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Influence of Characteristics of Inhaled Fibres on Development of Tumours in the Rat Lung

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The objective was to examine and quantify the influence of fibre dimensions, persistence in the lung, and dissolution and cell toxicity *in vitro*, on the risks of developing lung tumours in rats. Data were brought together from the studies carried out at the IOM under the Colt Fibre Research Programme, and from studies carried out in Switzerland and the USA under the programme of the Thermal Insulation Manufacturers Association. In both studies, groups of rats were exposed by inhalation to a range of airborne fibres. At the end of their lives they were examined for the presence of benign and malignant lung tumours and mesothelioma. The studies differed in a number of details, but were combined on the basis of approximate equivalence of cumulative exposure to airborne fibres. Logistic regression models were used to relate differences in carcinogenicity to fibre characteristics: dimensions, persistence in the lung after intratracheal injection, dissolution rates from bench-top flow-through experiments, measures of inflammation, and other cell responses to fibres *in vitro*. Despite the small number of data points, the results suggested a primary influence of the airborne concentrations of the numbers of fibres thinner than 1 μm diameter and longer than 20 μm, and of the measured dissolution rate of the fibres. While these results are based on only a small number of fibre types, the statistical model fits the data reasonably well, and enables some cautious insights into the quantitative influences of dimensions and biopersistence. Results were broadly consistent with those from intraperitoneal injection studies of the same fibres, in that the responses were dependent on both the durability of the fibres and the numbers of long thin fibres. *In vitro* and *in vivo* cell responses did not predict significantly the risk of cancer following inhalation. © 1999 British Occupational Hygiene Society. Published by Elsevier Science Ltd.

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INTRODUCTION

The characteristics of fibres that determine pathogenicity have been the subject of extensive experimentation, and currently the major factors that are considered to influence the pathogenicity of any respirable fibre preparation are length, diameter, durability and a debated influence of toxicity directly related to chemical composition and mineralogical structure (summarised by McClellan *et al.*, 1992). The Colt Fibre Research Programme (CFRP) was conceived to examine the interrelations between properties of fibres and their respiratory toxicity, and studies have been carried out on a range of fibres with regard to their performance in several tiers of investigations. Within this programme, Davis *et al.* (1996) have reported on long-term inhalation studies with a glass microfibre, a silicon carbide whisker and an amosite asbestos. Long-term inhalation studies of the carcinogenicity of other fibres have been carried out elsewhere, notably under the programme of the Thermal Insulation Manufacturers Association (TIMA) (Bunn *et al.*, 1993; Hesterberg *et al.*, 1993, 1995; Mast *et al.*, 1995; McConnell *et al.*, 1994), which included insulation wools, vitreous fibres and refractory ceramic fibres. The animals in that study were exposed and examined pathologically at Research and Consulting Company (RCC) in Switzerland, and fibres were

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counted and sized at the Johns Manville Technical Centre (JMTC) in Colorado.

Availability of the data from the long-term rat inhalation studies of the CFRP and TIMA programmes allowed the possibility of using regression models to relate the carcinogenicity of nine different fibres to dimensional characteristics of the fibres and to independent measures of their durability in the rat lung and dissolution rates *in vitro*.

Although there is general consensus that chronic inhalation studies are the most reliable for characterising toxicity and carcinogenicity of fibres, there is also considerable interest in the use of other shorter-term assays for this purpose, perhaps as part of a tiered testing strategy (Brochard and Bignon, 1995; McClellan *et al.*, 1992). Since the CFRP had examined these fibres in a number of *in vivo* and *in vitro* assays, the opportunity was taken to relate carcinogenicity of these fibres to the results of the shorter-term experiments.

OBJECTIVES

The aim of this work was, by bringing together data from a number of experiments, to investigate the relations between the development of lung tumours and fibre characteristics, including numbers of fibres in specific size ranges, biopersistence in the lung, dissolution, and markers of cellular activity from *in vitro* and *in vivo* assays.

THE EXPERIMENTAL DATA

The inhalation studies

The IOM's long-term inhalation studies in rats tested a glass microfibre, bearing the manufacturer's codes 100 (mean diameter 0.32 μm) and 475 (chemical composition relatively high proportion in boron and silicon, and low in aluminium and calcium); a silicon carbide whisker fibre which is commercially used for special applications; and an amosite asbestos sample which had already been shown to produce tumours in a previous experiment (Davis *et al.*, 1986), and which contained a significant proportion of long fibres (3% longer than 25 μm). Experimental details were described by Davis *et al.* (1996). Briefly, rats of the AF/HAN strain, in groups of about 40 animals, were exposed whole-body for 7 hours per day, 5 days per week, for 238 working days (i.e. almost one year), at a target airborne fibre concentration of 1000 WHO fibre ml^{-1} determined optically. The animals were then left for their full lifespan, except that the studies were terminated when the number of survivors in each group had fallen to around 15%. Lungs from all animals were examined histologically for the presence of neoplasms, and in the animals who lived longest, quantitative assessments were made of the degree of fibrosis present in the lung. The results of these assessments are presented in Table 1. Table 1 also shows (in italics) the results of the TIMA inhalation studies, which followed the same broad approach, but with a number of differences of experimental detail (Bunn *et al.*, 1993; Hesterberg *et al.*, 1993, 1995; Mast *et al.*, 1995; McConnell *et al.*, 1994). They used Fischer-344 rats, in groups mostly numbering over 110 animals, exposed nose-only for 6 hours per day, 5 days per week, for 104 weeks, at target concentrations mostly in the range 200–260 WHO fibre ml^{-1}. The total elapsed time of the exposures in the IOM and RCC studies was therefore in the ratio 1666:3120.

