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REPEATED DOSE INHALATION TOXICITY OF CINNAMIC ACID SMOKE

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Summary

The repeated dose inhalation toxicity of a white smoke, containing cinnamic acid, has been studied in mice, rats and guinea pigs. The smoke was of low lethal toxicity in the mice and rats, but there was an excess of early deaths amongst the guinea pigs dosed at the highest smoke concentration (304 mg/m^3). Organ-specific changes attributable to the test material were generally confined to the respiratory tract.

Introduction

White smokes have uses, both military and non-military, the latter including simulation in fire practice drills. Some widely-used pyrotechnic compositions, give rise, on ignition, to white smokes which have toxic properties, for example zinc oxide–hexachloroethane smoke. A fog of nascent hydrochloric acid is generated when this composition is ignited, producing, at high concentrations, toxic lung damage, including pulmonary edema [1,2]. In order to limit toxic hazards, there is a clear need for pyrotechnic compositions from which smoke of low toxicity can be generated.

A white smoke, which has proven to be of low toxic hazard in short term animal studies, is cinnamic acid (trans-β-phenylacrylic acid) smoke. Ballantyne and Clifford [3] studied the effects in rats and guinea pigs, of single doses of cinnamic acid smoke at concentrations of 920 mg/m^3 for 30 min or 1630 mg/m^3 for 45 min, these exposure concentrations being respectively 10 and 20 times the expected operational concentrations. All animals survived to the planned time of sacrifice (14 days). In the same paper, a study was described, in which mice, rats, guinea pigs and rabbits were exposed to 5 consecutive daily doses of cinnamic acid smoke (660 mg for 1 h): they all survived to euthanasia at the anticipated times, which were between 1 day and 8 weeks. Neither single nor repeated exposure produced smoke-related histologic changes. In the same study were reported results of a small number of human 10 min exposures to the smoke, at a concentration of 70 mg/m^3. Mild rhinorrhea, lachrymation and

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coughing were seen and small increases in the respiratory rate, tidal volume and minute volume, observed after exposure, lasted from 5 to 10 min. Forced vital capacity, forced expiratory volume over 1 s (FEV1) remained normal, while the peak flow rate showed an increase that was not statistically significant.

Cinnamic acid itself is a compound of relatively low acute toxicity [4]. Zaitsev and Rakhmanina [5] quoted an oral "average LD_{50} in mice, rats and guinea pigs" of 3.4 g/kg, and in general the toxicity of orally administered cinnamic acid or of its sodium salt is low [6,7].

Cinnamic acid is reported by Sax [8] to give rise to allergy and Peruvian balsam, which contains cinnamic acid, as well as benzoic acid, cinnamaldehyde and benzaldehyde, produces skin contact reactions in patients with dermatoses [9,10]. Direct evidence that pure cinnamic acid, or its inorganic salts can cause such reactions, is exiguous. Furthermore, there was no indication of idiosyncratic responses during the human volunteer [3] exposures discussed above.

For the foregoing reasons cinnamic acid smoke would appear a promising candidate to replace more toxic white smokes. Accordingly we have studied the effects on mice, rats and guinea pigs of repeated exposure to this smoke over a period of 40 weeks, the aim being to define the subchronic respiratory toxicity and to unmask any extra-respiratory organ-specific toxicity.

Method

Animals

Four hundred 27–33 d old Porton-strain female mice, 200 22–28 d old Porton Wistar-derived female rats and 200 22–33 d old Dunkin–Hartley female guinea pigs were supplied by the animal breeding unit, CDE (see Table 1). The animals were randomly allocated to equal sized groups designated controls, low, medium and high. Because of deaths after supply, but before the start of exposure, two of the mouse dose groups contained less than one hundred animals. In the case of the rats, group size was 50 in all cases. In the case of the guinea pigs, where 8 deaths occurred between supply and randomization, 48 were allocated to each dose group. The animals of all species were then divided into subgroups of five per cage. Special precautions were not taken to prevent hud-

TABLE 1

Mean weight of animals at the start of exposure (g)

Dose group	Mice	Rats	Guinea pigs
Control	25.1	93.5	272.5
Low	24.6	93.7	275.6
Medium	24.2	92.1	271.6
High	24.5	90.7	261.5

dling, since this phenomenon appears not to affect the inhaled dose of toxicant [11]. Between exposures, the animals were housed in a special accomodation near the inhalation unit, and then, after the last exposure, transfered to permanent accomodation.

