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The Effect of Chloroform Ingestion on the Growth of some Murine Tumours

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Abstract—*Chloroform was administered to mice in their drinking water at a dose level of either* 0.15 *or* 15 mg/kg/day. *At the lower dose level chloroform did not significantly alter the rate of multiplication of the Ehrlich ascites cells. Prolonged intake of chloroform at* 0.15 mg/kg *significantly increased the Lewis lung tumour protein levels although the growth and metastasis in the chloroform-ingesting animals was not significantly different from the controls. The percentage of animals with organs invaded by B* 16 *melanoma was increased by chloroform ingestion at* 0.15 mg/kg. *At the higher treatment level the rate of metastasis of the Lewis lung carcinoma and the number of Ehrlich ascites cells were increased.*

INTRODUCTION

THE ADMINISTRATION of chloroform at high dose levels has long been known to induce hepatomas in mice [1]. Recent experiments conducted to evaluate the carcinogenic risk of the use of chloroform in toothpaste and other products have led to the proposed controversial banning of its use in America [2, 3], although studies performed in Britain have emphasised the importance of the dose and the strain of the animals used [4, 5]. It has been estimated that brushing one's teeth twice daily with toothpaste containing 3.5% w/v chloroform is equivalent to a dose level of 0.15 mg/kg/day [6]. The evaluation of the potential carcinogenic hazard of chloroform exposure is now a question of the relevance of the large dose used in animal studies to human exposure especially from toothpaste. This experiment investigates another means by which chloroform may influence carcinogenesis, by affecting tumour growth. Thus, chloroform was administered to mice in their drinking water at this and a 100-fold increased dose level and the effect on the rate of growth and spread of 3 model murine tumours determined.

Lysozomal enzymes have been implicated in the spread of tumours and it has been demonstrated that carbon tetrachloride causes their release from liver slices [7]. Thus, in the case of the 0.15 mg/kg-dosed animals bearing the Lewis lung tumours only, the effect of the solvent on tumour β-glucuronidase activity was determined.

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MATERIALS AND METHODS

Chemicals

Analar chloroform was purchased from Fisons, Loughborough, Leics. and redistilled in 2.5 l portions at 60–62°C, discarding the first and last 250 ml portions. All other reagents were of the purest grade available and obtained from Sigma Chemical Co., Poole, Dorset.

Animals and treatment

Male C57 BL/10ScSn/01a and Theiller–Original (TO) mice of 20–22 g body weight were purchased from Olac, Bicester, Oxon, and maintained on PRM diet supplied by Dixons, Ware, Herts. A cage of 20 mice consumed 80–100 ml of drinking water daily and chloroform was therefore included to give a final dose of either 0.15 or 15 mg/kg/4ml/mouse/day. Any remaining chloroform/water was discarded and replenished with a fresh mixture each day. The water feed bottles were of all glass design and covered to protect the contents from the light.

Tumours

Ehrlich ascites. A group of 100 TO mice were divided into three approximately equal

sub-groups. In one sub-group termed pre-treated but including pre- and post-treated animals, each mouse received the equivalent of 0.15 mg/kg/day chloroform in the drinking water for 14 days before and after inoculation of the tumour cells. Another sub-group (post-treated) received a similar dose of chloroform only after inoculation of tumour cells. The third sub-group (controls) received no chloroform. Ehrlich ascites tumour cells were maintained in the peritoneal cavity of 8–10-week old male TO mice by weekly passage of 10^6 cells as described by Klein and Revesz [8]. Ascites fluid was collected aseptically 7 days after inoculation of the cells and diluted with phosphate-buffered saline (PBS). All the mice in the three treatment sub-groups were injected i.p. with 0.1 ml of the diluent containing 10^6 cells. Ten days after inoculation of the tumour cells, which was found in a previous experiment to coincide with the end of the exponential growth phase [9], the animals were sacrificed by cervical dislocation, the abdominal skin removed and all the ascites fluid withdrawn from the peritoneal cavity using a syringe. The peritoneal cavity was washed out with 3×5 volumes of PBS containing 100 U/ml heparin. The ascites fluid and the washings were pooled and the volume made up to 20 ml with PBS. Each suspension was then sonicated to disrupt the cells and the DNA per ml estimated by the technique of Burton [10] as a measure of total cell content. It was assumed that the vast majority of cells in the ascites fluid were malignant. This experiment was then repeated for a similar group of 100 TO mice, raising the chloroform ingestion level of the pre- and post-treated sub-groups 100-fold to 15 mg/kg/mouse/day.

