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Tumorigenicity of cellulose fibres injected into the peritoneal cavity

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Cellulose fibres, along with many other organic fibres are durable, and therefore, if inhaled, have the potential to persist within the lung. Persistent fibres may cause disease. This report describes the effects of injecting high purity cellulose fibres into the abdominal cavity of rats. This assay is used to investigate the potential of fibres to produce cancer in rats. Crocidolite asbestos was used as a positive control and saline (used to suspend the fibres for injection) as a negative control. Four doses, from 1 million to 1000 million fibres (defined as fibres longer than 5 μm) were tested for each fibre type.

The two higher doses of crocidolite asbestos caused greatly reduced survival compared to the saline controls. With cellulose there was a much less marked effect on survival. In the highest dose cellulose group, multiple large nodules (granulomas) and widespread adhesions (bands of new tissue connecting organs to each other and to the abdominal wall) were present in all animals. Granulomas were not observed in the 10^9 fibre crocidolite group. More than 80% of animals in the 10^8 and 10^9 crocidolite asbestos groups had mesotheliomas, a type of tumour sometimes found in the lungs of people exposed to asbestos. By contrast there were only two animals in the cellulose groups with mesothelioma tumours, one in the 10^7 and one in the 10^8 groups. However, nine (18%) of the 10^9 cellulose group had another type of malignant tumour called a sarcoma. This tumour type is not usually associated with fibres in this animal model.

This study has demonstrated that high doses of cellulose fibres are carcinogenic when injected into the abdominal cavity of rats.

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SUMMARY

INTRODUCTION

Cellulose fibres find use in many synthetic products such as cotton textiles, paper and cardboard, as a substitute for asbestos to give structural strength to cement and other construction products, and as insulation for buildings. The manufacture and use of these products can produce airborne cellulose fibres which may be inhaled. Like many other organic fibres, cellulose fibres are durable and thus have the potential to persist within the lung and hence may cause disease. A great deal is now known about the hazard posed by respirable mineral fibres such as asbestos and respirable, durable man-made fibres such as certain glass and ceramic fibres but, by comparison, relatively little is known about the hazards of inhaled cellulose fibres. A limited number of animal studies where cellulose fibres were instilled into the lung or inhaled, have shown pathological changes in the lung and one study in which cellulose was injected into the peritoneal cavity of rats resulted in mesotheliomas.

This report describes the results of intraperitoneal injection experiments in rats with a high purity fibrous cellulose. The aim was to investigate the possible carcinogenicity of cellulose fibres in the peritoneal cavity. Respirable crocidolite asbestos was used as a positive control.

AIM

The aim of the study was to investigate the carcinogenic potential of a sample of a commercial fibrous cellulose by means of a multi-dose intraperitoneal injection assay using crocidolite as a positive control and saline as a negative control.

MATERIALS AND METHODS

The experiments were carried out in SPF male Wistar rats.

A respirable fraction of cellulose fibre was collected from an aerosol of a thermally processed wood pulp. A sample of respirable crocidolite asbestos, known to produce mesotheliomas in rats, was used as a positive control. Four doses (10^6, 10^7, 10^8, 10^9 WHO fibres) for each fibre type were injected intraperitoneally as 3 weekly aliquots. A negative control was provided by phosphate-buffered saline used to suspend the fibres for injection. There were 50 rats per treatment group except for the 10^9 and 10^8 crocidolite groups which had 26 rats.

RESULTS

Granuloma and fibrosis

In the 10^9 fibre cellulose group, multiple large granulomas and widespread fibrous adhesions were present in all animals. The granulomas were typical of a response to fibrous dusts and made up of macrophages and fibroblasts with particularly frequent foreign body giant cells. Small granulomas with slight adhesions were usually recorded following the injection of 10^8

cellulose fibres but no macroscopic observation or histological finding of granulomas was made in the groups of rats that received 10^7 or 10^6 cellulose fibres.

Granulomas might have been expected in the higher dose crocidolite groups, but were not observed. This may have been because most of these animals died with advanced mesotheliomas where multiple tumour masses and blood stained ascites fluid would have made the detection of small granulomas impossible.

Tumours

More than 80% of animals in the 10^8 and 10^9 crocidolite groups had mesotheliomas. By contrast there were only two animals in the cellulose groups with classical mesothelioma, one in the 10^7 and one in the 10^8 groups. However, nine of the 10^9 cellulose group had sarcomas. Small numbers (1 to 3) of carcinomas were seen in some groups, including 2 in the saline controls; these tumours were not felt to be treatment related.

