THE EFFECT OF DOSE AND VEHICLE ON EARLY TISSUE DAMAGE AND REGENERATIVE ACTIVITY AFTER CHLOROFORM ADMINISTRATION TO MICE

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Abstract—The relationship between the acute toxicity of orally-administered chloroform and its long-term tumorigenic potential was studied in male mice of the CFLP outbred Swiss albino mouse strain. A single dose of approximately 18 mg $CHCl_3$/kg had no detectable acute toxic effect on the liver or kidneys and did not stimulate regenerative activity, whereas both toxicity and subsequent tissue regeneration were observed with single doses of about 60 mg/kg or higher. The severity of the toxic effects and regenerative changes was greater when corn oil was used as a vehicle for chloroform than when the vehicle was a toothpaste base. In earlier long-term studies in mice of the same strain, kidney tumours occurred in males given 60 mg/kg/day throughout life but not in mice given 17 mg/kg/day. The tumour response was greater when the 60-mg/kg/day dose was given in an oily vehicle than when it was given in toothpaste. The findings are consistent with the hypothesis that early acute toxic change and subsequent repair are a *sine qua non* for tumorigenesis in the kidney and liver in response to chloroform.

Introduction

For many decades, chloroform has been widely used as a preservative and flavouring agent in medicines and toothpaste, but the safety of these uses was questioned when, in some experiments (National Cancer Institute, 1976; Roe, Palmer, Worden & Van Abbé, 1979), the long-term administration of chloroform in high dosage led to enhanced incidences of liver or kidney tumours in laboratory rats and mice. From earlier work (Ilett, Reid, Sipes & Krishna, 1973; Klaassen & Plaa, 1967; Waters, 1951) it was known that the same tissues are the principal targets for the acute toxicity of chloroform. It seemed possible therefore that the later development of tumours was dependent on the much earlier production of tissue damage and/or on regenerative activity following such damage. The present study was carried out to establish the threshold dose for acute toxicity and repair, to determine whether this was influenced by the nature of the vehicle in which chloroform was administered and to test the hypothesis that kidney tumour development in CFLP mice exposed to chloroform depends on prior chloroform-induced damage and repair in the kidney.

Experimental

Dosage forms. Two chloroform formulations, one with corn oil as the vehicle and the other in a toothpaste base (see Roe *et al.* 1979), were prepared for administration to the mice. The chloroform was of pharmaceutical grade and the corn oil of food grade. The corn oil solutions contained 0, 0·45, 1·8 and 7·2% (w/w) chloroform. The toothpaste formulations contained 0, 0·325, 0·94 and 3·59% (w/w) chloroform, as shown by gas-chromatographic analysis, when administered.

Animals and treatment. Male 6-wk-old CFLP outbred Swiss albino mice (from Hacking and Churchill Ltd, Wyton, Huntingdon) were allocated to treatment groups at random. Groups, each consisting of at least three animals, were given single doses of one of the test materials by intragastric intubation, the dose levels of chloroform being as close as possible to a planned schedule of 0, 15, 60 and 240 mg/kg body weight. After 3 days, each mouse received an intraperitoneal injection of [6-^{3}H]thymidine (supplied by The Radiochemical Centre, Amersham; specific activity 83 mCi/mg and radiochemical purity 98%) to provide a 25·7-μCi dose. After a further 24 hr, the mice were anaesthetized with halothane, and blood samples were taken by cardiac puncture into heparinized tubes and centrifuged to separate the plasma. The mice were then killed and the liver and kidneys were dissected out and weighed.