Table 1 shows that, of the 38 animals exposed to the microfibre, four developed benign tumours in the lung, and none developed any malignant tumour. Sixteen of 42 animals exposed to amosite developed lung tumours, and in seven of these adenocarcinomas were found. In addition, two of these animals had mesotheliomas. In the 42 animals exposed to silicon carbide, there were ten animals with lung tumours, including five with adenocarcinomas; and ten animals had mesotheliomas at autopsy. Davis *et al.* (1996) give detailed descriptions of the IOM pathological findings. In the longest-lived animals, 9 amosite-exposed and 9 silicon carbide-exposed, there were extensive areas of advanced fibrosis, whereas in 11 microfibre-exposed animals there were only occasional very small areas, such as can be observed in unexposed rats at advanced ages (Davis *et al.*, 1986).

Characterization of exposure

Airborne concentrations in the IOM experiments were monitored and controlled by daily sampling, yielding size-selected gravimetric measurements of respirable dust mass. These were calibrated to a target fibre number concentration by counts made by phase contrast optical microscopy (PCOM). Table 2 shows the average airborne concentrations achieved. The main differences in length between the fibre types was that the number of fibres in the 10–15 μm length category was 50% higher for the code 100/475 microfibre than for the amosite and silicon carbide. The number concentrations of fibres >15 μm in length were quite similar.

The dimensions of the airborne fibres were assessed from airborne samples taken for one minute seven times in one day, repeated every two weeks. Fibre size distributions were estimated at the IOM from measurements of length and diameter of each fibre made by scanning electron microscopy (SEM). To better characterise the concentrations of the longer size fractions, for the amosite and 100/475 microfibre, additional measurements of fibres longer than 5 μm were made. The silicon carbide whisker fibres included a significant proportion of fibres with unusual shapes, resembling complexes of

Table 1. Pathology results from IOM and TIMA studies. Italics indicate RCC data from TIMA study.

		Inhalation pathology							Fibrosis (%)			
Fibre label	Concentration (mg m^{-3})	Animals	Lung cancers	% Lung cancers	All lung tumours	% Lung tumours	Mesotheliomas	% Mesotheliomas	Animals	Minimum	Mean	Maximum
ICI/475	5.9	38	0	0	4	11	0	0	11	0.0	0.2	0.7
SiC 1	11.4	42	5	12	10	24	10	24	9	6.6	8.7	20.2
Amosite	5.5	42	9	21	16	38	2	5	9	3.5	7.6	14.8
MMVF 10	*3*	*119*	*0*	*0*	*0*	*0*	*0*	*0*				
MMVF 10	*16*	*121*	*0*	*0*	*1*	*1*	*0*	*0*				
MMVS 10	*30*	*121*	*1*	*1*	*1*	*0*	*0*	*0*				
MMVF 21	*3*	*114*	*1*	*1*	*5*	*4*	*0*	*0*				
MMVF 21	*16*	*115*	*1*	*1*	*5*	*4*	*0*	*0*				
MMVF 21	*30*	*114*	*1*	*1*	*5*	*4*	*0*	*0*				
MMVF 22	*3*	*116*	*2*	*2*	*2*	*2*	*0*	*0*				
MMVF 22	*16*	*115*	*0*	*0*	*0*	*0*	*0*	*0*				
MMVF 22	*30*	*115*	*1*	*1*	*3*	*3*	*0*	*0*				
RCF 1	*3*	*125*	*0*	*0*	*2*	*2*	*0*	*0*				
RCF 1	*9*	*128*	*1*	*1*	*5*	*4*	*1*	*1*				
RCF 1	*16*	*126*	*1*	*1*	*2*	*2*	*0*	*0*				
RCF 1	*30*	*121*	*7*	*6*	*15*	*12*	*2*	*2*				
RCF 2	*30*	*121*	*5*	*4*	*8*	*7*	*3*	*3*				
RCF 4	*30*	*118*	*3*	*3*	*4*	*3*	*1*	*1*				

Table 2. Average concentrations (fibre ml^{-1}) of airborne fibres in IOM inhalation chambers, estimated from gravimetric samples and converted using data from PCOM counts

Fibre label	Fibre type	Length categories (μm)			
		All (>5)	5–10	10–15	>15
100/475	Glass microfibre	1139	655	243	222
SiC1	Silicon carbide whisker	984	615	167	201
Amosite	Amosite asbestos (long-fibre)	981	579	176	225

jointed fibres shaped, variously, like a 7, or a 'T', or a 'W'. Because of the difficulty of defining length for these complex shapes, no additional counts of longer fibres were made, but extra data were collected to characterise the diversity of the shapes. For whiskers composed of two or more branches, each branch was numbered and sketched. For each branch, the length, diameter, number of ends and number of free ends in the field of view were recorded. The length and breadth of the notional enclosing rectangle, with length parallel to the longest branch in the whisker, was also measured and recorded, as was the maximum diameter.

