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VINYL CHLORIDE: DOMINANT LETHAL STUDIES IN MALE CD-1 MICE

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Summary

The mutagenic activity of vinyl chloride (VC) at three exposure levels was assessed in fertile male CD-1 mice with the dominant lethal test.

Male mice were exposured by inhalation to VC at 3000, 10,000 and 30,000 ppm for 6 h a day for 5 days. By comparison with control males exposed to air, no mutagenic effects on any maturation stage of spermatogenesis in treated males were detected. There was no significant increase in the number of post-implantational early foetal deaths as shown by the number of females with one or more early deaths or number of early deaths/pregnancy or the number of early deaths/total implants/pregnancy. There was no evidence of pre-implantational egg losses as indicated by the total implants/pregnant female. There was also no reduction in fertility.

The lack of effect was not due to the insensitivity of the system used since a dominant lethal effect was clearly demonstated in male mice dosed i.p. with cyclophosphamide (CTX) at 200 mg/kg body weight and ethyl methanesulphonate (EMS) orally at 200 mg/kg body weight once a day for 5 days. During dosing these animals were housed under similar exposure conditions to those animals exposed to the test substances but with a flow of air through the exposure chambers.

Thus vinyl chloride is not mutagenic in the mouse at the stated exposure levels as measured by the dominant lethal test.

Introduction

Vinyl chloride (VC) used in the manufacture of polyvinyl chloride has been found to cause tumours in rats [12] and man [6]. It has also been shown to

produce chromosome breaks in exposed workers [7,10,13] and to cause mutations in *Salmonella typhimirium* [5]. We therefore carried out a dominant lethal study to determine if there were any mutagenic effects of this type in mice after vinyl chloride exposure at 3 different levels. At the same time, negative control animals, exposed to air, and positive control animals, also exposed to air and given ethyl methanesulphonate and cyclophosphamide, were assayed.

Materials and methods

Chemicals

VC was obtained from Air Products Limited, Worsley, Walkden, Lancs, and mixed with air which was metered from the compressed air taps in these laboratories. Ethyl methanesulphonate (EMS) was obtained from Koch-Light Limited, Colnbrook, Bucks, England, and cyclophosphamide B'' (Endoxana) was obtained from Ward Blenkinsop Limited, London, England. Both of these substances were prepared as aqueous solutions immediately before use.

Animals

CD-1 mice (Charles River, Manston, Kent) were used throughout the experiment. Undosed females were 8–10 weeks old when mated and male mice immediately after dosing were 10–12 weeks old. Males were caged individually and females in pairs. They received food and water ad libitum. All females were examined daily during the experiment for evidence of clinical or behavioural abnormalities.

Dosing of male mice

Dose levels for VC were selected on the basis of toxicity studies. Groups of 6 mice were given 5 daily 6 h exposures of VC at 30,000, 20,000, 15,000, 10,000 and 5000 ppm. Doses of 30,000, 10,000 and 3000 ppm were chosen for the main study. 30,000 ppm was chosen as the highest exposure level as this was found to be in the toxic range and it was desirable that the maximum tolerated dose or higher should be used. The required concentrations were generated by mixing known volumes of VC and air using rotameters as indicators.

During dosing the mice were housed individually in compartments in chambers made of stainless steel and glass with an internal capacity of 3 litres and they received food and water in agar cubes.

All groups, including positive and negative controls, were housed under similar conditions during the 5-day treatment period with a flow of air (mixed with the appropriate percentage of compound, where necessary) through the chambers.

Groups of between 15 and 25 mice (numbers shown in parentheses below) of proven fertility were treated in the following ways immediately before test-mating began.

Group 1 (20 mice), air.
Group 2 (20 mice), 3000 ppm VC for 6 h per day for 5 days.
Group 3 (20 mice), 10,000 ppm VC for 6 h per day for 5 days.
Group 4 (20 mice), 30,000 ppm VC for 6 h per day for 5 days.
Group 5 (15 mice), 200 mg cyclophosphamide in water per kg body weight once by i.p. injection on day 5.

