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Coalinga Fibre – A Short, Amphibole-Free Chrysotile

Part 2: Evidence for Lack of Tumourigenic Activity

Key Words
Short fibre chrysotile
Coalinga
Jeffrey
UICC/B
Tumours

Abstract
Controversy continues to surround the biological activity of short fibre chrysotile largely due to a lack of 'pure exposure' situations available for study: most human exposures are confounded by concomitant long fibre and/or amphibole exposure. This report presents the morphological and morphometric findings of a lifetime inhalation study of F344 rats exposed to three types of chrysotile. Fibres from the first sample, from Coalinga, Calif., are almost all less than 5 μm in length and do not contain amphibole types of asbestos. The other two, from Quebec, Canada, are a sample from the Jeffrey mine and the UICC/B standard. These are both long fibre preparations with a minor degree of amphibole contamination. Animals exposed to these fibres displayed no tumours above control levels following exposure to Coalinga chrysotile but gave significant tumourigenic responses with both types of Canadian fibres.

Introduction

The two major theories often cited to explain asbestos-related disease, the 'Stanton' [1, 2] and the 'amphibole' [3, 4] hypotheses, would lead one to assume that short, amphibole-free chrysotile is the least tumourigenic form of asbestos. This proposal, previously put forth by Davis [5], is supported by earlier studies especially those using injection techniques [5, 6]. Early inhalation studies [7–11] demonstrated that short fibre chrysotile was less biologically potent than long. However, the inability of researchers to obtain sufficient quantities of 'pure' short fibre chrysotile, devoid of significant numbers of long fibres, confounded interpretation of the experiments. One of the first long-term inhalation studies to use a highly purified, short fibre chrysotile sample was conducted by Platek et al. [12], who exposed rodents and primates to a preparation produced by intense milling. Although no tumours were obtained, other workers [6] felt that the study could have been confounded by a loss of crystallinity induced by the milling with a consequent loss of biological activity [13]. Davis and Jones [6] attempted to circumvent this problem by using a short chrysotile fibre sample prepared without milling but were unable to remove many of the long fibres, and a significant tumour response was still obtained. Although Davis and Jones [6] concluded that the shorter chrysotile fibres might have had some carcinogenic activity, they cast doubt on this interpretation since the highly purified, short fibre amosite preparation, used in one of their other inhalation studies [14], failed to produce any tumours. This was further confirmed by a reassessment based on transmission electron microscopy of all of Davis' inhalation studies [15] which led these authors to conclude that fibres less than 5 μm in length were not tumourigenic.

The present study is based upon a lifetime investigation of F344 rats exposed by inhalation to Coalinga chrysotile and two Canadian long fibre preparations, which

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was performed at the National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP) between 1978 and 1980. Coalinga chrysotile, a naturally occurring, amphibole-free, short fibre chrysotile, is probably the mineralogically purest sample ever to be tested experimentally and contains virtually no long fibres [Chatfield and Ilgren, in preparation]. The other two chrysotiles are from Quebec, Canada, and are samples from the Jeffrey mine and the UICC/B standard [16]. In the first part of this investigation we demonstrated that Coalinga chrysotile was not fibrogenic [16]. Here, we are able to show clearly that it also lacks tumourigenic potential. Taken together, these findings further support our earlier proposal [16] that short fibre chrysotile is innocuous and should be regarded and regulated as a nuisance dust.

Materials and Methods

Information pertaining to the source of materials including data and animals, the chrysotile preparations, exposure conditions, animal sacrifice, tissue distribution and sample preparation, as well as morphometric and statistical analyses are as described by Ilgren and Chatfield [16]. Histologically, the tumours reported here were classified after McConnell et al. [17] as adenomas, adenocarcinomas, squamous cell carcinomas or adenosquamous carcinomas.

For brevity, from hereon the different chrysotile samples are referred to by their descriptors.

Table 1. Incidence of pulmonary neoplasia in F344 rats on lifetime test exposed to Coalinga, UICC/B and Jeffrey chrysotile fibres

Treatment	Sex	Total animals with tumours						
		n1	n2	BAH	Ad	Ca	Meso	total
Control	**M**	**30**	**27**	**0**	**0**	**0**	**0**	**2 (7.4)**
[1998]	**F**	**30**	**26**	**0**	**0**	**0**	**0**	**0 (0)**
Control	M	28	24	1	0	0	0	0 (0)
[1984, MRC]	F	28	24	0	0	0	0	0 (0)
Control	M	30	27	0	1	2	0	3 (10)
[1984, NIEHS]	F	30	26	0	0	0	0	0 (0)
Control	M	2,320	–	nd	35	25	0	60 (2.7)
[1984, NIEHS]	F	2,320	–	nd	18	10	0	28 (1.3)
Control	M	529	–	nd	3	12	1	16 (2.7)
[1984, NIEHS]	F	529	–	nd	1	7	0	8 (1.5)
Coalinga	**M**	**30**	**27**	**3**	**0**	**2**	**0**	**2 (7.4)**
[1998]	**F**	**30**	**24**	**3**	**0**	**0**	**0**	**0 (0)**
UICC	**M**	**30**	**29**	**2**	**1**	**8**	**0**	**10 (35)**
[1998]	**F**	**30**	**25**	**1**	**1**	**3**	**0**	**4 (12)**
UICC	M	28	24	3	1	6	0	7 (29)
[1984, MRC]	F	28	24	2	0	5	0	5 (21)
UICC	M	30	29	2	3	6	0	9 (31)
[1984, NIEHS]	F	30	27	3	1	1	0	2 (7.4)
Jeffrey	**M**	**30**	**25**	**1**	**1**	**9**	**0**	**10 (40)**
[1998]	**F**	**30**	**24**	**2**	**0**	**1**	**0**	**1 (4.2)**

Pulmonary neoplasia = most severe (unquestionable) lesion; n1 = number of animals at risk at the start of the study; n2 = number of animals examined histopathologically at the end of the study; BAH = bronchiolar adenomatous hyperplasia (see McConnell et al. [17] for discussion); Ad = adenoma; Ca = carcinoma; Meso = mesothelioma. Figures in parentheses indicate percentages. MRC = Medical Research Council Pneumoconiosis Unit, Penarth, UK; NIEHS National Institute of Environmental Health Sciences, Research Triangle Park, N.C., USA; 1998 = this study (Ilgren and Chatfield, 1998) the data for which have been put in bold typescript; 1984 study of the control and UICC/B-treated animals from this investigation previously assessed by McConnell et al. [17] and Wagner et al. [18]; the results from Coalinga- and Jeffrey-treated animals in these studies have not previously been presented.