Airborne mass concentrations of total (i.e. not necessarily respirable) fibres in the TIMA experiments were measured from isokinetic samples taken at least four times per week. Fibre characterisation in the TIMA studies was based on five hour samples, taken quarterly (Hesterberg *et al.*, 1993). Size distributions were based on SEM analyses of these samples (but at a lower magnification than at the IOM).

Achieved exposures

Table 3 shows the bivariate size distributions estimated from the SEM assessments in the IOM experiments, scaled to airborne concentrations in fibre ml^{-1}. This table, and similar tables from JMTC for the airborne size distributions for the TIMA experiments, were converted to exposures, allowing for the differences in elapsed time and exposure day length described above, by multiplying IOM and JMTC concentration data by 1666 and 3120, respectively. There were many other differences in methodology between the two series (e.g. sampling times for airborne fibres, differences in microscopes, method of exposure, etc.), but the effects of these were either unknown or considered likely to be small, and adjustments were not made for these, nor for any differences in exposure-dose relationships which might have arisen due to clearance over different lengths of elapsed exposure time.

The exposures derived are shown in Table 4, summarised in two diameter classes (up to 0.9 μm, and 1.0 μm and over). Note that the length categories

Table 3. Bivariate size distributions from SEM analyses of IOM airborne samples, converted to estimated airborne fibre concentrations (fibre ml^{-1})

Fibre label	Diameter category (μm)	Length category (μm)						
		0.4–5	5–8	8–10	10–15	15–20	>20	All
100/475	≤0.1	223	0	0	1	0	0	224
	0.2–0.4	2498	397	114	165	37	13	3224
	0.5–0.9	191	155	52	70	15	18	501
	1.0–1.9	0	6	1	8	5	8	28
	≥2.0	0	0	0	0	0	0	0
	All	2911	558	168	244	58	38	3977
SiC1	≤0.1	30	4	0	0	0	0	34
	0.2–0.4	651	207	81	72	43	16	1075
	0.5–0.9	332	266	73	99	45	38	853
	1.0–1.9	0	6	0	8	2	0	16
	≥2.0	0	0	0	0	0	0	0
	All	1012	483	154	184	91	53	1978
Amosite	≤0.1	12	3	0	0	0	0	14
	0.2–0.4	1926	239	101	105	9	28	2478
	0.5–0.9	850	200	70	91	37	48	1296
	1.0–1.9	12	3	1	24	9	12	60
	≥2.0	0	0	0	0	0	1	1
	All	2869	445	172	220	55	89	3849

Table 4. Duration-weighted estimated exposure concentrations for IOM and TIMA/RCC inhalation experiments (fibre f^{-1} fibre cm^{-3}). Italics indicate JMTC data from TIMA study

Fibre label	Mass conc. (mg m^{-3})	Diameter class (μm)	Length (μm)					
			>0.4	>5	>8	>10	>15	>20
100/475	5.8	<0.95	6520	1710	810	533	138	52
		>0.95	46	46	36	34	21	12
SiC1	11.4	<0.95	3268	1581	786	530	237	89
		>0.95	26	26	16	16	9	9
Amosite	5.5	<0.95	6311	1551	814	530	204	127
		>0.95	102	83	58	77	36	21
MMVF10	*3*	<0.95	*27*	*22*	*16*	*13*	*8*	*5*
		>0.95	*73*	*70*	*58*	*50*	*34*	*24*
MMVF10	*16*	<0.95	*136*	*110*	*81*	*63*	*38*	*24*
		>0.95	*366*	*347*	*297*	*252*	*168*	*120*
MMVF10	*30*	<0.95	*218*	*178*	*129*	*102*	*61*	*38*
		>0.95	*586*	*535*	*475*	*404*	*269*	*192*
MMVF21	*3*	<0.95	*54*	*44*	*36*	*32*	*23*	*18*
		>0.95	*63*	*63*	*53*	*49*	*39*	*33*
MMVF21	*16*	<0.95	*238*	*195*	*160*	*140*	*102*	*77*
		>0.95	*286*	*276*	*236*	*216*	*172*	*146*
MMVF21	*30*	<0.95	*383*	*316*	*259*	*228*	*169*	*125*
		>0.95	*463*	*447*	*382*	*350*	*279*	*236*
MMVF22	*3*	<0.95	*58*	*47*	*39*	*32*	*23*	*17*
		>0.95	*49*	*47*	*42*	*40*	*31*	*25*
MMVF22	*16*	<0.95	*254*	*205*	*164*	*141*	*102*	*73*
		>0.95	*214*	*204*	*184*	*172*	*136*	*110*
MMVF22	*30*	<0.95	*414*	*334*	*266*	*229*	*165*	*119*
		>0.95	*348*	*332*	*299*	*280*	*232*	*178*
RCF1	*3*	<0.95	*53*	*37*	*25*	*23*	*13*	*11*
		>0.95	*47*	*46*	*40*	*39*	*32*	*27*
RCF1	*9*	<0.95	*153*	*106*	*73*	*65*	*43*	*31*
		>0.95	*135*	*131*	*115*	*111*	*91*	*77*
RCF1	*16*	<0.95	*234*	*169*	*117*	*105*	*69*	*50*
		>0.95	*216*	*210*	*184*	*178*	*146*	*122*
RCF1	*30*	<0.95	*395*	*296*	*222*	*186*	*135*	*106*
		>0.95	*300*	*293*	*265*	*258*	*189*	*157*
RCF2	*30*	<0.95	*431*	*276*	*176*	*141*	*96*	*59*
		>0.95	*434*	*425*	*369*	*337*	*290*	*235*
RCF4	*30*	<0.95	*198*	*58*	*22*	*9*	*2*	*0*
		>0.95	*496*	*441*	*300*	*220*	*116*	*59*