Group 6 (25 mice), 200 mg ethyl methanesulphonate in water per kg body weight orally once a day for 5 days.

Mating

Fertility testing

200 male mice were caged with 400 virgin female mice, 1 male and 2 female mice in each cage. After 5 days the females were transferred to other cages. The female mice were killed fifteen days after first introducing them to the males and examined for pregnancies. 106 males which survived dosing and were successful in fertilising at least 1 female in their cage were selected for continuation in the experiment.

Experimental mating

2 virgin female mice 8—10 weeks old were put into each of the 106 cages in which the males were individually housed. After 5 days, the females were removed and rehoused in pairs.

A week after the initial introduction the males were caged with another two virgin females and again left for 5 days. This process was repeated until the treated male mice had been mated at weekly intervals for 8 weeks with virgin females. The males were then killed and not examined further. No attempts were made to establish whether mating had occurred. Instead, it was assumed that most matings leading to fertilisation would occur 2 or 3 days after introducing female mice to the cages containing males.

Female mice were killed 13 days after the assumed date of fertilisation, i.e. 15 or 16 days after caging females with males.

Assessment

Uteri of the killed mice were examined for live implantations, early deaths and late deaths.

Statistics

The data have been statistically analysed as reported previously [1].

Results

Mating weeks after treatment are represented in Tables by numbers 1--8 and the mating week before treatment is represented by week 0. Assessment of females which became pregnant during the fertility test yielded the data for week 0 but only data from those animals that survived treatment have been included in week 0—8.

Survival data

The number of males surviving treatment are shown in Table I. The only significant mortality occurs in the groups exposed to the highest level of VC.

Successful mating frequency

The numbers and percentage of males successfully mating at each week is

TABLE I

NUMBER AND PERCENTAGE OF MALE MICE WHICH SURVIVED TREATMENT SUCCESFULLY MATING AT EACH WEEK

	Group 1 Air		Group 2 VC 3000 ppm (6 h X 5)		Group 3 VC 10,000 ppm (6 h X 5)		Group 4 VC 30,000 ppm (6 h X 5)		Group 5 CTX 200 mg/kg i.p.		Group 6 EMS 200 mg/kg oral X 5	
	No. of mice	%	No. of mice	%	No. of mice	%	No. of mice	%	No. of mice	%	No. of mice	%
Week 0[c]	20	100	18	100	19	100	9	100	15	100	25	100
1	20	100	18	100	18	100	7	77.8	14	93.3	3	12.2
2	20	100	18	100	19	100	9	100	14	93.3	23	92.0
3	20	100	18	100	19	100	9	100	14	93.3	23	92.0
4	20	100	18	100	19	100	9	100	15	100	25	100
5	19	95.0	18	100	19	100	7	77.8	15	100	25	100
6	20	100	18	100	19	100	9	100	No fs[a]		No fs[a]	
									14	100	24	100
7	20	100	18	100	19	100	9	100	14	93.3	25	100
8	20	100	18	100	19	100	8	88.9	1 ml[b] dead		25	100
									13	92.9		
% Survival after treatment	100% (20/20)		90% (18/20)		95% (19/20)		45% (9/20)		100% (15/15) except at week 8		100% (25/25)	

[a] No fs, females were not caged with males.
[b] ml, male.
[c] Data in week 0 only refers to animals surviving treatment.