Survival times

Median survival decreased with increasing dose of fibre for both crocidolite and cellulose. However, from the highest to lowest dose of cellulose, the range of values was only 56 days, and from the highest dose of cellulose to saline controls, the difference was 91 days. The two higher doses of crocidolite caused greatly reduced survival, because of tumour production, compared to the saline controls.

When comparing survival times for only those animals diagnosed with mesothelioma or sarcoma, there was again, a marked separation between crocidolite and saline groups. The 10^9 cellulose group showed significantly poorer survival than the saline group.

CONCLUSIONS

This study has demonstrated that high doses of cellulose fibres are carcinogenic when injected into the peritoneal cavity of rats. However, the tumours produced were recorded as sarcomas rather than mesotheliomas with only two mesotheliomas reported in the mid-dose (10^7 and 10^8 fibres) groups. The cellulose preparation used in the present study does not readily cause classical mesotheliomas, even in the extremely sensitive rat model.

1. INTRODUCTION

1.1 INTRODUCTION

Natural and synthetic organic fibres from a wide variety of sources and a wide range of compositions are used extensively by industry in applications ranging from traditional textiles and clothing, through building and other structural reinforcement materials, to modern high technology composites and specialist products. In some cases, manufacture, use and/or disposal of such products can be associated with the release of airborne respirable fibres.

Toxicological investigations of asbestos and man-made silicate fibres have shown that their pulmonary toxic effects are related to their fibrous morphology, with long thin fibres being particularly active, and to their persistence in the lung (Davis *et al.*, 1986; McClellan *et al.*, 1992; Bignon *et al.*, 1994; Miller *et al.*, 1999a; Searl *et al.*, 1999). However, by comparison, relatively little is known about the hazards of inhaled cellulose and other organic fibres (Davis, 1993). Many organic fibres are durable (Gruber, 1991) and thus have the potential to persist within the lung and cause disease.

We have tested a sample of cellulose using some of the protocols we have been applying to man-made mineral fibres in the Colt Fibre Research Programme (Davis *et al.*, 1996b; Cullen *et al.*, 1997; Miller *et al.*, 1999a,b; Searl *et al.*, 1999). Our initial experiments with cellulose have been published (Cullen *et al.*, 2000).

This report describes the results of intraperitoneal injection experiments in rats with a high purity fibrous cellulose. The aim was to investigate the possible carcinogenicity of cellulose fibres in the peritoneal cavity. Respirable crocidolite asbestos was used as a positive control.

1.2 BACKGROUND

1.2.1 Cellulose usage and health effects

Cellulose fibres are used in many synthetic products such as cotton textiles, paper and cardboard, as a substitute for asbestos to give structural strength to cement products, and as insulation for buildings. The manufacture and use of these products can produce airborne cellulose fibres. Cellulose dust is still generally regarded as a low toxicity material. Thus, in the UK, the maximum exposure limit for total inhalable cellulose dust is 10 $mg.m^{-3}$, higher than those for wood dust (5 $mg.m^{-3}$) and cotton dust (2.5 $mg.m^{-3}$).

Most of the cellulose fibre destined for manufactured products is derived from wood. Wood dust is a recognised carcinogen causing nasal cancer among hardwood furniture workers in Europe and, although with less strong evidence, in North America (Acheson, 1976; IARC, 1981, 1995; Blot *et al.*, 1997). Wood dust contains several compounds which may contribute to its carcinogenicity. In addition to polymeric components such as cellulose and lignin, wood also contains many lower molecular weight organic compounds with demonstrated biological activity such as terpenes, phenols, quinones, and stilbenes (IARC, 1995). Cotton dust also contains a small and variable fraction of respirable fibres and particles of cellulose and is associated with the human disease byssinosis. Studies with cotton dust have focused on non-cellulose components of cotton dust such as tannins derived from the bracts of the cotton plant, and endotoxin and proteases derived from the microbial flora on the plants and cotton fibres.