used here are cumulative; thus each column is a subset of the column to its left. Note also that the mass concentrations are for respirable fibres for the IOM experiments, but for total fibres in those from RCC. A small number of total dust samples taken from the IOM aerosols suggested that masses for total fibres tended to be higher than respirable by about 10–20%.

Inspection of Table 4 confirms that the silicon carbide, 100/475 microfibre and amosite fibre clouds contained mostly fibres thinner than 1 μm diameter (they were chosen partly for this reason), while the other vitreous fibres contained more equal proportions of thicker and thinner fibres. RCF 4 was particularly deficient in long thin fibres.

Biopersistence

In an intratracheal injection study (Searl *et al.*, 1999), fibres suspended in sterile physiological saline were injected via the exposed tracheas of anaesthetized rats, and the amounts and dimensional distributions of the fibres recovered from the lungs three days after injection and at three later time points were assessed by SEM methods similar to those described above, counting at least 100 fibres per animal. At each time, for each fibre type, four rats were sacrificed. Biopersistence was characterised by fitting an exponential decay model to the data from the four time points, and re-expressing this as the expected percentage of fibres remaining at twelve months. This parameter was calculated for the same cumulative size classes as used in Table 4; the results are shown in Table 5. These parameters are estimates, and sampling variation among the lungs at each time point means that values above 100% are possible. In particular, this is illustrated by the anomalously high values at >20 μm length in RCF 2 and RCF 4, which are probably products of high sampling variation, based on the relatively small numbers of very long fibres actually encountered and measured.

Table 5. Characteristics of fibres: physical characteristics, biopersistence estimates and results from toxicology assays

	Physical			Biopersistence at 12 months (%)						Toxicology results					
Fibre label	k_{eff} (ng cm^{-3} hr^{-1})	Adjusted k_{eff} (mg cm^{-3} hr^{-1})	Geometric mean diameter	All lengths > 0.4 μm	Length > 5 μm	Length > 8 μm	Length > 10 μm	Length > 15 μm	Length > 20 μm	Total cells (×10^6)	Macrophages (×10^6)	Granulocytes (×10^6)	% Granulocytes	TNF (units per 10^6 cells)	Macrophage toxicity (counts/min)
100/475	9.1	0.466	0.36	9.9	21.3	22.9	29.6	37.2	39.7	9.1	4.9	3.57	42.2	60	3736
SIC 1	0.2*	0.002*	0.44	52.6	53.7	47.1	49.2	54.6	59.2	13.9	10.0	3.99	30.2	1527	3869
Amosite	0.2*	0.002*	0.46	8.9	21.9	29.4	34.4	46.2	68.8	9.5	5.5	2.61	36.0	91	3605
MMVF 10	122.4	1.310	1.25	13.1	9.4	6.0	4.4	2.1	0.6	9.7	7.0	2.93	21.3	46	3318
MMVF 21	28.9	0.399	1.02	36.9	37.8	37.7	36.3	36.6	43.0	10.5	6.5	3.12	30.2	36	3661
MMVF 22	52.8	0.602	0.91	16.6	10.6	6.1	3.8	2.2	1.2	8.6	4.2	1.85	29.0	39	4034
RCF 1	4.4	0.063	0.86	49.3	42.8	44.9	46.6	49.6	50.5	7.6	4.3	2.61	35.0	39	4155
RCF 2	5.1	0.035	0.90	59.1	70.8	83.9	99.9	130.0	157.3	8.6	4.8	3.27	38.0	30	4324
RCF 4	0.5	0.005	1.24	72.2	81.3	95.7	108.4	113.1	142.7	2.8	1.8	0.73	27.0	50	3807

* Substituted values.

Dissolution rates

The ability of the fibres to resist dissolution was characterised by a continuous flow experiment with simulated physiological saline (Searl *et al.*, 1999). Dissolution rates k_{sa}, expressed as rate of mass loss of silicon per unit surface area, were estimated from silicon concentrations measured in samples collected from the flow. These were also expressed in terms of mass loss per unit initial mass, referred to here as 'adjusted k_{sa}'.

Because they were known to be of very low solubility, samples of silicon carbide and amosite were not included in the continuous flow experiment. Based on this knowledge, very low values of k_{sa} and adjusted k_{sa} were substituted as shown in Table 5.