Generation of the smoke

The pyrotechnic mixture was obtained from ROF, Glascoed (now, Royal Ordnance PLC, Ammunition Division, Glascoed, Gwent NP5 1XL, Wales). The pyrotechnic mixture (Table 2) was in the form of thick washers of mean weight 2 g. They were ignited on electrically heated nichrome wire, the concentration of the smoke being varied by using different numbers of washers. The resulting white smoke was mixed in a static 10 m^3 chamber with high velocity jets.

Exposure

The animals were exposed to the freshly generated cinnamic acid smoke, 1 h/d, 5 d/week, until they had undergone 200 exposures (i.e. 40 weeks). Corresponding groups of each species were exposed together, starting with exposure of the controls to air within the chamber and ending with the high dose group. As the concentration of smoke declined during exposure, fresh washers of the smoke composition were ignited, to maintain the desired concentration. During the half-hour interval between exposure of the various dose groups, the chamber was cleaned of ordure and detritus: at the end of the high dose exposure, the chamber was well washed twice.

During the exposures, the smoke was sampled by drawing air through absolute fiber-glass filters for a known time. Three samples of 20 min duration, 6 samples of 10 min and 8 samples of 5 min were taken in the low, medium and high dose exposures respectively. The impacted mass of smoke having been weighed, the concentration could be calculated. The cinnamic acid content of the smoke was determined, after elution with ethanol; the cinnamic acid content of eluate was determined spectrophotometrically at 272 nm, using a calibration curve, previously prepared with ethanolic solutions of pure cinnamic acid. The intended ratio of concentration and dose (concentration $\times$ time, Ct)

TABLE 2

Composition by weight of the pyrotechnic mixture used in the study (%)

Constituent	Wt.%
Cinnamic acid	20.0
Potassium chlorate	32.5
Lactose	32.5
Kaolin	15.0

was 0:3:10:30. The mass median diameter of the smoke was assessed using an Anderson particle sizer.

During the exposure period animals were examined for abnormal behavior or ill-health. They were weighed weekly during exposure, and monthly during the ensuing observation period. Animals showing signs of ill-health were sacrificed using pentobarbitone sodium: those alive at the end of the study (17 months after the start of exposure) were similarly killed. Animals sacrificed or found dead were examined post mortem. At autopsy lungs, larynx, trachea, liver, kidneys, spleen, thymus, adrenals, ovaries, cervical lymph node, stomach, small and large intestine were taken, as well as any other organ macroscopically abnormal. After fixation in neutral buffered formalin, sections 3 μm thick were cut and stained with haematoxylin and eosin. In addition any organ observed at autopsy to be abnormal was subjected to processing. Histologic data were recorded and analysed using a PLACES data acquisition system, Apoloco, 90 King Street, Newcastle-under-Lyme, Staffs, England, ST5 1JB. This package was run on a VAX minicomputer (Digital Equipment Corporation, Maynard, Mass., U.S.A.)

Statistics

The frequency of histologic changes in animals from the test groups was compared to the frequency in the corresponding control groups, using Fisher's exact test. In some cases the histologic changes were graded. In that case, since the data for the lower grades do not represent independent data, either the frequency of the severe form, or most severe two forms alone was analysed, or that of all grades. The effect of exposure to the smoke on weight gain was assessed as follows: the mean weight of the test group of animals was divided by the mean weight of the controls. This quantity was regressed against time, separately for the exposure and observation periods. The significance of these regressions was estimated using an F-test with a linear fit.