Table 2a. UICC/B-treated males and females – lifetime test: non-pulmonary neoplasia

Case No.	Animal No.	Fibrosis score	Primary tumour	Secondary tumour	Extrapulmonary tumours
Males (16.6%, 5/30, probably lethal non-pulmonary tumours; 33%, 10/30, lung tumours)					
3	130	7	AdCa (BAH)	none	testicular, ?mesothelioma
4	113	5	Ad	none	pituitary; testicular
5	177	5	AdCa	none	none
6	203	5	none	none	adrenal, malignant ganglioneuroma (2 cm)
7	207	6 + d	none	none	none
8	152	5	none	none	none
10	204	5	(BAH)	none	testicular
11	210	5	none	none	testicular
12	241	6	Ad	none	none
13	168	5	none	none	testicular
14	105	4	none	adrenal?	metastases to liver, pancreas, lung
15	36	6 + d	none	none	thymic lymphoma with metastases to liver, kidney, lung
16	211	6 + d	none	none	SqCa mouth; fibroma neck
17	219	5 + d	none	none	testicular
18	28	5	none	none	testicular
19	40	5	AdCa (metastasis)	none	thymic adenoma; probably metastases from lung to cervical spine
20	82	6 + d	(BAH)	none	none
22	133	6	none	none	testicular
23	99	6	none	none	testicular
24	72	6	none	none	none
25	56	5 + d	AdCa	none	pancreatic islet cell (small); pituitary
26	212	6 + d	AdCa	none	pituitary
28	79	5 + d	none	none	synovial sarcoma, arm; leukaemic infiltration liver
29	234	6 + d	AdCa	none	none
30	32	6	none	none	none
1 (L)[1]	176	–	AdCa	none	subcutaneous fibroma; leukaemia spleen, lungs
2 (MS)	236	–	AdCa	n.d.	
9 (A)	117	–	none	none	n.d.
21 (A)[2]	224	–	n.a.	n.a.	n.d.
27 (L)	225	–	none	none	leukaemia lungs, liver
Females (83.3%, 20/24, probably lethal non-pulmonary tumours; 12.5%, 3/24, lung tumours)					
1	304	6	(BAH)	mammary glands	splenic leukaemia; mammary AdCa, metastases
2	459	6	none	none	n.a. – largely cannibalized
3	325	6	none	none	liver leukaemic infiltration; splenic lymphoma, pituitary Ad
5	323	6 + dust	none	none	pituitary
6	364	4	AdCa	none	uterine/ovarian tumour (gross; no micro)
7	390	6 + d	none	none	cut keratoma; sarcoma,? site; pituitary
8	411	6	none	none	sebaceous gland; Ad; pituitary; ovarian cyst
9	548	6 + d	none	uterus	uterine AdCa; pituitary
10	321	5 + d	none	none	adrenal Ad; leukaemia; uterine (gross)
11	361	6	(?)!!	none	pituitary Ad; probably uterine and fallopian tube AdCa
13	496	4	none	uterus	uterine AdCa; pituitary
14	371	5 + d	none	uterus**	vaginal Ca; leukaemia; uterine (gross)
15	478	5	none	pancreas	pituitary; pancreatic Ca widely metastatic
16	398	6 + d	none	neuro-sarcoma	abdominal sarcoma, widely metastatic
17	313	5 + d	none	none	none
18	387	5 + d	AdCa	uterus	uterine AdCa, widely metastatic
19	439	5 + d	none	uterus	uterine AdCa, widely metastatic
20	381	5	none	none	none
21	435	5 + d	none	none	mammary tumour (gross); uterine AdCa, massive
23	318	5 + d	none	none	fallopian tube Ca
24	410	5 + d	adenoma	none	pituitary; uterine and fallopian tube Ca
26	363	5 + d	none	none	liver leukaemia; caecal nodule
22 (L)	345	–	none	none	lymphosarcoma, widely metastic
25 (A)	329	–	none	none	uterine AdCa, massive

Table 2b. Jeffrey-fibre-treated males and females – lifetime test: non-pulmonary neoplasia

Case No.	Animal No.	Fibrosis score	Primary tumour	Secondary tumour	Extrapulmonary tumours
Males (8.3%, 2/24, probably lethal non-pulmonary tumours; 36%, 9/25, lung tumours)					
1	178	4 + d	AdCa	none	testicular; jejunal nodule
2	84	6 + d	(BAH)	none	pituitary Ad
3	153	5 + d	AdSqCa	none	pituitary Ad
4	141	6 + d	none	none	splenic lymphoma
5	208	7 + d	none	none	colonic nodule
6	173	6 + d	none	none	none
7	44	6 + d	AdCa	none	testicular (mesothelioma)
8	114	4	none	none	tumour in inguinal area (no histopathology)
9	166	5	none	none	splenic lymphoma
10	209	6 + d	none	lymphoma	pituitary Ad; thymic lymphoma in liver
11	27	4 + d	none	none	none
12	185	5 + d	none	none	splenic lymphoma
13	53	5	AdCa	none	splenic lymphoma; testicular
14	137	6 + d	none	none	probable adrenal pheochromocytoma
15	214	5	none	none	thymic lymphoma; jejunal cyst; testicular
16	198	6	none	none	splenic lymphoma; testicular
17	144	7	AdCa	none	testicular
18	159	6 + d	none	none	none
19	52	6 + d	AdCa	none	lymphoma; testicular
20	142	7 + d	Sq cell Ca	none	testicular
21	184	6 + d	none	none	pituitary
22	216	6	AdCa	none	splenic lymphoma
24	141	4	none	none	none
25	192	6 + d	none	none	splenic lymphoma in liver; testicular
26	237	6 + d	AdSqCa	none	pituitary adenoma
Females (50%, 13/ 26, probably lethal non-pulmonary tumours; 3.9%, 1/26, lung tumours)					
1	479	5	AdCa	none	none
2	388	6	none	ovarian	thymic lymphoma; pheochromocytoma; ovarian AdCa and ++ hepatic metastasis
3	504	6 + d	none	none	none
4	358	6	none	none	splenic lymphoma (6 cm) in liver
5	352	6 + d	none	skin	*cutaneous tumour, 1 cm, near jaw*
6	489	5	none	none	uterine AdCa (6 cm)
7 –	462	4	none	none	splenic lymphoma (probable)
8	412	6	none	none	none
9	502	7	none	fallopian tube	none
11	480	6	none	none	ovarian AdCa (8 cm)
12	433	5	none	none	none
13	374	7	none	none	splenic lymphoma (massive) and uterine AdCa (massive)
14	468	4	none	uterus	uterine AdCa invading spleen, liver, ovaries
16	493	6	none	none	none
17	389	6 + d	none	none	none
18	344	6 + d	none	none	splenic lymphoma (massive)
19	485	6 + d	none	none	none
20	515	7 + d	none	none	ovarian AdCa (4 cm, probable)
21	399	6 + d	none	uterus	uterine AdCa (large); splenic lymphoma (probable)
22	326	6 + d	none	none	splenic lymphoma (probable)
23	408	7 + d	none	none	none
24	512	6 + d	none	pancreas	pancreatic AdCa with invasion of liver, stomach, uterus
25	317	6 + d	none	uterus	splenic lymphoma and uterine AdCa (massive) with metastasis to adrenal gland
15	487	MS	(none)	(none)	uterine and preputial AdCa (probable)
26	341	MS	(none)	(none)	no information – necropsy report missing