1.2.2 Epidemiological studies

A number of epidemiological studies have examined the health effects associated with working in the paper and pulp industries. Total dust levels in paper mills, especially in those making soft paper, have in the past been as high as 50 $mg.m^{-3}$ (Lundberg 1991). Fibre concentrations in one study were in the range 0.2 to 1.6 $fibres.ml^{-1}$ (Lundberg 1991). An excess frequency of cancers of the respiratory tract has been found in Finnish paper and pulp mill workers which could not be explained by smoking habits (Jappinen *et al.*, 1987). This and other epidemiological studies in the paper and pulp industry have also shown increased incidences of tumours in other organs such as stomach, colon, bladder, and lymphatic and haematopoietic systems (reviewed in Hogstedt, 1990). Some studies have reported increased respiratory symptoms and lowered respiratory function in paper mill workers (Ericsson *et al.* 1988; Jarvholm *et al.* 1988) whereas other studies found no such changes (Chan Yeung *et al.*, 1980; Ferris *et al.*, 1979). The agents responsible for the epidemiological findings have not been identified but workers in these industries can be exposed to a variety of chemicals such as bleaches, sulphites, sulphur dioxide, and chlorinated hydrocarbons, in addition to cellulose-containing dusts (Hogstedt, 1990).

1.2.3 Animal studies

From animal studies, purified cellulose causes no adverse health effects when ingested (Anderson *et al.*,1992). However, intraperitoneal injection of cellulose fibres caused mesothelioma in rats (Rosenbruch *et al* 1992). Other animal studies where cellulose fibres were instilled into the lung or inhaled, have shown pathological changes in the lung (Milton *et al.*, 1990; Hadley *et al.*, 1992; Tatrai *et al.*, 1995, 1996; Muhle, 1997; Adamis *et al.*, 1997). We have shown that inhalation exposure of rats to a concentration of 1000 cellulose fibres per ml caused a marked inflammatory response in the lungs which was greatest after one day of inhalation and thereafter declined, despite continuing exposure to the aerosol for about three weeks (Cullen *et al.*, 2000). In this study there were rounded epithelial cells and congregations of macrophages at the bifurcations of the terminal and respiratory bronchioles and small solid lesions formed by the interstitialisation of inflammatory cells. Cellulose fibres injected into the mouse peritoneal cavity also caused marked dose-dependent, but transient, recruitment of inflammatory cells (Cullen *et al.*, 2000).

1.3 INTRAPERITONEAL INJECTION ASSAY

The intraperitoneal injection assay is a useful technique for comparing the potency of fibrous materials in producing cancers of mesothelial tissue. The assay has the advantage of being able to produce very high tumour incidence, and thus can potentially produce highly significant differences with relatively small groups of animals. However, this assay has well-recognised limitations, namely, the peritoneal cavity does not have the particle clearance mechanisms of the lung and because the masses of fibre injected are large relative to animal size. Nevertheless, intraperitoneal injection is relatively inexpensive, compared to long term inhalation, and does allow comparison with published data for a wide range of fibre types (Pott, 1993). The assay is also currently accepted as part of testing protocols for fibre carcinogenicity within the European Community (CEC Directive, 1997). The highest dose used in the study reported here was 10^9 WHO fibres as used previously by ourselves and others (Pott, 1993, 1995; Davis *et al.*, 1996a; Miller *et al.*, 1999b) and as proposed for a German testing protocol (TGRS 905, 1994).

2. AIMS AND EXPERIMENTAL DESIGN

2.1 AIMS

The aim of the study was to investigate the carcinogenic potential of a sample of a commercial fibrous cellulose by means of a multi-dose intraperitoneal injection assay, using crocidolite as a positive control and saline as a negative control.

2.2 EXPERIMENTAL DESIGN

The experimental design for this study was based upon the recommendations of the International Cooperative Research Programme on Assessment of MMFs Toxicity (Bignon *et al.*, 1995) with regard to doses, controls, and group size. Groups of male Wistar rats were injected intraperitoneally with respirable fibres of cellulose or crocidolite asbestos, or phosphate-buffered saline (used to suspend the fibres). The total doses used were 10^9, 10^8, 10^7 and 10^6 fibres longer than 5 μm (as measured by scanning electron microscopy) administered by intraperitoneal injection in three, weekly aliquots (see section 3.5 below). To facilitate administration of the large number of injections whilst treating all levels concurrently, test groups were split into two approximately equal cohorts, spaced about 1 month apart. There were only 26 animals for each of the two highest doses of crocidolite because of the expected high incidence of mesothelioma in these groups; all other treatment groups contained 50 animals. The split between the two cohorts per treatment is shown in Table 2.1. The study was terminated when that cellulose group with the highest number of surviving rats had fallen to six rats.