Results

Analysis

The mean smoke concentrations achieved were in close accord with the intended concentrations (Table 3). The particulate matter contained 64% cinnamic acid by weight. The mass median diameter of the smoke was 1 μm, with a geometric standard deviation of 2, using a lognormal distribution.

Decedents

The number of decedents (Table 4), was similar in all dose groups of rats. Amongst the mice the largest number of early deaths was in the medium dose group. Of the three species only guinea pigs showed curtailment of longevity that appeared to be treatment related. In this species, 11/48 animals from the

TABLE 3

Concentration of cinnamic acid smoke during the study. The aimals were exposed 1 h/d, 5 d/week until they had received 200 exposures: for details of sampling see text. Total mass of particulate material: this contained 64% cinnamic acid by weight

Dose group	Total weight (mg/m^3)	
	Mean	S.D.
Control	0	0
Low	31.6	4.0
Medium	102.0	7.6
High	304.1	17.2

TABLE 4

Number of animals dying or sacrificed in extremis during the study as a proportion of the total at the start of exposure in that group

Species	Dose group			
	Control	Low	Medium	High
Mice	26/100	20/99	33/100	21/99
Rats	7/50	6/50	5/50	6/50
Guinea pigs	3/48	2/48	5/48	11/48

high dose group died before the end of the study, predominantly during the exposure period.

When examined histologically, changes in the mice appeared to be incidental; thus three alveologenic carcinomas were seen in both the control and low dose mice, whilst none was observed in the medium dose group and two in the high dose group. Leukemic infiltration was seen in a few animals, while non-neoplastic changes included some chronic murine pneumonia. In the rats, where the number of decedents was small, changes also appeared to be incidental.

The guinea pig decedents differed from the preceding species in that there was definite evidence of organ-specific toxicity. In the lungs there were considerably more changes in the high dose group, and, to a lesser extent, the middle dose group, than in the controls. The main change was moderate to severe consolidation accompanied by a variable degree of inflammatory cell infiltration. The cellular elements were mostly macrophages and lymphocytes whilst pseudoeosinophils were not prominent. This change affected the lungs of 7/11 and 3/5 decedents in the high and medium dose groups respectively.

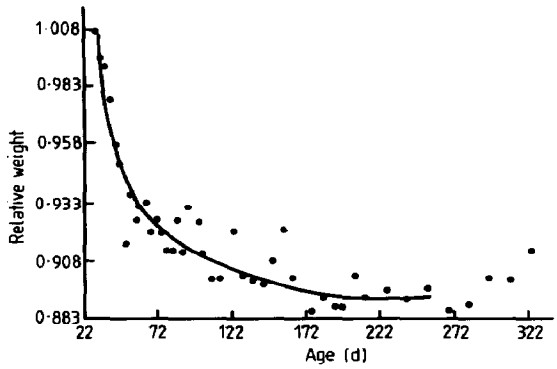

Fig. 1. Weight of high dose group of mice relative to the control group, during exposure and at the beginning of the observation period.

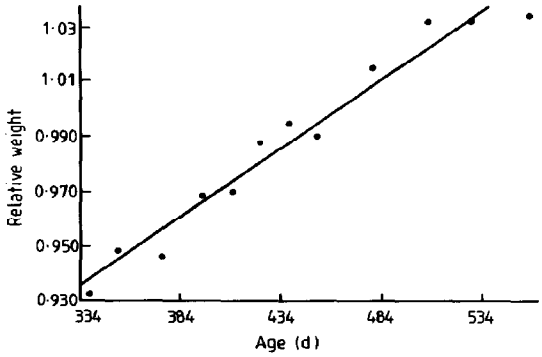

Fig. 2. Weight of the high dose group of mice relative to the controls, during the observation period.