Measures of cellular response

Cellular responses induced by these fibre samples have been reported by Cullen *et al.* (1997) They were intended to provide information on the activity of the fibre samples in macrophage cytotoxicity and activation, and bronchial epithelial cell damage and proliferative responses. The responses considered in this paper include the release of tumour necrosis factor (TNF), an inflammatory mediator released from rat alveolar macrophages when exposed to fibres *in vitro* (Cullen *et al.*, 1997); *in vitro* rat alveolar macrophage toxicity, as the release of ^{51}Chromium; and measures of the cellular response to intraperitoneal injection of fibres in the mouse, i.e. estimated numbers of total cells, granulocytes, macrophages (Donaldson and Miller, 1994; Donaldson *et al.*, 1994). These results are summarised in Table 5.

STATISTICAL ANALYSES

Methods

The incidence of tumours was treated as a binomial response variable. Its relationship with characteristics of individual fibre types was investigated by standard methods of multiple logistic regression (Collett, 1991), using the statistical software package Genstat (Genstat 5 Committee, 1993), which was also used for tabulations.

To avoid including the very large number of variables represented by a complete bivariate set of length and diameter variables, airborne fibre concentrations were summarised not only in cumulative length categories but also in two diameter classes, according to whether the fibre diameters (which were measured to the nearest 0.1 μm) were greater or smaller than 0.95 μm. This threshold was selected because evidence suggests that fibre diameters less than about 1 μm are more hazardous than thicker fibres (Stanton *et al.*, 1981; Pott *et al.*, 1997; Timbrell, 1984). Inspection of the observed fibre size distributions indicated that a threshold of 0.95 μm gave adequate numbers of fibres for statistical power and likely reliability, and illustrated some contrasts between fibre types. The other explanatory variables available were the durability variables from Table 5, i.e. length-specific clearance after intratracheal instillation and silicon dissolution *in vitro*, and the cell response data in Table 5. Standard techniques of forward stepwise selection were used to choose which (if any) of these variables best helped to explain the variation in the incidence of lung cancer. The logistic regression facilities of *Genstat* (Genstat 5 Committee, 1993) were used to compute, for each of the potential predictor variables, the contribution which it would make to the regression model if added on its own. One aim of regression modelling is, in general, to explain as much as possible of the variation in the response, and in logistic regression the unexplained portion is quantified by the residual mean deviance, an analogue of the residual mean square in linear regression.

Results

Table 6 shows, in the section labelled Step 1, the residual mean deviances achieved by models containing each of the potential predictors alone. The smallest residual mean deviances were obtained with variables representing dissolution rates, and the adjusted k_{sa} was a slightly better predictor than the unadjusted value. Both of these were better predictors than any of the exposure variables of numbers of thinner fibres. None of the exposure variables for the numbers of thicker fibres were useful predictors, and there was little evidence for an important predictive role for any of the *in vitro* toxicology results. At the end of this step, adjusted k_{sa} was added to the model and the search began for additional useful predictors.

At Step 2, the best additional predictors, which were also highly statistically significant, were variables representing the exposures to numbers of the longer, thinner fibres. The best of these was the number of thinner fibres of length over 20 μm. Again, the exposures to thicker fibres were not useful predictors. At the end of this step, the model included adjusted k_{sa} and exposure to thinner (diameter < 0.95 μm) fibres of length over 20 μm.

At step 3, no further terms gave a significant improvement to the model fit. In addition, the residual mean deviance after Step 2 was less than 1.0, the theoretical value in the case of purely binomial variation. With no evidence of extra-binomial variation, and no remaining significant predictors, it was concluded that further model building past Step 2 was not justified.

The coefficients for the logistic regression model achieved at the end of Step 2 are shown in Table 7, with their standard errors. These coefficients represent the change in the log-odds of disease for a unit change in each of the predictors, holding the

Table 6. Results of three steps of logistic regression modelling, evaluating potential contribution of each predictor variable by reduction in residual mean deviance

