8) and perchloroethylene (30). Increased cellular regeneration apparently preceded tumor development in every case in the bioassays of chloroform in mice.

The role of cellular regeneration in the chloroform bioassays in rats is not as clear. The NCI did not observe treatment-related tumors of the liver in Osborne-Mendel rats, and this is consistent with a much lower stimulation of cellular regeneration in the liver of rats following gavage with chloroform (20) as well as the report of Bull et al. (31) that the fatty infiltration of liver observed in B6C3F1 mice consuming drinking water with high levels of chloroform was not detected in Osborne-Mendel rats under similar conditions.

However, cellular regeneration was not greatly stimulated in the kidney of rats following acute treatment with 180 mg/kg chloroform (20), and this is a site where tumors did develop in the NCI bioassay. Clearly the effects of chronic chloroform treatment on the kidney of rats need to be better characterized.

Nevertheless, mice appear to be clearly more sensitive to the toxic effects of chloroform, including carcinogenicity, than rats. As discussed elsewhere (32), the toxicity of chloroform is apparently due to the production of a reactive metabolite produced by mixed function oxidases from the relatively inert chloroform molecule. Chloroform thus appears to belong to a class of chemicals which require metabolic activation for toxicity. Consequently, any species variation in the capacity to metabolize chloroform should dramatically affect the oncogenicity of this compound.

Mice have a much higher rate of oxidation of halogenated hydrocarbons to reactive intermediates than rats, and most of the halogenated hydrocarbons which produce tumors are more active in mice than rats. Since the relative activity of similar enzymes in man is less than either of the two rodent species (5,6) these considerations suggest that man should be less sensitive than either rodent species to any tumorigenic activity of chloroform.

Chang and Periera (33) have studied the alkylation of hemoglobin by 14C-chloroform in rats and mice, and they found that, in contrast to cytotoxicity and carcinogenicity, the levels of alkylation were very similar in the two species. The reason for this discrepancy is not clear, but it may be that the rate of bioactivation (relative to the rate of detoxification through some pathway such as glutathione conjugation) is more critical than the total amount of metabolite formed.

In summary, chloroform failed to demonstrate genotoxic potential in the following battery of tests: bacterial mutagenicity; mammalian cell mutagenicity; dominant lethal tests in D. melanogaster; DNA alkylation in vivo; DNA repair in vivo.

However, the carcinogenicity of chloroform, particularly in mice, did appear to be correlated with the production of recurrent cytotoxicity. When such cytotoxicity is produced through the lifetime of an
animal, increased frequencies and/or shorter laten-

cies of spontaneous tumors may be expected.

These considerations provide a firm basis for
discarding procedures such as the “one-hit” model for
risk estimation, since these assume a genotoxic
component. Increased tumor frequencies would not be
expected from exposure to levels of chloroform
below the cytotoxic threshold, since the genotoxic
potential of chloroform appears to be nil.

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