Growth

In the case of the low dose groups of all three species and in the medium dose guinea pigs effects on weight-gain were minimal. The general pattern in all other test groups was for the group weight relative to the weight of the control group to fall during exposure and the regressions were statistically significant in the cases of all three high dose groups as well as the medium dose groups of mice and rats ($p<0.01$). There was a greater or lesser degree of recovery throughout the observation period. The effect in a representative group, the high dose mice, can be seen in Fig. 1: there was a marked fall in relative weight especially early in the study. The recovery which followed can be seen in Fig. 2.

Survivors

The prevalence of selected pathologic changes observed in the mice killed at the end of the study is presented in Table 5. There was little evidence of test

TABLE 5

Selected pathology in mice surviving to the end of the study (total survivors in each group is in brackets)

Organ	Histologic change	Dose group			
		Control	Low	Medium	High
		(74)	(79)†	(67)	(78)†
Larynx	Lymphocytic infiltration	35	33	19	23
Trachea	Lymphocytic infiltration	4	1	3	0
	Pus in the lumen	3	0	1	0
Lungs	Alveologenic carcinoma	13	3[a]	8	15
	chronic murine pneumonia	16	7	13	5[a]
Kidneys	Cortical lymphocytic Infiltration				
	All grades of severity	22	44**	36*	41*
Liver	Hepatoma	1	5	1	3
	Hemangioma	0	1	0	0
Tumors in other organs					
Lymphoma		0	1	0	1
Lymphosarcoma		0	0	1	0
Ovarian sarcoma, ill-differentiated		0	0	1	0
Ovarian tubular adenoma		0	1	0	0
Serous cystadenoma		1	1	0	0
Papillary cystadenoma		1	1	0	0
Thyroid adenoma		0	0	1	0
Endometrial sarcoma (uterus)		0	1	0	0

*$p < 0.05$; frequency of change significantly greater than controls.
**$p < 0.01$; frequency of change significantly greater than controls.
[a]$p < 0.05$; frequency of change significantly less than controls.
†Group size 99.

material-related abnormality in the respiratory tract. Lymphocytic infiltration was seen in all the experimental groups, frequently in the larynx, but much less commonly in the trachea. Frank pus was found in the lumen of the latter organ at autopsy of a few animals and this was also seen on histologic examination. The lungs resembled the larynx and trachea in that histologic changes did not appear to be treatment-related; alveologenic carcinoma was most prevalent in the controls and high dose groups, less so in the medium group and distinctly infrequent in the low dose animals. Chronic murine pneumonia was more frequent in the controls than any of the test groups. Aggregations of lymphocytes in the lungs, generally perivascular or peribronchial were frequent in all groups.

The only indication of organ specific toxicity amongst the mice was in the kidney: in that organ lymphocytic infiltration of the cortex was significantly

more common in all the test groups than in the controls ($p<0.05$, high and medium versus controls; $p<0.01$, low versus controls). This was a reflection of a very high frequency of this change, graded very slight in the three test groups, for which only partial compensation was provided by a higher frequency of the same change graded slight in the controls (Table 6). Differences in the prevalence of lymphocytic infiltration in the calyx were mainly a reflection of the very high number graded as slight in the controls. No other indication of nephrotoxicity was found. There was a paucity of pathologic changes in the liver; nevertheless a small number of hepatomas was found together with an hemangioma. In the other organs examined neither neoplastic nor non-neoplastic changes were frequent, and no differences were observed in the frequency of their occurrence that approached statistical significance.

In contradistinction to the mice lymphocytic infiltration was rare in the rat larynx and trachea. Dilated mucus glands were frequently seen, in the former organ, in all groups (Table 7). In the lungs, lymphocytic aggregations were often observed; perivascular ones were significantly more frequent in the high dose animals than the controls ($p<0.01$). A few instances of macrophage infiltration were seen, some of the macrophages being granular. Alveolitis, sometimes focal was found in all three test groups but was absent from the control group: the difference between the controls and test was statistically significant in the case of the high dose group animals ($p<0.05$).