Ad = Adenoma; AdCa = adenocarcinoma; BAH = bronchiolar adenomatous hyperplasia (see McConnell et al. [17] for discussion); d = dust; Sq = squamous; Ca = carcinoma; L = leukemia; n.d. = not detected; n.s. = not stated; A = autolysis; n.a. = not available; !! = lung tumour reported in necropsy report but not found in slides; MS = missing slides.

[1] Cases on lifetime test that could not be read due to autolysis.

[2] Cases on lifetime test that could not be read due to leukaemia.

Table 3. Alveolar epithelial type I cell volume (mm^3), number ($\times 10^6$) and surface area (μm^2) in F344 rats

	Sex	Control	Coalinga	UICC/B	Jeffrey
Cell volume					
3 months	M	81±6	112±3[a]	93±8	95±2
		0.08±0.01 cm^3 [a]	0.11±0.00[a] cm^3 [a]	n.d.	0.09±0.00[a] cm^3 [a]
		1,469±116 μm^3 [a]	2,523±156[a] μm^3 [a]	n.d.	1,877±109[a] μm^3 [a]
	F	78±7	80±6	63±5	79±5
12 months	M	83±3	113±2[a]	113±12[a]	155±7[a,b,c]
	F	57±7	64±8	67±8	133±9[a,b,c]
24 months	M	84±8	114±15	116±7	121±22
	F	66±6	66±8	79±5	87±8[c]
Cell number					
3 months	M	53±1 [a, b, c]	43±4 [a]	56±3	48±3 [a, c, d]
	F	39±5 [b, c]	36±3	43±3	32±2 [c, d]
12 months	M	62±6 [b, c]	57±2	57±5	102±4[a,b,c] [c, d]
	F	47±4 [b, c]	38±3	44±1	60±4[c] [c, d]
24 months	M	59±7 [b, c]	46±3	67±5[a,c]	74±9[a,c] [c]
Cell surface area					
3 months	M+F	8,919±941 [e]	9,898±581 [e]	8,348±544 [e]	9,259±418 [e]
12 months	M+F	8,013±562 [e]	8,832±276 [e]	7,412±605 [e]	6,200±660[a] [e]
24 months	M+F	9,189±538 [e]	8,480±777 [e]	6,090±569[a] [e]	6,608±604[a] [e]

All data are means ± SEM; n = 4 for each group and time period; [a] $p < 0.05$ when comparing to the age-matched control values using Duncan's multiple-comparison test after first testing for significance using a one-way ANOVA; [b] $p < 0.05$ when comparing the group exposed to Jeffrey chrysotile with all other treatment groups; [c] $p < 0.05$ when comparing one treatment group to its corresponding treatment group of the preceding time point; n.d. = not done.

[ ] = References in which these data have previously been presented in part or wole are replicated here for the sake of comparison: a = these data were in the quarterly report 'Effects of Chronic Exposure to Airborne Environmental Agents', August 1 to October 31, 1978, Becton Dickinson Research Centre, CS Stone; there are small discrepancies between these and those presented by Pinkerton [19]; b = Pinkerton et al. [21], table 7; c = Pinkerton et al. [22], table 3; d = Pinkerton et al. [23], figure 1; e = NTP archives data obtained by the author (E.B.I.) in December 1995.

Results

The incidence of primary pulmonary tumours in Coalinga-treated animals on a lifetime test (3.9%, 2/51) was the same as that of untreated controls (3.8%, 2/53; table 1). In contrast, 22% of the animals exposed to Jeffrey and 26% of those exposed to UICC/B had primary pulmonary tumours: 11/49 (10 carcinomas, 1 adenoma) and 14/54 (12 carcinomas, 2 adenomas), respectively (table 1). There were no mesotheliomas and only one instance of distant metastasis from a pulmonary primary tumour.

The lungs of all tumour-bearing, UICC/B- and Jeffrey-treated animals demonstrated evidence of fibrosis with scores between 5.0 and 6.0. The 2 Coalinga-treated, tumour-bearing animals had fibrosis scores of 3.0 which were probably overestimates due to concomitant uraemic pneumonitis and leukaemic infiltration.

The incidence of primary pulmonary tumours was greater in males than females in both the Jeffrey and the UICC/B treatment groups (table 1). By contrast, the incidence of non-pulmonary tumours was higher in females than males (tables 2a, b). The incidence of non-pulmonary neoplasia was not treatment related.