Table 2.1
Numbers of rats per treatment per cohort

Treatment	Number in cohort 1	Number in cohort 2
Cellulose 10^9 fibres	24	26
Cellulose 10^8 fibres	24	26
Cellulose 10^7 fibres	24	26
Cellulose 10^6 fibres	24	26
Crocidolite 10^9 fibres	9	17
Crocidolite 10^8 fibres	12	14
Crocidolite 10^7 fibres	24	26
Crocidolite 10^6 fibres	24	26
P-B Saline	23	27

3. MATERIALS AND METHODS

3.1 ANIMALS

Specific-pathogen-free outbred, male Wistar rats, about 9-12 weeks old at entry to the study, were used in the experiments. All animals were obtained from Charles River UK Ltd. Throughout the study, all animals were supplied with Edinburgh community tap water and pelleted standard maintenance diet *ad libitum*.

3.2 TEST FIBRES

The cellulose fibre was a thermally processed wood pulp supplied by Laxa Bruks AB of Sweden (Inorphil Thermocell mechanically processed woodpulp) normally used as a bitumen stabiliser in road construction. It was supplied as a high purity cellulose material containing no anti-rot or fire retardant impregnating substances and less than 0.1% of a surfactant (principally dialkyl dimethyl ammonium chloride).

For comparison, a sample of respirable crocidolite asbestos was obtained from the Thermal Insulator Manufacturing Association (TIMA) fibre repository.

3.3 GENERATION OF CELLULOSE FIBRE AEROSOL AND COLLECTION OF A RESPIRABLE FRACTION

We generated cellulose dust clouds from Inorphil Thermocell Mechanical Wood Pulp using a Jet-O-Mizer mill (Fluid Energy Aljet, Plumsteadville, Pa, USA) according to the manufacturer's instructions. Briefly, the wood pulp was passed from a hopper via a screw feed into the mill where high velocity jets of air broke up the material. The cellulose dust was then passed in a stream of compressed air into a $1m^3$ chamber and a respirable fraction of the generated aerosol was collected on filters using an elutriator attached to a high volume pump (200 litre.minute^{-1}). The dust collected on the filters was pooled and mixed before use.

3.4 CHARACTERISATION OF RESPIRABLE FIBRES

3.4.1 Fibre size distribution and number to mass ratio

The respirable cellulose fibres were counted and sized by scanning electron microscopy (SEM) and the data used to calculate the fibre number to mass ratio for use in determining dose in the intraperitoneal injection experiments. A total of six SEM samples were prepared from aqueous suspensions of three precisely weighed samples of the respirable fibre. The length and diameter of fibres was measured at a magnification of x10K following a modified version of the WHO method (WHO 1985). Measurements were recorded to the nearest 0.1 μm. For each sample, the counting of fibres longer than 0.4 μm continued until at least 100 fibres longer than 0.4 μm and 15 fibres longer than 20 μm were sized, thus giving a total (over 6 samples) of at least 600 fibres longer than 0.4 μm, and 90 fibres longer than 20 μm. The same procedure was used to count and size the positive control fibre, crocidolite asbestos.

The data obtained by scanning electron microscopy were used to calculate the numbers of fibres longer than 5 μm per unit mass. Thus, masses of 0.178 mg and 11.587 mg were required to give 10^8 fibres of crocidolite and cellulose respectively. In terms of mass dose, the amount of cellulose injected for the 10^8 group was 66 times greater than the mass injected to give 10^8 crocidolite fibres. This was because the cellulose contained more thick fibres. The volume of cellulose injected was approximately 150 times greater than that of crocidolite because of its lower density.

3.4.2 Cellulose purity

It was expected that the wood pulp, being a natural product, would be likely to be contaminated by bacteria or fungi and could contain substances such as endotoxin. The respirable cellulose was microbiologically screened to identify the nature and extent of any bacterial or fungal contamination. The amount of endotoxin present was determined by washing samples of both the bulk cellulose and the respirable fraction in sterile, endotoxin-free water for 2 hours at room temperature (2 mg dust.ml^{-1}). The fluid was then recovered and endotoxin measured using a "Coatest" kit (Chromogenix AB, Molndal, Sweden). The purity of the respirable cellulose was further checked by undertaking X-ray diffraction analysis of ashed samples to determine whether any crystalline mineral phases were present.