Step 1 Candidate model change	Residual mean deviance	Step 2 Candidate model change	Residual mean deviance	Step 3 Candidate model change	Residual mean deviance
Add Dissolution k_{du} (adjusted: log)	1.866	Add Thinner Fibres (> 20 μm)	0.833	Add Biopersistence (> 15 μm)	0.777
Add Dissolution k_{du} (log)	1.889	Add Thinner Fibres (> 15 μm)	1.151	Add Thicker Fibres (> 20 μm)	0.795
Add Thinner Fibres (> 8 μm)	2.326	Add Thinner Fibres (> 10 μm)	1.353	Add Biopersistence (> 20 μm)	0.793
Add Thinner Fibres (> 10 μm)	2.330	Add Thinner Fibres (> 8 μm)	1.414	Add Thicker Fibres (> 15 μm)	0.794
Add Thinner Fibres (> 15 μm)	2.396	Add Mean Diameter	1.512	Add Biopersistence (> 10 μm)	0.808
Add Thinner Fibres (> 5 μm)	2.565	Add Thinner Fibres (> 5 μm)	1.540	Add Thicker Fibres (> 10 μm)	0.818
Add Mean Diameter	2.624	Add Thinner Fibres (> 0.4 μm)	1.581	Add Biopersistence (> 8 μm)	0.822
Add Thinner Fibres (> 0.4 μm)	2.867	Add Biopersistence (> 0.4 μm)	1.611	Add Macrophage Toxicity	0.825
Add Thinner Fibres (> 20 μm)	3.197	Add Granulocytes	1.627	Add Thicker Fibres (> 8 μm)	0.825
Add % Granulocytes	3.825	Add % Granulocytes	1.628	*No Change*	0.823
Add TNF	3.951	Add Total Cells	1.645	Add % Granulocytes	0.839
Add Biopersistence (> 20 μm)	3.998	Add Biopersistence (> 5 μm)	1.657	Add Biopersistence (> 5 μm)	0.843
Add Biopersistence (> 15 μm)	4.089	Add Biopersistence (> 8 μm)	1.689	Add Thicker Fibres (> 5 μm)	0.851
Add Granulocytes	4.105	Add Biopersistence (> 10 μm)	1.724	Add Thinner Fibres (> 10 μm)	0.855
Add Biopersistence (> 10 μm)	4.259	Add Thicker Fibres (> 20 μm)	1.727	Add Thinner Fibres (> 8 μm)	0.855
Add Biopersistence (> 8 μm)	4.304	Add Macrophage Toxicity	1.788	Add Thinner Fibres (> 5 μm)	0.857
Add Total Cells	4.325	Add Macrophages	1.805	Add Thicker Fibres (> 0.4 μm)	0.859
Add Biopersistence (> 5 μm)	4.356	Add Thicker Fibres (> 15 μm)	1.856	Add Thinner Fibres (> 0.4 μm)	0.865
No Change	4.426	Add Biopersistence (> 15 μm)	1.863	Add Biopersistence (> 0.4 μm)	0.868
Add Biopersistence (> 0.4 μm)	4.599	*No Change*	1.866	Add Granulocytes	0.880
Add Macrophages	4.603	Add Biopersistence (> 20 μm)	1.883	Add Thinner Fibres (> 15 μm)	0.882
Add Macrophage Toxicity (> 5 μm)	4.622	Add Thicker Fibres (> 10 μm)	1.930	Add TNF	0.886
Add Thicker Fibres (> 0.4 μm)	4.655	Add Thicker Fibres (> 0.4 μm)	1.939	Add Mean Diameter	0.886
Add Thicker Fibres (> 8 μm)	4.663	Add Thicker Fibres (> 5 μm)	1.970	Add Dissolution k_{du} (log)	0.890
Add Thicker Fibres (> 15 μm)	4.665	Add Thicker Fibres (> 8 μm)	1.988	Add Macrophages	0.891
Add Thicker Fibres (> 20 μm)	4.665	Add TNF	1.988	Add Total Cells	0.893
Add Thicker Fibres (> 10 μm)	4.666	Add Dissolution k_{du} (log)	1.995	Drop Thinner Fibres (> 20 μm)	1.866
Add Thicker Fibres	4.672	Drop Dissolution k_{du} (adjusted: log)	4.426	Drop Dissolution k_{du} (adjusted:log)	3.197
Chosen action: Add Dissolution k_{du} (adjusted:log)		Chosen action: Add Thinner Fibres (> 20 μm)		Chosen action: *No Change*	

Table 7. Results of logistic regression modelling: coefficients and statistical significance after two steps of Table 6

Terms in regression model	Coefficient	Standard error	z-statistic ratio
mean	−6.40	0.45	
ability (log)	−0.4907	0.0803	−6.11
Thinner fibres (> 20 μm)	0.01649	0.00423	3.90

other constant, and are invariant to the order of selecting the terms in building the model. The ratio of the coefficient to its standard error is expected to have a Gaussian or Normal distribution, so it provides a z-statistic for testing the significance of that term added last, i.e. adjusted for the other terms present. Critical values for particular significance levels are obtained from standard tables of the Normal distribution. Thus, for example, the 5% two-tailed critical value is 1.96. Both terms make highly statistically significant contributions to the model.

This model predicts the risk, r, that an individual animal will develop lung cancer as a function of x_1, the log of the adjusted dissolution rate, k_{adj}, and x_2, the number of thinner fibres > 20 μm in length, using the logistic function

$$r = [1 + \exp(6.40 + 0.4907x_1 - 0.01649x_2)]^{-1}$$

Table 8 compares the predictions from this model with the observed data, in terms of the number of animals with cancers within each treatment group. Also shown is the standard error of each predicted value, expressing the statistical uncertainty in the position of the fitted function. Overall, the agreement is reasonable.

The analyses were rerun taking the logarithms of the fibre numbers and biopersistence variables. Two steps of model fitting gave a similar residual mean deviance (0.838), and the same selection of predictors, and the conclusions were unchanged.

DISCUSSION

Fibre dimensions

Fibre length and diameter are well known to influence carcinogenicity (WHO, 1986). In the pleura and peritoneum the evidence from systematic comparative animal studies of both asbestos and man-made mineral fibres has shown that the potential to cause mesothelioma appears to be confined to fibres longer than about 8 to 10 μm (Stanton *et al.*, 1981; Pott *et al.*, 1997; Timbrell, 1984) and to those long fibres only if they are thin; diameters less than 0.25 μm, or possibly 1.5 μm (Stanton *et al.*, 1981; Pott *et al.*, 1997). Small-scale but elegant human clinical studies of asbestos have tended to confirm that the upper threshold for fibre diameter for mesothelioma is about 0.5 μm or less (Timbrell, 1983).