TABLE II

NUMBER OF MATED FEMALES BECOMING PREGNANT

	Group 1 Air		Group 2 VC 3000 ppm (6 h X 5)		Group 3 VC 10,000 ppm (6 h X 5)		Group 4 VC 30,000 ppm (6 h X 5)		Group 5 CTX 200 mg/kg i.p.		Group 6 EMS 200 mg/kg oral X 5	
	No. preg.	No. mated	No. preg.	No. mated	No. preg.	No. mated	No. preg.	No. mated	No. preg.	No. mated	No. preg.	No. mated
Week 0	35	40	30	36	28	38	15	18	25	30	37	50
1	35	40	33	36	32	No fs [a]	11	18	26	30	4 [c]	50
2	36	40	36	36	33	36	15	18	25	30	31 [d]	50
3	34	40	30	36	34	38	16	18	26	30	40	50
4	38	40	34	36	33	38	17	18	30	30	46	50
5	35	40	34	36	36	38	12	18	28	30	47	50
6	36	40	33	36	36	38	18	18	26	No fs [a]	47	No fs [a]
										28		48
7	37	40	31	36	37	38	18	18	22	30	41	50
8	37	40	32	36	31	1 f. dead	14	18	23	1 ml [b] dead	45	50
						37				28		

[a] No fs, females were not caged with males.
[b] ml, male.
[c] $P < 0.001$.
[d] $P < 0.01$.

also shown in Table I. Numbers remained high during the experiment. No statistically significant differences in the mating frequency were found between VC treatment groups and the control at any week using a Chi-squared test. There was, however, a significant difference between the EMS-treated group and the control in week 1.

Pregnancy frequency

The numbers of females in each group which became pregnant at each week of mating is shown in Table II. Statistical differences between treated groups and the control group were only found in the EMS treated group at weeks 1 and 2 using a Chi-square test.

These results indicate that VC does not decrease fertility at the administered doses.

Total implantations

The total number of implants per pregnant female in each group is shown in Table III. The mean values were adjusted to take account of the unequal numbers of pregnant females per male and were compared statistically at each week using an analysis of variance and a Dunnett's '*t*' test. There were statistically significant differences between the positive control groups (5 and 6) and the negative control. The differences were evident in week 1 in the cyclophosphamide-treated group and weeks 1 and 2 in the EMS-treated group. A significant difference ($P < 0.05$) in week 4 was also found between the group exposed to the highest dose of VC (Group 4) and the negative control.

Early deaths

Early deaths are considered to be important in the assessment of a dominant lethal effect [4], and there are various ways in which the results may be evaluated, each producing some bias.

TABLE III

AVERAGE TOTAL IMPLANTS PER PREGNANT FEMALE

	Group 1 Air	Group 2 VC 3000 ppm (6 h X5)	Group 3 VC 10,000 ppm (6 h X5)	Group 4 VC 30,000 ppm (6 h X5)	Group 5 CTX 200 mg/kg i.p.	Group 6 EMS 200 mg/kg oral X5
Week 0	11.90	12.61	11.97	13.05	11.33	11.78
1	12.93	13.56	13.36	12.68	8.82 [b]	4.00 [b]
2	12.85	12.67	13.03	13.67	10.93	8.43 [b]
3	12.50	12.53	12.39	11.16	10.92	12.50
4	12.38	12.14	13.66	10.00 [a]	12.60	12.68
5	13.68	12.72	14.13	12.79	12.30	12.66
6	11.75	11.94	12.50	12.56	12.46	12.10
7	11.80	11.56	11.76	11.94	12.50	12.36
8	12.68	12.14	12.56	12.13	12.46	12.52

[a] $P < 0.05$.
[b] $P < 0.01$.

TABLE IV

NUMBER OF PREGNANT FEMALES WITH 1 OR MORE EARLY DEATHS

	Group 1 Air		Group 2 VC 3000 ppm (6 h X5)		Group 3 VC 10,000 ppm (6 h X5)		Group 4 VC 30,000 ppm (6 h X5)		Group 5 CTX 200 mg/kg i.p.		Group 6 EMS 200 mg/kg oral X5	
ED [a]	0	>1	0	>1	0	>1	0	>1	0	>1	0	>1
Week 0	18	17	21	9	16	22	10	5	22	3 [b]	21	26
1	15	20	16	17	10	21	6	5	1	25 [b]	1	3
2	19	17	18	18	13	20	6	9	2	23 [b]	5	26 [b]
3	18	16	16	14	19	15	9	6	8	18	15	25
4	16	22	17	17	20	13	9	7	14	16	19	27
5	17	18	18	16	14	22	3	9	12	16	25	22
6	17	19	11	22	21	15	7	11	13	13	24	23
7	18	19	13	18	21	16	8	10	10	12	22	19
8	13	24	14	18	15	16	8	6	13	10	22	23

[a] ED, early deaths.
[b] $P < 0.01$.