3.5 ADMINISTRATION OF TEST SUBSTANCES

Samples of the respirable dusts were weighed and suspended in sterile phosphate-buffered saline to give the required dose of WHO fibres in a 2 ml volume. The suspensions were sonicated in an ultrasonic bath for 1 minute to break up clumps of fibres and thoroughly mixed immediately prior to injection into the rat peritoneal cavity. As the total amount of cellulose fibre to be injected per rat exceeded the 50 mg limit for a single injection recommended in the Paris Protocol (Bignon *et al.*, 1995), the dusts were administered as a total of 3 injections spaced at weekly intervals. Negative control rats received 3 injections of the carrier liquid, phosphate-buffered saline (PBS).

3.6 ANIMAL WEIGHTS AND THE MAXIMUM TOLERATED DOSE

As the high dose cellulose group were receiving such a high mass (or volume) of material there was concern that the Maximum Tolerated Dose (MTD) might be exceeded. The National Toxicology Program's (NTP) Bioassay Program defines the MTD as the dose that causes a 10% reduction in normal weight gain in a 90-day study (Eaton and Klaasen, 1995). Accordingly, all rats in the 10^9 and 10^8 crocidolite and cellulose groups, together with the saline control animals, were weighed on each injection day, and then at 5, 7, 9, 11 and 12 weeks after the first injection. All other rats were weighed on each injection day and at 12 weeks after the first injection.

3.7 AUTOPSY AND HISTOLOGY PROCEDURES

At autopsy the contents of both peritoneal and pleural cavities of all the rats were examined and the presence of any abnormalities, particularly visible lumps that could be tumours, were recorded. Routinely, four large blocks of tissue were taken for histological examination from sites where mesotheliomas have been most frequently found in previous intraperitoneal

injection studies in this laboratory. These sites were: the liver with diaphragm, the spleen plus pancreas and pieces of small and large intestine with large amounts of surrounding connective tissue included. In addition, any abnormality not included in the routine blocks was taken as a separate specimen. Tissues were fixed in buffered formal saline and paraffin sections were stained with haemotoxylin eosin. Histology slides were examined by an experienced animal pathologist.

In a few cases where rats had died spontaneously and fixation was delayed, autolysis was found to have left peritoneal tissues in a state too poor for detailed histological examination.

3.8 STATISTICAL ANALYSES

The times to death were summarised as survival functions, using Kaplan-Meier estimates (Collett, 1994). This technique estimates the value of the survival function at each time point at which a death occurs (for cause-specific analyses, a death from the chosen cause). The estimate is a function of survival at the previous time of death, number of deaths, and number of animals surviving to that time. Deaths from other causes or sacrifices of healthy animals give rise to censored data, that is where we know only that at the truncation of follow-up the animal had not yet died from the chosen cause. The Kaplan-Meier estimates make allowance for censoring events, by reducing the numbers surviving. The estimates were calculated by the statistical program BMDP1L (Dixon, 1992), which also provided estimates of mean and median survival times, with approximate 95% confidence intervals for the latter.

Test statistics of the general null hypothesis, that there are no differences between a set of treatment groups, lack power to detect a trend with dose. Accordingly, dose-response relationships were investigated by fitting proportional hazards regression functions (Collett, 1994) using the program BMDP2L (Dixon, 1992). The fibre types were analysed separately, using a log-linear transformation of dose levels with 10^9 = 1, 10^6 = 4, PBS = 5. Additional models were fitted, including a term distinguishing the two experimental cohorts of animals, to test for any possible confounding from cohort differences.

3.9 QUALITY ASSURANCE

Although this study was not required to be GLP compliant, the practices and procedures adopted during its conduct were consistent with the OECD Principles of Good Laboratory Practice (as set forth by the United Kingdom Department of Health), except that this report was not subject to full QA procedures.

4. RESULTS

4.1 CHARACTERISATION OF TEST FIBRES

4.1.1 Fibre size characteristics

SEM analysis of cellulose samples indicated that there were 8.6 x 10^6 WHO fibres per mg of respirable cellulose (i.e. fibres longer than 5 μm). About three quarters of the fibres sized were greater than 5 μm in length (Table 4.1) and half of the fibres had a branched "hairy" appearance (Figure 4.1).