The fibre dimensions influencing lung cancers have not been studied so systematically. The uncertainties have included the extent to which these critical dimensions apply also to lung cancer, and to man-made fibres. Systematic comparative inhalation studies on the scale of the intracavitary studies would represent a huge exercise, but the combined analysis we report here of studies performed at IOM and at RCC Geneva, both of which included the essential counts of fibre numbers within bivariate size categories, permit a reasonably effective comparative approach, based on a modest number of fibre types.

Table 8. Results of logistic regression modelling for lung cancer incidence: numbers of animals with lung cancers observed and predicted by fitted model, with standard error of prediction.

Fibre label	Mass conc (mg m^{-3})	Animals	Observed with lung cancers	Predicted with lung cancers	Standard error of prediction
100/475	5.8	38	0	0.2	0.07
SiC 1	11.8	42	5	5.5	1.24
Anosite	5.5	42	9	9.3	2.16
MMVF 10	3	119	0	0.2	0.08
MMVF 10	16	121	0	0.3	0.13
MMVF 10	30	121	1	0.3	0.13
MMVF 21	3	114	1	0.4	0.14
MMVF 21	16	115	1	1.1	0.30
MMVF 21	30	114	1	2.3	0.81
MMVF 22	3	116	1	0.3	0.13
MMVF 22	16	115	0	0.8	0.25
MMVF 22	30	115	1	1.7	0.62
FF 1	3	123	0	0.9	0.33
FF 1	9	128	1	1.3	0.38
RCF 1	16	126	1	1.8	0.41
RCF 1	30	121	7	4.3	0.91
RCF 2	30	121	5	2.7	0.53
RCF 4	30	118	2	2.6	1.09

The present results are consistent with the hypothesis that, for inhalation studies, lung carcinogenicity of man-made fibres in rats is a function of fibre length. Parallel to the work on asbestos of Berman *et al.* (1995), we found evidence that man-made fibres longer than 20 μm, longer than would be easily engulfed by macrophages, had the greatest effect on carcinogenicity. It appears that the influence of length in man-made fibres is similar to that of the asbestos minerals.

Clarification of the fibre diameters below which the toxicity is concentrated is of interest for the identification of potentially toxic fibre types, and for the definition of appropriate measurement methods. Mean fibre diameter was a weak predictor of toxicity in this series, and our estimate of the number of fibres longer than 20 μm and thinner than 1 μm was a better indicator. The 1 μm diameter differentiation is a little smaller than would be expected if the influence of diameter were due purely to the respirability of thin fibres. If respirability were the sole factor, a slightly higher critical range might be expected, say below 1.5 μm or 2 μm. For example, a 20 μm long fibre with diameter 1.7 μm and density 2.5 g cm^{-3} has an aerodynamic diameter of 5 μm, which is just below the estimated approximate upper limit of rat respirability (Jones, 1993). Intraperitoneal studies (e.g. Miller *et al.*, 1999; Stanton *et al.*, 1981) have also shown some influence of fibre diameter, independently of respirability.

The tenor of discussions on the subject of the ranges of critical dimensions which determine carcinogenicity has been one of justifiable caution. The concept of 'thresholds' has usually, although not invariably, been avoided (Timbrell, 1983) and, instead, gradients of risk have been described over ranges of fibre dimensions. This has been necessary, not least because fibre preparations invariably include fibres of a range of sizes, and it is difficult to distinguish precisely the effects of one size of fibre from another. An understanding of the mechanisms of the influence of dimensions on risk can inform the choice of statistical model and the likelihood of a threshold. Thus the concept of some fibres being harmful because they are too long for macrophages to clear them provides some insights into the likely range of lengths critical to this effect. In practice, the determination of the threshold range of critical length is not too important for product design, since most practical applications require fibre lengths to be much longer than the minimum of the carcinogenic length range.

On the other hand, the mechanism for the influence of fibre diameter is not clear, and this makes it difficult to judge whether a narrow threshold range is likely. The critical value for mesothelioma induction suggested by Stanton *et al.* (1981) was 0.25 μm for fibres longer than 8 μm (with some difficulty in distinguishing statistically from an alternative conclusion of 1.5 μm for fibres longer than 4 μm). It has been suggested that thin fibres are more likely than thick to penetrate cell membranes without killing the cells (Stanton *et al.*, 1981; Allison, 1977). Some measurements of the quantitative influence of diameter on uptake by cells would be informative in judging the relationship of this phenomenon to the diameter thresholds demonstrated by toxicological studies, and the likely range of this critical threshold.

The fibre types selected for the present study provide contrasts in distributions of fibre diameters which are informative about the diameters in the critical threshold range. However more precise definition of this threshold range is likely to require an improved ability to generate size-separated fibre samples for use in animal studies. Our findings may help in the selection of size separations for future studies.

Dissolution and biopersistence

The persistence of long fibres in lungs is known to be influenced by the dissolution properties of fibres *in vitro* (Morgan *et al.*, 1982; Morgan and Holmes, 1986; Bernstein *et al.*, 1984; Eastes and Hadley, 1995), implying that dissolution *in vitro* should help to predict carcinogenicity. Eastes and Hadley (1996) recently described a mathematical model of the influence of dissolution of relatively soluble vitreous fibres, measured *in vitro*, on the carcinogenicity of long fibres in chronic inhalation and intraperitoneal studies. These and other workers have pointed out that biopersistence in the lung, which depends on other factors as well as on dissolution, may in fact be a more fundamental parameter (Morgan *et al.*, 1982; Bellmann *et al.*, 1987; Davis, 1994).