Nephropathy was frequent in the rats and often accompanied by deposition of calcium. This change, which also occurred independently, was more prevalent in the low dose animals than the controls ($p<0.05$, Table 7). It was also seen more often in the low dose group than in the two highest dose groups but the difference did not approach statistical significance at the 5% level. Hepatic fibrosis of some degree was observed with moderate frequency in all dose groups. In some cases it verged on cholangiofibrosis. There were one or two instances

TABLE 6

Frequency of lymphocytic infiltration, in the renal cortex and calyx, in mice surviving to the end of the study, graded according to severity

		Control	Low	Medium	High
	Total	74	79	67	78
Cortical	Very slight	4	27	18	26
	Slight	14	6	8	5
	Moderate	2	7	9	5
	Severe	2	4	1	5
Calyceal	Very slight	9	33	28	39
	Slight	37	4	8	4
	Moderate	1	10	10	10
	Severe	1	6	1	2

TABLE 7

Selected pathology in rats surviving to the end of the study (total survivors in each group is in brackets)

Organ	Histologic change	Dose group			
		Control	Low	Medium	High
		(43)	(44)	(45)	(43)[a]
Larynx	Mucus glands-dilated	10	19	17	12
Trachea	Mucus glands-dilated	6	2	2	0
Lungs	Peribronchial lymphocytes	35	42*	41	41
	Perivascular lymphocytes	7	2	15	23**
	Alveolitis	0	2	4	7*
Kidney	Nephropathy	38	33	32	26[b]
	Calcium deposition	30	40*	33	33
Liver	Fibrosis	15	20	19	13
Tumors in other organs					
Adenoma of mammary gland		0	1	1	1
Fibroadenoma of mammary gland		1	1	0	0
Lipoma in abdominal cavity		2	2	0	1
Mesenteric lipoma		0	0	0	1
Adenocarcinoma of salivary gland		0	0	0	1

*$p < 0.05$; frequency of change significantly greater than controls.
**$p < 0.01$; frequency of change significantly greater than controls.
[a]Excluding one animal.
[b]$p < 0.05$; frequency of change significantly less than control.

of lymphocytic foci in the liver. Tumors were only infrequently seen. Cystic change in the thymus was significantly more frequently seen in the high dose group thymuses than the controls ($p < 0.05$, data not shown).

Inflammatory changes of any sort were uncommon in the larynx or trachea in the guinea pigs (Table 8). By contrast the lungs were frequently abnormal. Thus consolidation, most often focal, was significantly more frequent in the low dose group than the controls, but it was often seen in all dose groups. Alveolitis was infrequently observed, but lymphoid foci were extremely common. Some degree of alveolar thickening was present in approximately equal proportions of all groups of guinea pigs, but when graded, the most severe form of it was significantly more frequent in all the test groups than the controls ($p < 0.01$, except high vs. controls, $p < 0.05$).

Chronic interstitial nephritis and calcium deposition were very frequent, the latter being more common in the controls than in the test animals (Table 7). In the case of both these renal changes, the majority of instances were graded slight in degree, most of the remainder being moderate and only a few severe. Neoplasms were extremely uncommon in the guinea pigs.