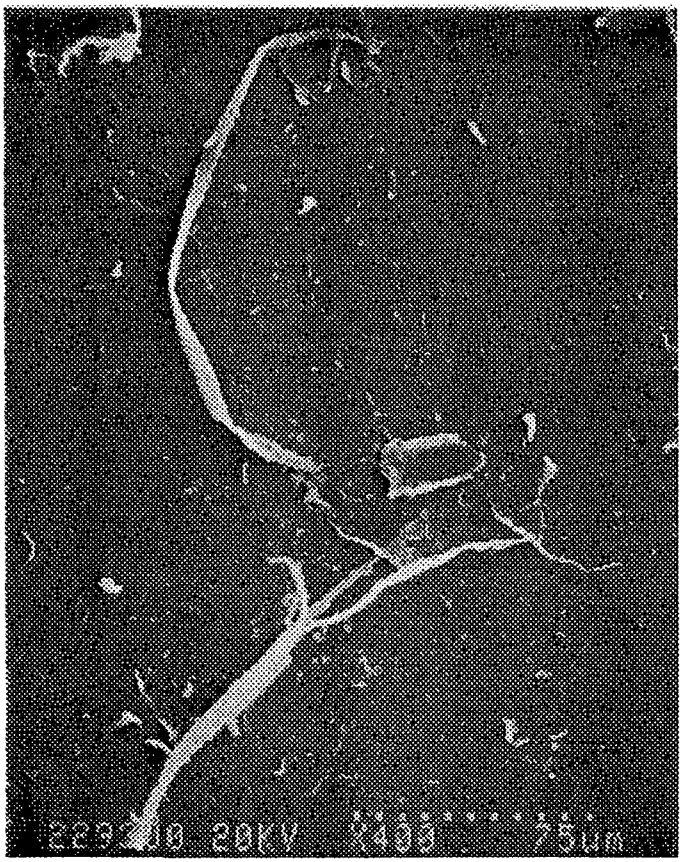

Figure 4.1
Scanning electron micrograph of cellulose fibres

Bivariate size distributions for cellulose and crocidolite are shown in Table 4.1 where the margins summarise the length and diameter distributions. With respect to fibre length there was a great deal of similarity between the two materials, the main differences being the greater number of long fibres (> 15 μm) in the respirable cellulose sample, 24.3% compared to 11.7% for crocidolite, and fewer cellulose fibres in the 1 to 5 μm category, 44.8 % compared to

58.5%. Cellulose fibres tended to be thicker than crocidolite fibres, especially for the long fibres. The cellulose samples contained significant numbers of non-fibrous particles at a ratio of particles to fibres of approximately 2:1.

The cellulose samples were produced by elutriation of airborne dust with a sampler designed to correspond to the human respirable range. Therefore, injection tests with these samples are relevant to fibres that might deposit in human lungs.

Table 4.1
Bivariate size distributions from scanning electron microscopy of respirable fibre samples of cellulose and crocidolite showing the actual number of fibres counted per size category

Fibre	Diameter category (μm)	Length category ≤ 0.9	>0.9 - 5	>5 - 10	>10 - 15	>15 - 20	> 20	All	All%
Cellulose	0.1	3	4	0	0	0	0	7	1.0
	0.2	2	90	14	3	1	2	112	15.9
	0.3	1	85	25	6	3	5	125	17.8
	0.4 - 0.7	0	125	70	19	13	23	250	35.6
	0.8 - 1.5	0	11	48	14	19	47	139	19.8
	> 1.5	0	0	3	9	6	52	70	10.0
	All	6	315	160	51	42	129	703	
	All %	0.9	44.8	22.8	7.3	6.0	18.3		
Crocidolite	0.1	6	137	37	19	7	14	220	24.3
	0.2	7	318	101	36	24	30	516	56.9
	0.3	0	57	29	13	4	12	115	12.7
	0.4 - 0.7	0	19	16	5	7	7	54	6.0
	0.8 - 1.5	0	0	0	1	0	0	1	0.1
	> 1.5	0	0	0	0	0	1	1	0.1
	All	13	531	183	74	42	64	907	
	All %	1.4	58.5	20.2	8.2	4.6	7.1		

4.1.2 Microbiological contamination of cellulose

The microbiological screening of the respirable cellulose found very low numbers (<140 per mg) of pasteurisation-resistant bacteria to be present. Endotoxin was detected in the cellulose but at the low level of 0.012 ng endotoxin per mg cellulose. The mineral content of the ashed cellulose samples was 1.1%.

4.2 MAXIMUM TOLERATED DOSE

To check that the maximum tolerated dose (MTD) had not been exceeded, animals were weighed regularly throughout the first few months of the study. Mean animal weights in the saline, and the 10^9 fibre groups for cellulose and crocidolite are shown in Table 4.2 for the first few weeks following the first injection and at week 12.