The number of pregnancies with one or more early deaths (Table IV)

Cyclophosphamide and EMS treatment caused increases in the number of pregnancies with early deaths. The effect was significant (Chi-square) in weeks 1 and 2 for the cyclophosphamide-treated group and in week 2 for the EMS-treated group. No differences from the control group were seen in the VC-treated group.

Number of early deaths per pregnancy (Table Va)

The number of early deaths per pregnancy was only increased in the cyclophosphamide and EMS-treated positive controls. A statistical analysis of the data is shown in Table Vb. A large number of low or zero values was encountered so it was necessary to stabilise the variance prior to analysis. The variance ratio test on the transformed data showed that groups 5 and 6 were different from group 1. Differences were evident in weeks 1 and 2 with cyclophosphamide treatment and week 2 with EMS treatment, whereas VC treatment induced no increase in early deaths.

TABLE Va

AVERAGE EARLY DEATHS PER PREGNANT FEMALE

	Group 1 Air	Group 2 VC 3000 ppm (6 h X5)	Group 3 VC 10,000 ppm (6 h X5)	Group 4 VC 30,000 ppm (6 h X5)	Group 5 CTX 200 mg/kg i.p.	Group 6 EMS 200 mg/kg oral X5
Week 0	0.77	0.33	0.54	0.40	0.16	0.59
1	0.86	0.91	1.00	0.45	4.27	3.50
2	0.83	1.00	1.15	0.93	4.84	2.58
3	0.91	0.73	0.88	0.93	1.54	1.45
4	0.89	0.79	0.61	0.69	1.13	0.85
5	1.06	0.71	1.03	1.00	1.11	0.72
6	0.97	0.91	0.75	1.39	0.65	0.85
7	0.76	0.87	0.68	0.89	0.62	0.63
8	1.03	0.88	1.00	0.71	0.70	0.73

TABLE Vb

TRANSFORMED EARLY DEATHS PER PREGNANT FEMALE

	Group 1 Air	Group 2 VC 3000 ppm (6 h X5)	Group 3 VC 10,000 ppm (6 h X5)	Group 4 VC 30,000 ppm (6 h X5)	Group 5 CTX 200 mg/kg i.p.	Group 6 EMS 200 mg/kg oral X5
Week 0	1.92	1.49	1.64	1.59	1.66	1.74
1	2.08	2.08	2.21	1.61	4.18 [a]	3.06
2	1.9	2.02	2.21	2.1	4.57 [a]	3.28 [a]
3	2.02	1.89	2.01	2.08	2.52	2.38
4	2.05	1.92	1.61	1.7	2.16	1.99
5	2.10	1.82	2.10	2.32	2.16	1.81
6	2.00	2.09	1.90	2.30	1.76	1.91
7	1.89	2.04	1.76	1.99	1.91	1.69
8	2.19	2.07	1.99	1.85	1.90	1.85

[a] $P < 0.01$.

TABLE VIa

EARLY DEATHS AS A PERCENTAGE OF TOTAL IMPLANTS PER GROUP

	Group 1 Air	Group 2 VC 3000 ppm (6 h X5)	Group 3 VC 10,000 ppm (6 h X5)	Group 4 VC 30,000 ppm (6 h X5)	Group 5 CTX 200 mg/kg i.p.	Group 6 EMS 200 mg/kg oral X5
Week 0	6.49	2.67	4.42	3.09	1.42	5.08
1	6.65	6.67	7.49	2.69	49.12	77.78
2	6.41	7.89	9.57	6.90	44.65	31.50
3	7.35	5.87	6.99	7.82	14.08	11.58
4	7.23	6.55	4.48	6.55	8.99	6.70
5	7.81	5.54	7.28	7.95	9.06	5.73
6	8.16	7.59	6.02	11.06	5.21	7.04
7	6.36	7.34	5.66	7.44	6.98	5.10
8	8.02	7.18	8.09	5.88	5.67	5.92