The incidence of tumours in the interim sacrifice animals could not be determined precisely since some of the records were missing. However, for the 24-month interim sacrifice animals, the available data [19, 20] strongly suggest an absence of tumours in the untreated controls, probably no more than 1 tumour-bearing Coalinga-treated animal and 4 Jeffrey-treated tumour bearing animals in addition to the 2 UICC/B-treated rats with tumours already known to exist in that group [17].

Examination of the lungs of the Coalinga-treated rats sacrificed at 3-, 12- and 24-month intervals by electron microscopy did not reveal ultrastructural epithelial ab-

Table 4. Alveolar epithelial type II cell volume (mm^3) and number ($\times 10^6$) of F344 rats

	Sex	Control	Coalinga	UICC/B	Jeffrey
Cell volume					
3 months	M	36±9	132±14[a]	79±12	**123±27[a]**
		0.04±0.01 cm^3 [a]	0.13±0.01[a] cm^3 [a]	n.d.	**0.12±0.03[a] cm^3 [a]**
		436±104 μm^3 [a]	974±133[a] μm^3 [a]	n.d.	**937±176[a] μm^3 [a]**
	F	25±1	68±6[a]	66±7[a]	**67±5[a]**
12 months	M	30±4	82±4	100±14[a]	**156±37[a]**
	F	20±3	56±11[b]	70±9[a]	**99±7[a,c]**
24 months	M	28±9	62±15	96±21[a]	**73±26[c]**
	F	18±5	29±9[c]	41±11	**70±16[a,c]**
Cell number					
3 months	M	79±7 [b, c]	131±10[a]	121±10[a]	**127±16[a] [c, d]**
	F	49±7 [b, c]	80±11[a]	94±9	**75±5[a] [c, d]**
12 months	M	58±7 [b, c]	115±3[a]	114±9[a]	**220±29[a,b,c] [c, d]**
	F	56±5 [b, c]	92±7[a]	77±4	**138±7[a,b,c] [c, d]**
24 months	M	57±8 [b, c]	118±50	110±7[a]	**124±13[a,c] [c, d]**
	F	50±4 [b, c]	61±9[c]	96±11[a]	**116±12[a,c] [c, d]**

All data are means ± SEM; n = 4 for each group and time period; [a] $p < 0.05$ when comparing to the age-matched control values using Duncan's multiple-comparison test after first testing for significance using a one-way ANOVA; [b] $p < 0.05$ when comparing the group exposed to Jeffrey chrysotile with all other treatment groups; [c] $p < 0.05$ when comparing one treatment group to its corresponding treatment group of the preceding time point; n.d. = not done.

[ ] = References in which these data have previously been presented in part or whole are replicated here for the sake of comparison: a = these data were in the quarterly report 'Effects of Chronic Exposure to Airborne Environmental Agents', August 1 to October 31, 1978, Becton Dickinson Research Centre, CS Stone – there are small discrepancies between these and those presented by Pinkerton [19]; b = Pinkerton et al. [21], table 7; c = Pinkerton et al. [22], table 3; d = Pinkerton et al. [23], figure 1.

normalities. In contrast, prolonged exposure to Jeffrey and UICC/B resulted in dramatic ultrastructural changes in type II epithelial cells at 12 and 24 months which included megalamellar bodies and markedly dilated rough endoplasmic reticulum cisternae (also, for further description of changes noted on untreated and Jeffrey-treated animals see Pinkerton [19], NTP [20] and Pinkerton et al. [21–23] respectively). These qualitative electron-microscopic findings clearly discriminate Coalinga from the Canadian fibres and were confirmed morphometrically using data parameters related to changes in the epithelium as a whole and its type I and type II cellular constituents. Changes in the thickness of the whole epithelium, referenced here as alterations in 'total epithelial volume', are not shown for the sake of brevity but are available upon request from the authors. Data referable to changes in the number, surface area and volume of type I and type II cells are shown in tables 3 and 4. The time course of events within the epithelium and its cellular components during and following exposure to each of the three chrysotiles is classified here as 'acute', 'subchronic' and 'chronic' events for alterations taking place after 3, 12 and 24 months of exposure, respectively.

Over the first 3 months of exposure, acute changes (see above) were generally noted within the total epithelial volume in response to all three types of chrysotile largely due to type II epithelial responses. After 12 months of exposure, all three chrysotile types significantly increased the total epithelial volume though this was far greater for Jeffrey than for UICC/B and Coalinga. Again, this was largely due to type II cell responses. Although the degrees of cellular changes observed with Coalinga and UICC/B were generally similar, UICC/B increased type II cell volume significantly over controls whilst Coalinga did not. By contrast, at 12 months, Jeffrey dramatically increased both type I and type II cell responses, not only above control values, but also above the preceding 3-month time point and the other two treatment groups as well. The Jeffrey-induced type II cell responses were greater than those noted in type I cells. By 24 months, Coalinga was the only

fibre that failed to produce a persistent, significant increase in any epithelial cell parameter. In contrast to Coalinga, the majority of epithelial changes noted following exposure to Jeffrey remained significantly above controls by 24 months, indicative of persistent injury. Some Jeffrey-related, epithelial parameters significant at 12 months fell to non-significant levels by the end of the study suggestive of regression or resolution. By 24 months, the majority of UICC/B-related epithelial tissue measurements were also significantly greater than controls, but the pattern of epithelial changes noted following cessation of exposure was clearly different to those seen with the other two fibre types. Thus, the pattern of changes in the type I and II epithelium in the UICC/B treatment groups displayed, for the most part, either no change or progression. The latter was evident in the significant increase in type I cell numbers over the preceding 12-month values (table 3). In fact, there was only one measured parameter, type I cell volume, that was not significantly greater than in controls by the end of the study period in the UICC/B treatment group. Throughout the 24-month time course, the severity of the observed epithelial changes following UICC/B was greater than that noted with Coalinga but less than with Jeffrey. Irrespective of fibre type, changes which occurred in the type II epithelium were more pronounced than those seen in type I cells. This is reflected in percent changes in the number of alveolar type I and type II cells for the three different fibres. Although the type I cell responses appear to be generally attenuated, the differential responses in type I cell surface area (table 3) still serve to distinguish Coalinga from the two Canadian fibres. Finally, the type II cell responses in females appeared to be less than those noted in males irrespective of fibre type.