Sample preparation and measurements. Plasma glutamic–pyruvic transaminase (GPT) and glutamic-oxalacetic transaminase (GOT) activities were measured on a Union Carbide Centrifichem analyser using the appropriate Roche diagnostic test kits at a working temperature of 30°C. Plasma urea was measured using the Smith Kline SK1 test kit. Liver and kidney samples were preserved in 10% buffered

Table 1. *Liver and kidney weights and [³H]thymidine uptake in male CFLP mice given single intragastric doses of chloroform in corn oil or toothpaste*

Group	CHCl₃ dose (mg/kg/body weight)	Liver weight (g)	Kidney weight (g)	Radioactivity uptake from [³H]thymidine (% of dose in tissue)	
				Liver	Kidney
Vehicle: Corn oil					
Control	0	2·41 (0·37)	0·56 (0·05)	2·46 (0·49)	0·61 (0·17)
Low	17·3 (0·9)	2·44 (0·10)	0·58 (0·02)	2·87 (0·19)	0·70 (0·01)
Intermediate	65·6 (9·4)	2·57 (0·37)	0·70 (0·11)	3·87 (1·03)	3·02 (0·30)**
High†	273 (30·6)	2·61 (0·25)	0·87 (0·08)***	6·24 (3·43)*	2·53 (1·21)**
Vehicle: Toothpaste					
Control	0	2·38 (0·37)	0·58 (0·14)	2·66 (0·18)	0·65 (0·11)
Low	18·2 (0·6)	2·48 (0·15)	0·58 (0·04)	2·70 (0·43)	0·63 (0·08)
Intermediate	59·2 (2·1)	2·16 (0·09)	0·53 (0·04)	2·44 (0·60)	0·85 (0·37)
High‡	199 (6·5)	2·40 (0·38)	0·89 (0·18)*	3·87 (1·18)	2·88 (0·85)**

†Group of five mice.
‡Group of four mice.
Values are means for groups of three mice (except where indicated otherwise) with standard deviations in parentheses, and those marked with asterisks differ significantly (by Student's t test) from the corresponding control value: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

formalin and embedded in paraffin wax; sections 5 μm thick were stained with haematoxylin and eosin. For radioactivity measurements, further samples of liver and kidney (about 0·1–0·2 g) were burned in oxygen using a Model 306 automatic sample oxidizer (Packard Instrument Co. Ltd, Reading) and the combustion products were absorbed in Monophase-40 scintillator solution. The efficiency of the oxidizer was checked by combustion of tritium standards (obtained from the Radiochemical Centre). The dose, in terms of radioactivity, given to the mice was checked by mixing aliquots of the [³H]thymidine dosing solution with a toluene–Triton X-100 (2:1, v/v) scintillator solution (10 ml) containing 0·39% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) p-bis-(o-methylstyryl)benzene. Radioactivity was measured using a Philips automatic liquid scintillation analyser (Philips NV, Eindhoven, The Netherlands). Counting efficiencies were approximately 25–30% for all samples. Samples were counted for 4 min, thereby accumulating at least 10⁴ counts, with a counting error <1% of the mean gross count. Recovery of radioactivity from combusted standards was 99·1 ± 3·0%.

Results

No signs of toxic effects on the liver or kidneys were detected in mice given chloroform at the low dose level (means of 17·3 mg/kg in corn oil solution, 18·2 mg/kg in toothpaste). Liver and kidney weights, [³H]thymidine uptake and values for plasma urea, GPT and GOT all closely approximated to those for the control mice (Tables 1 and 2) and histological examination revealed no abnormalities (Table 3).

With the intermediate dose of chloroform (mean 65·6 mg/kg) in corn oil, there was little sign of toxicity in the liver, no increase in liver weight, a minimal and non-significant increase in [³H]thymidine uptake by the hepatocytes, no significant change in GPT or GOT and no histological abnormality. However, an increase in kidney weight approached significance at

the 5% level and this was accompanied by a marked increase in [³H]thymidine uptake in the kidneys, conspicuous tubular necrosis and areas of tubular basophilia indicative of tubular regeneration, but there was no rise in plasma-urea concentrations. With the intermediate dose of chloroform (59·2 mg/kg) in toothpaste, however, there was little sign of either liver or kidney toxicity and [³H]thymidine uptake in the kidney was not significantly increased.