TABLE 8

Selected pathology in guinea pigs surviving to the end of the study (total survivors in each group is in brackets)

Organ	Histologic change	Dose group			
		Control	Low	Medium	High
		(45)	(46)	(43)	(37)
Larynx	Lymphocytic infiltration	1	0	0	1
Trachea	Lymphocytic infiltration	2	0	0	0
Lungs	Focal consolidation	38	46*	41	36
	Alveolitis	1	1	0	0
	Lymphoid foci	41	46	42	36
	Alveolar thickening (all grades)	44	46	43	37
	Alveolar thickening — moderate + severe grades	8	34**	22**	19*
Kidneys	Chronic interstitial nephritis	41	36	38	32
	Calcium deposition	43	35[b]	30[b]	31[a]
Tumors in other organs					
Thyroid adenoma		1	0	1	0
Skin papilloma		1	0	0	0
Abdominal lipoma		0	2	0	0

*$p < 0.05$; frequency of change significantly greater than controls.
**$p < 0.01$; frequency of change significantly greater than controls.
[a]$p < 0.05$; frequency of change significantly less than controls.
[b]$p < 0.01$; frequency of change significantly less than controls.

Discussion

In two out of the three species cinnamic acid smoke had little effect on survival. The exception, the guinea pig, probably exemplifies that species' sensitivity to inhaled particulates [12]. The phenomenon was, however, less severe than in some previous smoke or aerosol inhalation studies, conducted in this laboratory [13,14], where exposure of the high dose group had to be stopped early, because of high mortality in this species.

In the case of the mice and rats, the observed no effect concentration, with respect to growth, was 30 mg/m^3. For the guinea pig it was probably higher.

Most of the pathologic changes observed in the mice were incidental, since they were seen as often in the control as the test groups. Moreover many were indicative of well-recognised spontaneous lesions in mice: thus alveologenic carcinoma of the lung is frequent in the Porton mouse [13,14], as in many other strains [15,16]. Chronic murine pneumonia is also a common feature of laboratory mice, which may be viral in origin [17]. Renal disease is frequent

in all small laboratory animals, and chronic interstitial nephritis is seen in a high proportion of Porton mice [13]. The finding of a statistically significant excess of renal cortical lymphocytic infiltration is difficult to explain as an incidental finding. Its biologic significance is hard to assess, for its impact is blunted when the graded findings are considered. Moreover other renal pathologic change was not present. Nor was there, in the low dose group, impairment of growth, a common accompaniment of functional renal impairment.

Most of the changes that were observed in the rats were also, doubtless, incidental. Thus nephropathy, which has been called by a variety of names including chronic nephritis, chronic interstitial nephritis, glomerulonephrosis as well as nephrosis, is a common condition in rats [18–21]. Unlikely to be an incidental finding was the high frequency of perivascular lymphocytic infiltration in lungs of the top dose rats; this indicated some smoke induced chronic inflammatory change.

In the guinea pigs there were both incidental and a few test material-related changes. Amongst the latter may be pulmonary consolidation and alveolar thickening, seen in the survivors as well as the consolidation seen in the decedents. Amongst the incidental findings in the guinea pigs were lymphoid nodules [22], a phenomenon still unexplained [23] and chronic interstitial nephritis [22]. Tumors were, as usual, rare in the guinea pigs [24].

Cinnamic acid smoke thus gave rise to only mild and non-specific changes in the respiratory tract. Only in the kidney was there any suggestion of organ-specific toxicity outside the respiratory tract. This renal lesion, seen in the mice, was not demonstrated by the other two species: even in the mice its impact was blunted when grades of severity were considered. Previous studies of the toxicity of cinnamic acid do not indicate major toxicity from this source [3–7]: the other components of the pyrotechnic mixture, potassium chlorate and lactose, simply give rise to carbon dioxide, water and potassium chloride [25], all relatively innocuous. Furthermore, studies of the gaseous effluent after detonation of cinnamic acid grenades [3] showed only carbon dioxide and monoxide with small amounts of methane and oxides of nitrogen.

Other white smokes, particularly those generated from zinc oxide and hexachloroethane, have notable acute toxicities in animals [1,2], which cinnamic acid smoke appears to lack [3]. Moreover repeated exposure in the present study only curtailed the life span of the guinea pigs, and that at the highest concentration. It is likely that cinnamic acid smoke would be significantly safer than existing white smokes.

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