Survival and Tumour Formation

Statistical analysis (F test for two-sample variance) did not demonstrate significant differences in length of survival between control and/or Coalinga treated animals with or without primary and/or secondary tumours. However, when the UICC/B and Jeffrey male and female animals without primary tumours were compared with those with primary neoplasms, the observed reduction in survival duration just missed attaining statistical significance ($p = 0.08$). Moreover, when UICC/B- and Jeffrey-exposed females were examined separately with regard to secondary neoplasms, there was a suggestion that the secondary tumours also had reduced survival though again this failed to attain significance [$p = 0.14$ (UICC) and 0.23 (Jeffrey)].

Discussion

Our findings demonstrate that long-term, high-dose exposure to Coalinga fibre (a short, amphibole-free chrysotile) does not induce either tumour formation or persistent epithelial responses. In contrast, the two long fibre Canadian samples tested are both tumourigenic and capable of inducing severe attendant epithelial abnormalities. These results were based upon our own analysis of animals on lifetime test, plus careful review of interim sacrifice data, analysed both morphologically using light microscopy and morphometrically with electron microscopic methods, provided to us by Drs. Kent Pinkerton and Gene McConnell.

Other Studies with Coalinga Chrysotile

The investigation by Muhle et al. [24] is the only other inhalation study of Coalinga chrysotile, and they too failed to find tumours. These workers chose Coalinga as their 'positive' control since they were unable to obtain sufficient amounts of Canadian chrysotile for an inhalation bioassay and they stated that 'when we started our inhalation experiment we expected a carcinogenic potency comparable to that of the UICC chrysotile samples'. Muhle [pers. commun., 1988] was indeed very surprised that 'significant tumour rates were found neither in the inhalation experiment nor after intraperitoneal injection of 0.5 mg'. Although Muhle et al. [24] did not use long fibre chrysotile in these experiments, they did employ a concurrent crocidolite positive control. Unfortunately this also failed to induce tumours, a finding that led some [25] to argue against the claim of Muhle et al. [24] that Coalinga lacked carcinogenic potential. However, Muhle et al. [24] have countered this by stating that 'the high rate of bronchiolar alveolar hyperplasia of 74%, one case of squamous metaplasia, and one adenocarcinoma may indicate a tendency of the crocidolite fibres used to induce neoplasms'. We would support this interpretation and suggest that the high percentage of short fibres in the UICC crocidolite preparation [26] was the most likely explanation for the very low tumour rates noted by the German group and by others [27, 28]. Oberdorster [29] has argued that Coalinga's lack of carcinogenicity is due to its low biopersistence. The evidence to date suggests that Coalinga's failure to persist is due more to its short length and increased mechanical clearance than to intrinsic hypersolubility [30] though further research in this area is clearly warranted [see Chatfield and Ilgren, in prep., for detailed discussion].

Table 5. Intraperitoneal injection studies with Coalinga, UICC/A (Rhodesian) and UICC/B (Canadian) chrysotile

Source	n	Sex	Dose mg	Study duration weeks	Tumor yield %	Author(s)	Year
Coalinga	50	F	0.5	116	6 (2/32)	Muhle et al. [24]	1987
Coalinga	50	F	0.5	142	6.3 (2/32)	Pott et al. [31]	1987
Rhodesian	50	F	0.5	142	56.3 (18/32)	Pott et al. [31]	1987
Canadian	24	M	0.5	99	91.7 (22/24)	Davis [33]	1984
Rhodesian	33	M	0.5	99	81 (26/33)	Bolton et al. [34]	1983
Coalinga	50	F	1.0	131	2 (1/50)	Rittinghausen et al. [32]	1991
Canadian	50	F	1.0	112	62	Rittinghausen et al. [32]	1991
Canadian	36	F	1.0	103	83.35 (27/32)	Pott et al. [31]	1987
Canadian	32	F	1.0	51	84 (27/32)	Muhle et al. [24]	1987
Rhodesian	32	F	1.0	51	84.4 (27/32)	Pott et al. [31]	1987
Coalinga	50	F	3.0	131	10 (5/50)	Rittinghausen et al. [32]	1991
Rhodesian	24	M	2.5	144	97.1 (22/24)	Davis [35]	1988

Coalinga = Fibre from the Coalinga deposit mined by Union Carbide Corp. (Calidria ^{T14}).

Muhle et al. [24], and other German workers [31, 32], conducted three separate, well-controlled injection studies with Coalinga and Canadian chrysotiles all of which have convincingly demonstrated Coalinga's lack of carcinogenicity (table 5). All of these studies utilized large numbers of animals (n = 50) and dose levels low enough to avoid non-specific, high-dose, mass effects [29, 36–38]. An Italian group, represented by Maltoni and his colleagues [39, 40], found results opposite to those of Muhle and his colleagues. However, their chrysotile sample was labelled 'California', not Coalinga, and could thus have been from one of the many serpentine ore bodies in that State. Even assuming that the sample was Coalinga, the data of Maltoni et al. [39] are severely undermined by a total lack of 'internal' and 'external' consistency. Thus, in 1982, Maltoni et al. [39] obtained tumour yields of 10–25% with their 'California' sample whilst in 1988, Maltoni and Minardi [40] induced a 72.5% incidence presumably with the same sample. The major difference was that the earlier study ran for 67 weeks whilst the later one lasted 104. However, study duration is probably not sufficient to account for the dramatic difference in tumour yields since Davis et al. [41] and Davis and Jones [6] produced tumours well in excess of 50% by 67 weeks with six different types of chrysotile using the same dose (25 mg) and the same mode of administration. Large inconsistencies have also been noted in other chrysotile injection studies conducted by Maltoni et al. Thus, in their 1982 study, untreated Canadian and Rhodesian preparations induced 0–5% tumours whilst, in their 1988 investigation, tumour yields of 80% were obtained. Some of this could be attributable to non-specific mass dose effects from the enormous 25-mg inoculum. Oberdorster [29] notes that 'large injected doses, even of short fibres and lower doses of large, non-fibrous particles can readily overwhelm and block' clearance mechanisms so that the dosage must be 'appropriately considered' if this mode of administration is to be used in an attempt 'to identify a potential hazard of a fibre for inducing mesothelioma'. As Oberdorster [29] also points out, 'the dose defines the mechanism', 'the challenge (being) to reach an agreement as to what is unacceptably high'. As the maximum total fibre dose (250 mg) recommended by the International Co-Operative Research Program for injection, normalized over the lung's surface area, would weigh more than an entire rat's lung if given by inhalation [29], it is obvious that such doses are grossly excessive. We would argue that the same is true for the maximum recommended amount of 50 mg per single injection and that this should not exceed 10 mg (see Ilgren [36] for comparative tumour yields at this dose level). If this is done, the 'possibility of the tumours being due to secondary events and mechanisms associated with high-dose toxicity' [29] will be greatly lessened. Still, the discrepancies in the data of Maltoni et al. are so great that factors other than dose must also be operative though at the present time these are not apparent. For example, the low (0–5%) tumour yields obtained by Maltoni et al. are totally inconsistent with every other intraperitoneal injection chrysotile study ever performed in the rat at the 25-mg dose level (68–