In mice given the high chloroform dose in either vehicle (means of 273 mg/kg in corn oil, 199 mg/kg in toothpaste), there was an increased uptake of [³H]thymidine in the kidneys, plasma urea was elevated and kidney necrosis was seen in all animals. All

Table 2. *Biochemical indices of tissue damage in male CFLP mice given single intragastric doses of chloroform in corn oil or toothpaste*

Group	Mean plasma concentrations		
	Urea (mg/100 ml)	GPT (mU/ml)	GOT (mU/ml)
Vehicle: Corn oil			
Control	48	32	78
Low	49	31	80
Intermediate	47	35	64
High†	378***	71*	66
Vehicle: Toothpaste			
Control	68	25	60
Low	54	44	56
Intermediate	63	34	62
High‡	348‡	55	64

†Group of five mice.
‡Group of four mice. Individual urea values were 119, 145, 361 and 768; the difference from the control group was not statistically significant.
Values are means for groups of three mice (except where indicated otherwise) and those marked with asterisks differ significantly (Student's t test) from the corresponding control values: *$P < 0.05$; ***$P < 0.001$.

Table 3. *Histological findings in the liver and kidneys of male CFLP mice given single intragastric doses of chloroform in corn oil or toothpaste*

Group	No. of mice examined	Liver findings* Minimal centrilobular enlargement	Liver findings* Areas of degenerate or necrotic cells	Tubular basophilia Occasional areas	Tubular basophilia Widespread	Tubular necrosis Occasional areas	Tubular necrosis Widespread
			Vehicle: Corn oil				
Control	3	0	0	0	0	0	0
Low	3	0	0	0	0	0	0
Intermediate	3	0	0	1	2	2	1
High	5	4	1	0	3	0	5
			Vehicle: Toothpaste				
Control	3	0	0	0	0	0	0
Low	3	0	0	0	0	0	0
Intermediate	3	0	0	1	0	0	0
High	4	2	0	0	4	0	4

*No. of mice affected.

the kidneys were enlarged and pale compared with those of the control mice. Plasma GPT and the uptake of [³H]thymidine in the liver were both increased, particularly in the mice given chloroform in corn oil.

Discussion

Reitz, Quast, Watanabe & Gehring (1979) contrasted the negative findings for chloroform in bacterial mutagenicity studies with the positive findings recorded in some tumorigenicity bioassays. This group (Reitz, Quast, Stott et al. 1980) also reported their own investigations in which they gave single doses of chloroform dissolved in corn oil to male B6C3F1 mice and male Osborne-Mendel rats, in order to determine a no-effect level for liver and kidney damage and for tissue repair. They reported a no-effect level of 15 mg/kg. We have described here the outcome of similar experiments in which we examined the responses to single doses of chloroform given to males of the CFLP outbred Swiss albino mouse strain previously used in our own tumorigenicity bioassays. We had earlier reported (Roe et al. 1979) the development of an excess of kidney tumours in male CFLP mice given chloroform at 60 mg/kg/day for 80 wk though not in those given 17 mg/kg/day. The response had been numerically greater when chloroform was given in oil rather than in a toothpaste vehicle. The work described here offers a comparison of the effects produced by single doses of chloroform in these vehicles.

The intended dose levels of chloroform in this study were 15, 60 and 240 mg/kg. Although actual dose levels differed slightly in practice, we do not attach any importance to the minor discrepancies involved (e.g. between a nominal dose level of 60 mg/kg and actual levels of 59 mg/kg in toothpaste or 66 mg/kg in corn oil).

With both vehicles, we found that effects were seen in the kidney at a lower dose level than was needed to produce changes in the liver. These effects were more noticeable with corn oil as the vehicle than with toothpaste, a water-miscible vehicle. No detectable changes in either organ were recorded with single doses of 17 mg CHCl₃/kg in corn oil or 18 mg CHCl₃/kg in toothpaste.