B16 melanoma. A group of 100 C57 BL mice comprising the pre-treated, post-treated and control sub-groups were inoculated s.c. in the region near the base of the tail with 10^6 B16 melanoma cells suspended in sterile isotonic PBS (0.1 ml). The inoculum had been freshly prepared in the manner of Geran *et al.* [11] from another C57 BL mouse which had received a transplant of the syngeneic B16 melanoma, maintained by i.m. passage every 14 days. The animals were sacrificed by cervical dislocation 21 days after inoculation of the tumour cells when the primary implant, which was measured using calipers, had achieved dimensions of approximately 4 $\times$ 4 mm. The spleen, mesenteric lymph nodes and lungs were examined for metastatic invasion by the B16 melanoma, the number of tumour foci on the latter organ were counted manually. As with the previous tumour, this experiment was also repeated at the higher chloroform ingestion level of 15 mg/kg/mouse/day.

Lewis lung carcinoma. Lewis lung tumour cells were maintained by serial i.m. transplantation into one flank of 8-week old C57 BL mice. A group of 100 mice comprising the 3 treatment sub-groups which were being used to investigate the effect of 15 mg/kg/day ingestion were injected i.m. in one flank with (2×10^6) freshly prepared tumour cells suspended in PBS (0.1 ml). These animals were sacrificed by cervical dislocation 14 days after inoculation of the tumour. The skin was removed from the tumour-bearing and normal thigh which were then severed at the knee and hip. The weight of the tumour was determined by subtracting the weight of the normal from that of the tumour-bearing thigh. The lungs of the mice were removed and fixed in Bouin's solution overnight and the number of pulmonary tumour foci determined manually on the following day. For the mice in which the effect of the lower dose chloroform intake was investigated, a group of 100 was divided into sub-groups of 20 which were pre-treated with 0.15 mg/kg/day chloroform in the drinking water before, for periods of 8, 6, 4 or 2 weeks, and after inoculation of 2×10^6 Lewis lung carcinoma cells. These mice were sacrificed 16 days after inoculation of the tumour cells. Primary tumour size and lung metastases were determined in the manner described for the higher level of chloroform ingestion. In these animals only, the primary tumours were homogenised in acetate buffer (pH 4.8) and the protein content determined by the method of Lowry *et al.* [12]. The remainder of the homogenate was then used for β-glucuronidase estimation and sufficient Triton X-100 added to bring the final concentration of detergent to 0.2% (v/v). The enzyme was extracted using a modification of the technique of Barrett [13] for cathepsins. The semi-purified enzyme was dialysed for 48 hr to remove inhibitors before β-glucuronidase activity was assayed essentially as described by Fishman [14] using 4-nitrophenyl glucuronide as substrate.

RESULTS

The effect of chloroform on the growth of the three murine tumours is given in Tables 1, 2 and 3. Chloroform intake did not significantly reduce the body weight of any of the treated sub-groups when compared with their

Table 1. *The effect of oral chloroform ingestion on the growth of Ehrlich ascites tumour**

Dose	Treatment group	No. of animals per group	Average body wt (g)	Tumour DNA (μg/ml)	Significance
	Control	33	38.3±3.7	661±222	
0.15 mg/kg/day	Post-treated	33	39.4±2.9	724±254	ns†
	Pre-treated	33	37.9±3.2	770±283	ns†
	Control	43	39.4±3.4	637±221	
15 mg/kg/day	Post-treated	37	37.5±3.0	1143±324	$P<0.001$
	Pre-treated	30	37.0±3.9	827±245	$P<0.001$

*Results expressed are the means±S.D.
†Not significant, $P>0.05$.

Table 2. *The effect of oral chloroform ingestion on metastic 'tumour takes' with B16 melanoma*

Dose	Treatment	No. of animals per group	Animals with B16 melanoma invasion in organs (%)			
			Spleen	Mesenteric lymph nodes	Lung (a)	Lung (b)
	Control	26	15	13	12	3
0.15 mg/kg/day	Post-treated	31	35	10	10	5
	Pre-treated	28	36	29	18	10
	Control	30	15	12	6	4
15 mg/kg/day	Post-treated	32	31	25	19	6
	Pre-treated	32	31	32	20	20

(a) Percentage of animals with tumour foci on the lungs.
(b) Average number of lung metastases.

appropriate control sub-groups. With the number of cells inoculated in this study the number of takes were complete, i.e., 100% for each tumour, neither did the chloroform intake increase the mortality rate, all the animals being sacrificed at the times indicated above. Ingestion of chloroform at 0.15 mg/kg produced no significant increase in Ehrlich ascites tumour cells (as estimated by peritoneal DNA content). The animals which ingested chloroform in the drinking water at 15 mg/kg/day had significantly more tumour cells than the control groups.