100%, [36]). Finally, a New York/Japanese collaborative group, represented by Suzuki and Kohyama [42, 43], assessed the carcinogenicity of Coalinga using the injection method. Although these workers were able to induce tumours with Coalinga, their studies are subject to serious criticism. Firstly, the use of the mouse is problematical since mineral fibre injection studies employing this species give far less reliable results than those which use the rat [Davis, pers. commun., 1994]. Secondly, the studies of Suzuki [42] and Suzuki and Kohyama [43] are confounded by supra-maximum-tolerated-dose effects [29] indicated by the high number of very sick or dead mice (192 of 586) found before 7 months. Suzuki and Kohyama [43] also recognized that they should have used much lower doses to avoid these untoward effects stating that 'animals receiving large doses of asbestos ... died of peritoneal fibrosis followed by intestinal obstruction before the tumour was induced. To perform long-term observations of the development and course of experimental peritoneal mesothelioma, doses of the carcinogenic mineral fibres must be carefully chosen'. The high percentage of tumours with sarcomatous histology, a variety more apt to be associated with damage-related, reparative effects and scarring than the epithelial form, is also probably another manifestation of the very high doses employed by these workers. Again, Suzuki and Kohyama [43] would appear to concur with this stating: 'It is not known why, unlike human mesothelioma, the epithelial form is so rare and the fibrous form so common in the induced mouse mesothelioma, although the severe focal fibrosis which constantly occurred around the mesothelium might be related to the high incidence of fibrous malignant mesothelioma.' By contrast, Rittinghausen et al. [32, 44] found that 80% of their Coalinga-induced tumours were partially or totally epithelial, a finding consistent with the lack of observed, untoward mortality (median life-spans for Coalinga identical to controls [24, 32]) and the lower doses employed (0.5–3.0 mg). The studies of Suzuki and Kohyama were also confounded by technical problems such as the accidental administration of fibre into the abdominal wall, diagnostic problems, as in the inability 'to differentiate plasmacytomas from mesothelioma', and viral infection.

Other Comparable UICC/B and Jeffrey Inhalation Studies

McConnell et al. [17] examined the same untreated controls and UICC/B treated groups as we did for this report and found nearly identical tumour incidences in both cases (table 1, see 1984 NIEHS). Wagner et al. [18] also examined untreated and UICC/B-treated animals maintained in a manner identical to those of McConnell et al. [17] and observed tumour yields similar to ours (table 1, see 1984 MRC). Small discrepancies in tumour yields between our own analysis and those of McConnell et al. [17] and Wagner et al. [18] are potentially attributable to interobserver variation. This is well supported by the findings of 13 independent pathologists from 9 institutions[1] who reviewed 20 tumour cases from the comparative study [17]. Diagnostic variation was particularly notable (Appendix).

Wagner [45] and Wagner et al. [46] appear to be the only other group to have administered UICC/B to rats via inhalation using approximately the same duration and intensity as was used in the present study. The incidences of primary pulmonary tumours noted by Wagner [45] (30%, 8/23) and Wagner et al. [46] (22%, 5/23) were consistent with those noted by us. However, 3 mesotheliomas were reported by Wagner [45] whilst none were found either by us in this study, by McConnell et al. [17] or by Wagner et al. in other studies [18, 46]. One of our cases displayed marked pleural invasion from an underlying pulmonary neoplasm thus resembling the type of peripheral tumour described by McConnell et al. [17] 'that could have been confused with mesothelioma if the primary tumour had not been observed' (also note case 18 and the footnote in the Appendix). McConnell and Adkins [47] were the only other group to study Jeffrey fibres via inhalation and used the same exposure duration and intensity as the present investigation. They found a far higher incidence of bronchiolar adenomatous hyperplasia and lung tumours (30 and 20%, adenomas and carcinomas combined, no mesotheliomas, respectively) than we did, and it is presently not possible to reconcile the observed differences with our own findings.

Sex-Related Differences

The incidence of primary pulmonary tumours was greater in males than females (table 1). This finding is not only consistent with that of McConnell et al. [17] but also with the sex-related differences noted in many of the morphometric epithelial responses seen in this study and in

[1] Drs. Wagner (MRC), Cobb and Hulse (AEA, Oxford), Smith (Los Alamos), McConnell and Boorman (NIEHS), Hollander (the Netherlands), Lamb (Scotland), Keysser and Smith (Rutgers), Kotin (JM). Kuschner (SUNY) and Mohr (Hannover) were brought to the MRC Pneumoconiosis Unit (Penarth, UK) on December 2, 1981, to review 20 cases from rats exposed to UICC/B, rockwool and glasswool with and without resin. Their diagnoses were subsequently tabulated and a final classification given after the meeting. The appendix gives the findings of pathologists from three of the institutions, but the full data sets are available upon request from the authors.

various interstitial changes previously observed by Ilgren and Chatfield [16]. Neither Wagner et al. [18] nor Wagner [45] noted sex-related differences in the incidence of lung tumours, although the former failed to stratify their data by fibre type. Nonetheless, Wagner [45] did find that the distribution of adenocarcinomas, squamous cell carcinomas and the number of mesotheliomas differed between the sexes. Wagner et al. [46] did not state the sex of the animals used whilst McConnell and Adkins [47], Davis et al. [14, 27, 48] and Davis [49] used only males. The sex-related differences in tumour incidence noted by us and McConnell et al. [17] are difficult to explain. The comparatively high incidence of malignant, non-pulmonary tumours in females might 'competitively' inhibit the growth of a primary lung tumour [50] or simply kill the animal before one has time to grow. Also, the high incidence of tumours arising in endocrine productive and/or responsive tissues, e.g. ovarian, uterine, pituitary adenomas and carcinomas, noted at autopsy might reflect important conditioning, age-related hormonal changes. These might also account for sex-related differences in lung tumour incidence since hormones can directly and indirectly affect the growth of normal [51] and neoplastic pulmonary tissue [52].