TABLE VIb

TRANSFORMED EARLY DEATHS PER TOTAL IMPLANT PER PREGNANT FEMALE

	Group 1 Air	Group 2 VC 3000 ppm (6 h X5)	Group 3 VC 10,000 ppm (6 h X5)	Group 4 VC 30,000 ppm (6 h X5)	Group 5 CTX 200 mg/kg i.p.	Group 6 EMS 200 mg/kg oral X 5
Week 0	0.55	0.41	0.46	0.45	0.35 [a]	0.50
1	0.59	0.56	0.61	0.44	1.56 [b]	1.64 [b]
2	0.55	0.57	0.65	0.59	1.49 [b]	1.25 [b]
3	0.58	0.54	0.57	0.71	0.78	0.68
4	0.58	0.56	0.43	0.55	0.61	0.57
5	0.57	0.51	0.57	0.65	0.63	0.51
6	0.59	0.60	0.54	0.66	0.49	0.56
7	0.55	0.69	0.51	0.59	0.56	0.50
8	0.62	0.59	0.56	0.53	0.55	0.52

[a] $P < 0.05$.
[b] $P < 0.01$.

Percentage of total implants recorded as early deaths per pregnancy (Table VIa)

Again it was necessary to stabilise the variance prior to analysis (Table VIb). The variance ratio test on the transformed data showed that groups 5 and 6 were different in weeks 1 and 2 from the negative control, whereas VC-treated groups were not. Group 5, however, also showed a difference from the negative control in week 0 (week 0 being the pre-treatment week).

Percentage induced pre- and post-implantational dominant lethality

These values are shown in Table VII. The typical variation of the data falls between −14 and +12. Only groups 5 and 6 show large values in weeks 1 and 2 and group 5 also shows a slightly increased value in week 3. A similar value is also seen in week 4 for the highest VC exposure group. The remaining values lie within the typical variation range suggesting that VC shows no induced pre- and post-implantational dominant lethality.

TABLE VII

PERCENTAGE INDUCED PRE- AND POST-IMPLANTATIONAL DOMINANT LETHALITY

	Group 2 VC 3000 ppm (6 h X5)	Group 3 VC 10,000 ppm (6 h X5)	Group 4 VC 30,000 ppm (6 h X5)	Group 5 CTX 200 mg/kg i.p.	Group 6 EMS 200 mg/kg orial X5
Week 0	−10.3	−3.0	−13.7	−0.4	−0.5
1	−4.8	−2.4	−6.5	+62.3	+95.9
2	+2.9	+1.2	−6.0	+40.3	+51.3
3	−1.8	+0.7	+11.7	+19.1	+4.7
4	+1.2	−13.6	+19.0	+0.2	−2.1
5	+4.8	−3.8	+6.6	+11.3	+5.4
6	−2.3	−9.0	−3.6	−0.6	−4.4
7	+2.3	+3.3	−0.1	+3.3	−6.3
8	+3.3	+0.8	+2.0	−0.9	−1.2

Late deaths

Late deaths are not as important as early deaths in the assessment of the mutagenic potential of a test substance [4]. Late deaths were randomly distributed throughtout the groups and did not appear to be treatment-related. This would suggest that the induction of late deaths is independent of the males and that males and females contribute to the effect which is not increased by treatment of the males either with the mutagens or test substance.

Conjoined placentae were seen in this strain. They were the result of very close implantation sites and were not monozygotic twins [3]. In this experiment they were classified as double implantations. Since they occurred with equal frequency in all groups, they did not appear to be correlated with treatment.