Within any treatment group the rats in the first cohort were lighter at the start of the study than those in the 2nd cohort due to a slight (about 10 days) difference in age, but by week 12 there

was no significant difference between the cohorts. Over the first 3 weeks, on average, rats in the fibre-treated groups tended to be lighter than those in the saline group. However, only the cellulose 10^9 group at week 3 was 10% lighter, on average, than the saline animals. We conclude that an MTD effect, as determined by weight measurement, was not significant in this study. Furthermore, as shown in the following sections, there was no indication that lifespan was being shortened by factors other than tumours in this group. This is also an indicator of the absence of MTD effects.

Table 4.2
Mean weights of rats at intervals following the first injection in the saline and the highest dose groups for cellulose and crocidolite

		Mean weight in grams (standard deviation)				
Cohort	**Treatment**	**week 0**	**week 1**	**week 2**	**week 3**	**week 12**
1	PB-saline	279(14)	319(17)	362(20)	391(26)	559(44)
1	Cellulose 10^9	282(17)	307(22)	330(25)	350(33)	541(42)
1	Crocidolite 10^9	289(18)	319(20)	349(25)	377(29)	541(37)
2	PB-saline	344(23)	383(32)	414(36)	ND*	560(62)
2	Cellulose 10^9	343(23)	363(29)	380(35)	393(39)	542(56)
2	Crocidolite 10^9	342(19)	376(27)	404(30)	420(34)	567(58)

** ND = no data available*

4.3 INITIAL TISSUE REACTION TO FIBRES

4.3.1 Crocidolite

In the present study no evidence of an early tissue reaction to crocidolite was obtained since no animals from the highest dose (1.8 mg) died during the first few months of the study. Most animals from this dose died with advanced mesotheliomas where multiple tumour masses and blood stained ascites fluid made the detection of small granulomas impossible.

4.3.2 Cellulose

The reaction to the intraperitoneal injection of 10^9 fibres of cellulose in rats was extremely vigorous. One animal was sacrificed at the end of two months because of weight loss. In this animal, multiple large granulomas had been produced, some almost 1 cm in diameter, but in addition there were widespread fibrous adhesions between the peritoneal contents themselves or between these organs and the body wall or the diaphragm. In this first animal sacrificed, the adhesions had caused restrictions of the intestines resulting in partial blockage. Multiple large granulomas and widespread fibrous adhesions were present in all animals subsequently examined from this treatment group including those from the final kill at 28 months after injection. The granulomas were typical of a response to fibrous dusts and made up of macrophages and fibroblasts with particularly frequent foreign body giant cells. Cellulose fibres were visible among the cellular reaction but were much less obvious than was the case with crocidolite. The peripheral regions of cellulose granulomas remained cellular until the final kill and cellulose fibres were still visible at this time. In the largest granulomas, however, central regions often became calcified from about nine months from the date of injection.

Multiple large granulomas with fibrous adhesions were found in all animals that had received 10^9 cellulose fibres. Small granulomas with slight adhesions were usually recorded following the injection of 10^8 cellulose fibres but no macroscopic observation or histological finding of granulomas was made in the groups of rats that received 10^7 or 10^6 cellulose fibres.

4.3.3 Saline

No granulomas or adhesions were observed in saline-treated control animals.

4.4 TUMOUR DEVELOPMENT

4.4.1 Summary of tumour incidence

Table 4.3 summarises the autopsy results, showing for each treatment the size of the initial group, and the numbers dying without tumours and with each of the tumour types. The fourth column (from the left) shows animals in which detailed histological examination was not possible because of autolysis, and in which we therefore cannot be certain that no tumour was present. There was no difference in tumour incidence between cohorts.

The PBS-injected controls had only two carcinoma deaths. The crocidolite treatments, particularly at the higher doses, had numerous deaths with mesothelioma, confirming that they had been effective as positive controls. The results for the cellulose treatments showed an interesting contrast. There was one mesothelioma at 10^8 fibres and one at 10^7 fibres, while the 10^9 fibre treatment produced no carcinomas or mesothelioma, but 9 sarcomas. Note that spontaneous mesothelioma are rare in this rat strain (see Section 5.5). The 10^6 fibres cellulose group had one sarcoma and three carcinomas. We do not regard the carcinomas as being treatment-related. The details of the pattern of tumour production are discussed in more detail for each fibre type below.