Survival: Tumour Lethality, Metastasis and Competing Causes of Death

McConnell et al. [17], in their analysis of the same untreated and UICC/B-treated animals, reported that 'approximately half of the rats with pulmonary tumours died from mononuclear cell leukaemia (monocytic leukaemia, Fischer rat leukaemia)' and thus claimed that the 'pulmonary tumours grow relatively slowly and may not always be the cause of death'. In this study, there was a strong suggestion that the primary pulmonary tumours reduced survival. Fibrosis was also previously shown to reduce survival independently of tumour formation, and indeed the two may thus be working in concert. Since definite metastasis from a pulmonary primary was only noted in one instance in our study, and not observed at all by McConnell et al. [17] in their review of the same group of animals, it is unlikely that distant spread of tumour was an important cause of death. McConnell and Adkins [47], in their study of Jeffrey-treated animals, also failed to find metastasis from the primary pulmonary tumours although metastasis was noted, almost in a dose related fashion, by Wagner [45] and Wagner et al. [46] following exposure to UICC/B (carcinomas: 1/4 at 3 months; 2/5 at 6 months; 5/12 at 12 months; 3/5 at 24 months).

Morphological and Morphometric Correlation of Tumour Formation with Epithelial Changes

The failure to observe tumours after exposure to Coalinga clearly correlates with the lack of persistent, morphometrically detectable, epithelial changes. Conversely, the significant tumour formation noted with the two Canadian fibres was associated with severe, persistent epithelial changes. This was particularly so for type II cells attempting to repair injured type I epithelium. The acute epithelial changes noted with Coalinga were not manifestations of injury but reversible, non-specific, high-dose effects [29]. Thus, exposure to exceedingly high levels of Coalinga chrysotile from 3 to 12 months, even though it was continuously applied, was still not able to prevent epithelial resolution and healing from taking place during this period of time. In contrast, continuous exposure to Jeffrey over the same 3- to 12-month time period produced dramatic epithelial abnormalities that frequently progressed to eventual irreversible, neoplastic changes. In contrast to either Coalinga or Jeffrey, exposure to UICC/B produced clear progression of type I epithelial changes over the final 12 months of the study. This temporal pattern of epithelial changes resembled the interstitial abnormalities previously shown to be unique to this fibre type [16] which again emphasizes the interdependence of epithelial and interstitial responses noted by others [53].

Possible Potentiation of Long Fibre Related Effects by Short Fibre

Davis [54] has stated that 'human fibre exposures associated with workplace dust will always be to a mixture of fibres and particulate materials of several kinds'. Since short fibre chrysotile will frequently make up a substantial proportion of many such occupational exposures, it is important to ask if it could contribute to fibre carcinogenesis by potentiating the effects of long fibre. Davis [54] has indicated that particle-mediated potentiation could occur either by producing a modified tissue reaction and/or by modifying fibre clearance rates through macrophage overload and the alteration of fibre transport. Although the long-term, high-dose administration of insoluble, non-fibrous, nuisance dusts (reviewed by Oberdorster [29] and Ilgren [37]) is able to induce lung tumours, the findings of this report and others [12] indicate that this would not be the case for short fibre chrysotile either for primary pulmonary neoplasms or mesothelioma. Indeed, Oberdorster [29] has argued that 'in the rat, any *persistent* particle at sufficiently high lung burdens appears to induce lung tumours' though only 'in association with lung particle overload' of the sort that occurs 'at *extremely high* partic-

ulate lung burdens' (also see Lehnert et al. [55]). However, as short fibre chrysotile does not persist in the lung [6, 24, 56] but is largely, if not totally, cleared, this would not apply to this fibre type. Oberdorster [29] has also stressed that solubility is also probably more important for humans than rodents in this regard stating that: 'because mechanical clearance rates for highly insoluble particles are slower in humans by a factor of 8–10 compared to rats, it can be predicted that high solubility rates of fibres have a much greater impact on lowering the overall biopersistence of fibres in human lungs than in rodent lungs'. As for the potentiation of fibre carcinogenesis and specifically mesothelioma induction [48, 54], the evidence assembled to date would indicate that this can only be brought about by insoluble particles able to persist within macrophages (e.g. TiO_2) and/or by those which directly affect the pleural lymphatic endothelium (e.g. quartz) so as to enhance pleural fibre transport. As it is 'highly unlikely' that short fibre chrysotile can cause overload [38], it is equally unlikely that short fibre chrysotile will potentiate long-fibre-mediated mesothelioma induction. Its inability to cause overload is largely related to rapid mechanical clearance and short length, possible hypersolubility (see above) and a lack of pleural lymphatic endothelial cell toxicity.

Short fibre chrysotile should therefore be regulated as a 'nuisance dust' but must be distinguished from those nonsoluble 'nuisance dusts' able to induce pathogenic effects either when administered alone under long-term, high-dose conditions or in association with long fibres.