We could not detect any kidney necrosis after giving the intermediate dose level (approximating to 60 mg CHCl₃/kg) in toothpaste but the appearance of tubular basophilia, indicative of regenerative activity, in one animal suggested that this dose may have reached a threshold at which regeneration following toxic action was beginning to appear. At the higher dose (approximating to 240 mg CHCl₃/kg), both tubular necrosis and basophilia were pronounced in all the mice.

With chloroform dissolved in corn oil, tubular necrosis and basophilia were readily seen at the intermediate dose level in all the mice and these changes became more marked with the high dose.

Corresponding data for [³H]thymidine uptake in the kidneys (indicative of regenerative activity) corroborated the histological findings, showing a significant increase with the intermediate chloroform dose in corn oil.

With respect to the liver, histological evidence for toxic changes after a single chloroform dose was seen only at the high dose level. It was more noticeable with the corn-oil vehicle than with toothpaste, in terms of both centrilobular enlargement and [³H]thymidine uptake, and was probably also reflected in the elevation of plasma GPT.

Taken as a whole, our findings agree with those of Reitz et al. (1980), confirming that toxic changes due to single doses of chloroform appear at lower dose levels in the kidney than in the liver. However, we have also shown that the changes are accentuated when chloroform is given in corn oil rather than in toothpaste.

Thus no toxic or regenerative changes were detectable following single-dose chloroform administration at approximately the dose level (17 mg/kg) at which no excess of tumours was found after long-term daily administration. However, toxic and/or regenerative changes began to appear when single doses of chloroform were given at the dose level (approximating to 60 mg/kg) at which long-term daily administration led

to the development of excess kidney tumours in elderly male mice of the strain used. A higher proportion of these mice (12/48 compared with 5/47) developed kidney tumours when the chloroform was given to them in an oily vehicle (arachis oil) than when the same chloroform dose was given in toothpaste. This appears to parallel our observation of a greater toxic effect and regenerative activity after the giving of a single dose of chloroform in corn-oil rather than in toothpaste.

Chloroform dose levels in our long-term studies with male CFLP mice and in the NCI study (National Cancer Institute, 1976) with Osborne–Mendel rats did not differ greatly, and excess liver tumours were not seen under these conditions. The present work and that of Reitz et al. (1980) point to the fact that little or no hepatotoxic effect would occur at these dose levels. However, at the much higher chloroform dose levels given to B6C3F1 mice (National Cancer Institute, 1976) hepatotoxic changes and regenerative activity would be expected, especially as a corn-oil vehicle was used, and these mice had a high incidence of liver tumours.

Numerous short-term studies using a wide variety of experimental techniques have shown that chloroform has little or no genotoxic activity (de Serres & Ashby, 1980; Diaz Gomez & Castro, 1980; Kirkland, Smith & Van Abbé, 1981; Perocco & Prodi, 1981; Petzold & Swenberg, 1978; Simmon, Kauhanen & Tardiff, 1977; Sturrock, 1977; Styles, 1979; Uehleke, Werner, Greim & Krämer, 1977; Van Abbé, Green, Jones et al. 1982). In looking for a non-genotoxic mechanism to explain the excess tumours observed when chloroform is given repeatedly to laboratory rodents, it is interesting to note the apparent relationship between chloroform dose level, choice of vehicle, onset of toxic changes in the organs concerned and early signs of regenerative activity even with single-dose administration. This suggests the possibility that a relatively modest toxic insult frequently repeated may suffice to increase opportunities for "spontaneous" development of neoplasms in those tissues where regeneration is repeatedly provoked. On the other hand, if dosage is insufficient to provoke regenerative activity, the risk of developing excess tumours may be negligible with a compound having little or no genotoxic potential. The limited evidence from the work reported here appears to be consistent with such a hypothesis.

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