Discussion

The best indication of mutagenic activity of a substance in the dominant lethal test is an increase in the number of post-implantational foetal early deaths [4]. From the early deaths data there was no evidence in this study of a mutagenic effect with VC at the administered exposure levels. This did not appear to be a result of lack of sensitivity of the animals used, since there was a marked response to CTX and EMS.

Different evaluation methods were used for the early death data because there is some conflict concerning the commonly-used mutagenic index (early deaths or dead implants/total implants). Epstein et al. [9] have reported a lack of statistical correlation between dead implants and total implants using a weekly mean, whereas Green and Springer [11] do find a correlation, although not a strong one. (The positive correlation indicates that dead implants per female should be adjusted by the total implants for that female — that is essentially what the index does.) The U.S. Food and Drug Administration has analysed all data in dead implants in two ways (both on dead implants/total implants/female and dead implants/female basis) and infers that equally significant data are provided by both [11]. Since there are no standard methods of

analysis both methods have been included in this report, but in an attempt to increase the sensitivity, early deaths only were considered and late deaths were not included. A third method was also used (number of females with one or more early deaths). With all three methods of evaluating the results the lack of effect of VC was consistent. This was in spite of the fact that exceptionally high levels of VC were used in the present study where only 9 out of 20 animals survived at the top exposure level.

Pre-implantation egg losses, whilst representing some of the mutagenic effect, are not as important as the post-implantational losses [4]. Pre-implantation egg losses in the present study have been considered on their own or pooled with post-implantational losses. Pre-implantation egg losses have been indicated by comparing values of total implants in females mated with treated males and those mated with control males, as suggested earlier [8] (as opposed to counting corpora lutea). There was no pre-implantation egg loss by comparison of treated groups with the control except at the highest VC exposure group in week 4. This loss was at a low level of significance ($P < 0.05$). When a positive control is significant there is a uniform reduction of implants, whereas in the highest VC exposure group in week 4 the low result was due to a large extent to the result from one female. Without this female the mean value for total implants per pregnancy would be 11.19 which is not significantly different from the control, and for percentage induced pre- and post-implantational dominant lethality would be 8.7 which lies within the range of normal variation. Therefore the results in group 4 week 4 are not biologically significant.

There was no reduction in fertility as measured by the mating and pregnancy frequency at any week at these exposure levels of VC, suggesting no antifertility effect.

Much of the data was subjected to various methods of statistical analysis, including an analysis of variance. When an analysis of variance is performed, it is assumed that the data are normally distributed, variances are equal in all groups and results are independent. The data for early deaths for individual females had a skewed distribution since there were many females with zero early deaths, so a variance stabilising transformation was used both for the method of determining early deaths/pregnancy and early deaths/total implants/pregnancy. This helped to reduce the skewedness of the distribution of early deaths for individual females. Since the male was the test organism (and the female the indicator organism) the necessary independence of results was provided by taking the male as the basic unit. The statistical analysis then involved estimating missing values for non-pregnant females and using the "between male" variability for assessing statistical significance. The only criterion which was not completely satisfied was the normality of the distribution. An analysis of variance however, is relatively robust to departure from normality, whereas it is not so to departure from independence.

A fertility study was undertaken prior to the experiment because previously unmated males react differently in the first week of mating when compared with the remaining weeks of the study [11] and also to ensure a high fertility throughout the remainder of the experiment. From these initial fertility data the background dominant lethality of all groups was determined, primarily to ascertain that there were no initial differences between groups.

The purpose of the high doses of CTX and EMS used in the present study was to obtain a highly significant positive result. However, the dominant lethal assay in our hands is at least as sensitive as that reported for other studies. We have shown EMS to give a positive result in a single i.p. dose of 150 mg/kg body weight which is comparable to that reported previously for the same mouse strain [14] and HN_2 to give a positive result where it has previously been reported negative [9].

In conclusion, therefore, whilst mutagenic effects of vinyl chloride, as measured by chromosomal aberrations in exposed workers, have been reported [7, 10,13], no such effects as determined in the present study occur in the germ cells of male CD-1 mice.

Acknowledgment

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