The rankings of the fibres on our measure of *in vitro* dissolution were similar to their rankings on biopersistence of long fibres (Searl *et al.*, 1999). Contrary to expectation, we found that the dissolution measure was a somewhat better predictor of carcinogenicity than was the direct biopersistence measure. However, these estimates of biopersistence were based on lung burden measurements which have been found by others as well as ourselves to be subject to large uncertainty due to variations between animals (Bernstein *et al.*, 1996). The measurement error in the quantification of biopersistence, which might be considerably greater than in a bench-top experiment, might have obscured an underlying relationship.

Intrinsic toxicity of fibres

The possible additional influence on carcinogenicity of toxicity generated by the chemical composition or mineralogical structure of fibres has for long been debated (Donaldson *et al.*, 1993; McClellan *et al.*, 1992). Cytotoxicity has been found

by others to be characteristic of asbestos fibres (Goodglick and Kane, 1990; Hahon *et al.*, 1986). *In vitro* toxicity of asbestos is confined to the long fibres (Donaldson *et al.*, 1992, 1989) but also appears to be related to surface structure, since modification of the surface reduces the activity (Goodglick and Kane, 1990; Hahon *et al.*, 1986; Galumian *et al.*, 1989). There is relatively little other information on the relative toxicity of long man-made vitreous fibres and long asbestos fibres. Proliferation of bronchiolar/alveolar epithelial cells after short term inhalation appears to identify a property associated with long fibres of asbestos and silicon carbide not shared by long fibres of glass microfibre (Cullen *et al.*, 1997). This has been examined so far in only three fibre types, but seems worthy of further exploration. Complement activating activity of glass fibre does not appear to be different from that of asbestos (Warheit *et al.*, 1989)

In none of our models did the *in vitro* cell responses appear to be related significantly to risks of cancer, either before or after allowing for the influence of fibre dimensions and biopersistence. This is disappointing, in that it suggests that these short-term toxicity tests are not a useful predictor of carcinogenicity in chronic inhalation studies, and therefore cannot be used as a substitute for such studies. The search for tests which will predict carcinogenicity continues.

Predictions from the model

The statistical model fitted the data reasonably well. Predictions from it must be treated with caution, but they nevertheless provide some insight into the relative influences of dimensions and biopersistence.

For example, exposure to a fibre with high airborne concentrations, dimensional size distributions, numbers and biopersistence characteristics similar to the silicon carbide fibre tested is predicted by the model to be associated with an approximate 13% risk of lung cancer (in addition to the mesothelioma risks). An hypothetical fibre with the same biopersistence but a quarter of the number of long thin fibres would be predicted to be associated with about a 5% risk of lung cancer, approaching background rates. On the other hand, another hypothetical fibre with the same fibre size distribution as the silicon carbide but with an adjusted k_{dis} similar to that of MMVF 22, would be predicted to be associated with a cancer risk of only 1%.

Validation of the model will require independent comparisons with other fibre types. One comparison will be provided by comparable studies of glass microfibre 104E, currently in progress. However, there is a need to verify the relationship with fibre number in experiments using different exposures to the same fibres, particularly those which have shown the greatest toxicity.

Comparisons with results from intraperitoneal injection

Our companion paper (Miller *et al.*, 1999) describes the differences between these fibre types in their ability to induce mesothelioma production in the rat peritoneum, following injection of fixed numbers of fibres. Both that study and this have demonstrated relationships between fibre characteristics and cancer risks. For the intraperitoneal injection studies, numbers of long thin fibres and their biopersistence in the lung were the most important determinants. For the inhalation studies, the number of long thin fibres and the adjusted dissolution rate k_{dis} were the principal determining characteristics.

It is perhaps surprising that biopersistence in the lung appears more relevant, from these studies, to risks of peritoneal mesothelioma than to lung cancer, given the different clearance mechanisms in the lung and peritoneum. Fibres in the peritoneum are probably cleared only by dissolution, and the large deposited doses become bound up to varying degrees in the development of fibrosis, while in the lung the dose of deposited fibres is much lower, and macrophage clearance is important, at least for the shorter fibres. While large measurement errors in assessing biopersistence might obscure the role of this parameter in inhalation studies, it seems unlikely that the same measurement error would artificially promote biopersistence as a predictor of mesothelioma risk. This may be a further warning against over-interpretation of the analysis of very small data sets such as that from the injection study.

A simple comparison of the ranking of tumorigenicity of fibre types according to the two assays, unadjusted for the dosage of long thin fibres, would be artificial. Some of the apparent contrasts in response may possibly be explained by differences in the administered numbers of long thin fibres, or by differing mechanisms of response. In particular, the high intraperitoneal mesothelioma rate for MMVF 21 (Miller *et al.*, 1999) might appear inconsistent with the low cancer rate in the inhalation study, but the number of long thin fibres injected into the peritoneum was much greater than for any other fibre type, while the estimated number of long thin fibres in the aerosol was within the range of the other types; and at the other extreme, the low intraperitoneal mesothelioma rate for RCF 4 might be attributable to the low number of long thin fibres in the preparation.

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