Relevance of these Findings to the 'Amphibole Hypothesis' and Mesothelioma Induction

The failure to observe mesotheliomas in this study was due, in no small part, to the absence of amphibole in the Coalinga fibre [Chatfield and Ilgren, in prep.] and the presence of only very low amounts in the two Canadian samples. Although Wagner et al. [46] failed to find tremolite in their UICC/B sample (only some talc), 'traces of tremolite' were found in their sample from the Bells mine (Quebec), one of the eight 'contributors' to the UICC/B standard (also see Addison and Davies [57]). Wagner [45] did not mention the amphibole content of their UICC/B sample, but Wagner et al. [58] indicated that tremolite had been found in a crude ore grade 7 sample from the Bells mine and also in the lungs of rats dusted with the 'super-fine asbestos' sample from the Canadian Normandie mine that had been used by these same workers in their earlier study of this sample [46]. As both Bells and Normandie were used to produce the UICC/B standard sample, further analysis of UICC/B-exposed rat lungs would probably have revealed significant tremolite concentrations though many of these lung tissues no longer appear to be available for study [58]. The level of tremolite in the first 1974 UICC/B sample may have been higher than that of the later ones, an idea consistent with the batch variation described by Elmes [26]. The general failure to observe mesotheliomas in rats exposed to UICC/A (Rhodesian chrysotile), as opposed to UICC/B [59], is probably related to the lower amounts of tremolite in the former (tremolite probably <0.05% [60, 61]) than the latter (probably <1.0% [60, 61]; though see Wagner et al. [58]). Nonetheless, UICC/A, despite its reduced tremolite content, may still be able to induce mesotheliomas in circumstances that greatly facilitate fibre release and local deposition (e.g. with unusual chrysotile preparations like exp. WDC, [41]) or that markedly enhance fibre transport to the pleura (e.g. when co-administered with quartz [48]). The relative thinness of the rat's visceral pleura [48] and biological consequences of the comparatively short life-span of the rodent [62] are also probably important contributory factors to the 'anomalous' induction of mesothelioma with chrysotile in rodents exposed via inhalation. Nonetheless, long-term exposure to *short* fibre chrysotile contaminated with very low levels of tremolite similar to the material composition of many end products (also see Sebastien et al. [63] for evidence that tremolite levels are reduced during the processing of chrysotile ore) is not able to induce mesotheliomas in humans. Good support for this comes from other investigations such as the NIOSH rat and primate inhalation study where long-term, high-dose exposures to short fibre chrysotile failed to produce tumours even though the animal lungs contained significant amounts of tremolite [Salomon and Stettler, pers. commun.]. Sluis-Cremer [59] also stated that 'unless there is evidence to the contrary, no one can be certain that pure chrysotile fibres can cause mesothelioma when inhaled at all. Final proof that chrysotile fibre can cause mesothelioma can only come by the experimental inhalation of absolutely pure chrysotile'. The present study, in failing to find mesothelioma with such a pure form of chrysotile, thus goes a very long way to support Sluis-Cremer's proposal.

The evidence in support of the 'amphibole hypothesis' is far clearer in humans than in animals though the constraints of space preclude detailed discussion of this subject here. Although several recent review articles claim a clear, causal link between chrysotile and mesothelioma [64–66], their claims are based upon numerous false assumptions (see rebuttal in Mossman and Gee [67]),

which include the contention that asbestos usage and consumption patterns are reliable indicators of exposure, that lung tumours are appropriate 'surrogate measures' of mesothelioma incidence and that pleural fibre burdens and 'early events' can predict mesothelioma risk. There has probably never been an attributable, clinically and pathologically proven case of mesothelioma in any manufacturing industry, e.g. cement, friction products, or textiles, amongst the many tens of thousands of workers where chrysotile alone has been used. The McDonalds [68–71] have clearly shown, in critical, comprehensive analyses of the available data over the last 20 years, that small amounts of amphibole are sufficient to account for the appearance of mesotheliomas in such settings. Those less inclined to believe this should be reminded of the following parable [Browne, personal commun., 1997]:

'A man drank whisky and water the first night and awoke with a hangover; the next night he drank gin and water, and again awoke with a hangover; the third night vodka and water with the same result. The fourth evening he decided he must avoid water since this was clearly the cause of his symptoms.'

Appendix

Independent pathologists' review (the full data set is available from the authors)

	Wagner (MRC Pn)	McConnell, Boorman (NIEHS)	Kotin/ Kuschner (JM/SUNY)	Diagnoses reclassified after meeting
1 UICC/B	AdCa	AdCa	AdSqCa	AdCa
2 UICC/B	Ad	not sent	–	BAH
3 UICC/B	AdCa + Ad	AdCa	AdCa	AdCa + BAH
4 UICC/B	AdCa	AdCa	metaplasia	AdSqCa
5 UICC/B	–	–	metaplasia	–
6 UICC/B	SqCa	AdCa + Sq diff	AdSqCa	AdSqCa
7 UICC/B	Ad (?)	–	metaplasia	BAH
8 UICC/B	–	–	metaplasia	–
9 UICC/B	SqCa	AdCa + Sq diff	AdSqCa	AdSqCa
10 Rockwool	Ad	AdCa or Ad hyper	metaplasia	Ad
11 Glasswool/sR	Ad(dys)	AdCa	metaplasia	Ad + BAH
12 Rockwool	Ad	AdCa	metaplasia	Ad
13 Glasswool/sR	Ad	–	metaplasia	BAH
14 Glasswool/sR	Ad	not sent	–	BAH
15 Glasswool/sR	Ad	–	metaplasia	BAH
16 Glasswool/sR	AdCa	AdCa	AdCa (prob)	AdCa
17 Glasswool/sR	AdCa + SqCa	AdCa + Sq diff	AdSqCa	AdSqCa
18 Glasswool/sR	AdCa	AdCa or mesothelioma[2]	AdCa	AdCa[2]
19 Rockwool	Ad	AdCa or Ad hyper	metaplasia	BAH
20 UICC/B	Ad	AdCa or Ad hyper	metaplasia	AdCa + BAH

MRC Pn. = MRC Pneumoconiosis Unit, Penarth, UK; NIEHS = National Institute of Environmental Health Sciences, Research Triangle Park, N.C., USA; JM = Johns Manville Company; SUNY = State University of New York, N.Y., USA.

Ad = Adenoma; Ca = carcinoma; Sq = squamous; BAH = bronchiolar alveolar hyperplasia; diff = differentiation; hyper = hyperplasia; dys = dysplasia; – = no lesion found; prob = probably; / = or; sR = without resin.

[1] Selected 4 out of the 9 groups (see text).

[2] Smith (Los Alamos), Hollander (TVO) and Smith (Fairleigh) diagnosed this case as mesothelioma.

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