The results in Table 2 indicate that a greater percentage of the organs of the animals receiving chloroform at either dose level had been invaded by the B16 melanoma cells than those of the controls. The lungs of the animals which had received chloroform in their drinking water also had a greater number of larger B16 melanoma tumour foci than those of the control mice. Prolonged intake of chloroform at 0.15 mg/kg for periods of up to 8 weeks produced no significant increase in either Lewis lung tumour size or metastasis. The apparent decrease in β-glucuronidase levels of these tumours is a result of the increase in tumour protein caused by the prolonged chloroform ingestion, so that the amount of enzyme present in terms of wet weight of tissue is largely unaffected. The chloroform-induced increase in protein synthesis could possibly be the result of increased tumour cell growth at this dose level, and the method of quantitation (i.e., weighing) was not sensitive enough to detect these changes. At the 100-fold higher level of chloroform ingestion there were significantly more metastases in the chloroform-treated animals than in the control sub-groups.

*Table 3. The effect of oral chloroform ingestion on the growth and spread of the Lewis lung tumour**

Dose	Treatment	No. of animals per group	Average body wt (g)	Tumour wt (g)	Lung metastases	Significance	β-glucuronidase activity‡	Protein content§
0.15 mg/kg/day	8†	20	30.6 ± 3.8	3.5 ± 0.81	165 ± 36	ns	0.33 ± 0.56	78.2 ± 4.2
	6	20	30.6 ± 3.8	3.3 ± 0.72	170 ± 41	ns	0.27 ± 0.79	66.8 ± 2.7
	4	20	29.0 ± 3.6	3.3 ± 0.54	154 ± 39	ns	0.38 ± 0.073	60.1 ± 5.1
	2	20	29.0 ± 3.5	3.2 ± 0.72	147 ± 44	ns	0.49 ± 0.070	60.3 ± 4.7
	0 (control)	20	29.3 ± 2.7	3.1 ± 0.41	142 + 34		0.58 ± 0.094	50.8 ± 6.2
15 mg/kg/day	Control	33	23.6 ± 1.3	1.6 ± 0.34	44 ± 26			
	Post-treated	33	24.5 ± 2.3	1.7 ± 0.51	57 ± 19	$P<0.05$		
	Pre-treated	33	24.4 ± 2.2	1.8 ± 0.42	61 ± 19	$P<0.01$		

*Results expressed are the means ± S.D.
†Duration of treatment (weeks).
ns = not significant, $P>0.05$.
‡Expressed as μmole product/mg protein/min.
§Milligrams of protein after extraction mg/g wet wt.

DISCUSSION

In a previous experiment alcohol ingestion at 2 dose levels has been demonstrated to retard the growth and spread of 3 murine tumours, probably through the creation of nutritional imbalances [9]. Conversely in this experiment chloroform at the higher dose level appeared to enhance the growth of these tumours and there was no obvious evidence of any nutritional imbalance.

The principal organ concerned with chloroform metabolism in the body is the liver and ingestion of large quantities of this solvent result in severe necrosis [1, 15]. It has been demonstrated that metabolism of the related chlorinated alkane, carbon tetrachloride, enhances its hepatotoxicity [16]. Whether chloroform exerts its toxicity by a similar mechanism is unclear although the highly electrophilic phosgene has been identified as a minor metabolic product of microsomal chloroform oxidation [17]. As a result of the obviously injurious effects of large doses of chloroform on the liver (and therefore, ultimately, the kidney) most of the safety evaluation studies conducted have concentrated on the effect of sub-lethal doses in animals in order to determine possible mutagenic or carcinogenic levels. Clearly, the dose level and duration of treatment of chloroform is important as is shown from the differing results that have been obtained in the British [4, 5] and American [2, 3] experiments. Similarly, in this experiment there was no significant difference between the control and treated animals at 0.15 mg/kg with the Lewis lung and ascites tumours, whereas the 100-fold level of ingestion significantly enhanced tumour growth. However, the development of a tumour from a primary chemical carcinogenic stimulus is a multistage process [18]. Therefore, experimentation designed to determine whether chloroform is carcinogenic *per se* could overlook the possibility that the solvent might alter other metabolic functions which also have a role, not only in carcinogenesis, but in tumour growth. It has been demonstrated that chloride ions present in hyperchlorinated water depressed macrophages in peritoneal exudates [19]. Possibly the observed enhancement of tumour growth of chloroform is mediated through a similar reduction in the immunological defence mechanism, since it does not appear to affect lysozomal stability. In summary, it is seen that chloroform, even in low doses for relatively short periods of ingestion, can influence tumour growth and this should be considered in future safety evaluation trials.

These results demonstrate that chloroform ingestion, even at the low dose levels employed in this study can influence the growth and spread of experimental tumours. The extent to which the solvent can exert its effects will depend upon the dose, duration of exposure, type of tumour and strain of mouse.

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