Table 4.3
Summary of causes of death in study of rat survival following intraperitoneal injection

Injection treatment (number of fibres)	Number of animals	Number in final kill	Diagnosis at death				
			No histology	Carcinoma	Mesothelioma	Sarcoma	No tumour
Crocidolite 10^9	26	0	0	0	22	0	4
Crocidolite 10^8	26	0	0	0	21	0	5
Crocidolite 10^7	50	4	1	0	14	0	35
Crocidolite 10^6	50	2	2	3	4	2	39
Cellulose 10^9	50	4	1	0	0	9	40
Cellulose 10^8	50	6	2	1	1	0	46
Cellulose 10^7	50	5	0	1	1	0	48
Cellulose 10^6	50	5	0	3	0	1	46
PB Saline	50	6	3	2	0	0	45

4.4.2 Crocidolite

Intraperitoneal injection of crocidolite fibres in the peritoneal cavity of rats produced typical mesotheliomas in all four treatment groups. A clear dose response was recorded (Table 4.3) with the highest doses producing the highest proportion of tumours and the shortest median survival times (section 4.5). To provide comparison with the 10^9 cellulose group, where median survival could not be calculated, the mean survival times of animals with tumours were as follows: crocidolite 10^9, 437 days; crocidolite 10^8, 508 days; crocidolite 10^7, 665 days; crocidolite 10^6, 743 days; and cellulose 10^9, 476 days.

With all advanced mesotheliomas from the crocidolite treatment groups, the rat peritoneal cavity was found at autopsy to be filled with blood-stained ascites fluid, and multiple tumour nodules or masses were present. With early mesotheliomas, however, there was often nothing to be seen at autopsy by macroscopic observation and the tumours were subsequently diagnosed by histological examination. As is typical of rat peritoneal mesotheliomas the histological pattern of tumours varied from widespread papillary growths to multiple small pedunculated nodules, to sheets of sarcomatous growth covering large areas of the peritoneal contents on the body wall. In the papillary pattern, cells of mesothelial type were supported by loose fronds of connective tissue. The nodules and sheets consisted mainly of spindle cells with varying amounts of connective tissue fibres although any free surfaces of these lesions were often covered with several layers of more rounded cells of mesothelial type.

Two animals from the 10^6 crocidolite group developed peritoneal sarcomas. One was a small spindle cell tumour clearly originating within the wall of the intestine while the other was a mass attached to the omentum consisting of blood-filled spaces separated by loose connective tissue walls. This was classed as an angiosarcoma.

4.4.3 Cellulose

Intraperitoneal injection of cellulose fibres did not result in the production of typical widespread mesotheliomas in rats. Only one animal (from the 10^9 cellulose group) developed blood-stained ascites fluid with widespread tumour masses in the peritoneal cavity. This was, however, a lymphoma with typical masses of small mononuclear cells present in many of the body organs. Only two animals treated with cellulose fibres developed peritoneal tumours classified clearly as mesothelioma. One was from the 10^8 treatment group. This tumour, diagnosed only by histology, was mainly of a sarcomatous pattern growing as sheets in the region of the pancreas but with the surface of the tumour masses consisting of rounded cells of mesothelial type. This animal died at 744 days post-treatment. The second mesothelioma was in an animal from the 10^7 treatment group. This tumour was also diagnosed only by histology and consisted of a single mass on the surface of the spleen. The histological pattern was a papillary growth very similar to the spontaneous mesotheliomas that occasionally occur in old male rats in the region of the epididymis. While on this occasion the epididymis was macroscopically normal and therefore no histology was taken, we cannot rule out the possibility that what was found was very early spread from an early spontaneous source (see also the discussion in Section 5.5). This animal died at 400 days post-treatment.

Nine animals from the 10^9 cellulose injection group developed large peritoneal tumours. These were sometimes single masses up to 4 cm in diameter but in other animals the tumours grew as sheets invading the peritoneal organs or the body wall. The histological pattern of these tumours was usually of spindle cell/fibrosarcomatous type. In contrast to the usual pattern of mesothelioma development following treatment with mineral fibres in rats, the nine tumours

showed no obvious involvement of mesothelial tissues and were not associated with blood-stained ascites fluid. They developed relatively early in the study with a mean time to death of only 476 days and none were found during the last nine months of the study. To draw attention to these differences, these nine tumours are described as sarcomas, but it is possible, as explored in the discussion, that they do form an extreme end of the mesothelioma range.

One animal from the cellulose 10^6 treatment group developed a single nodule attached to the omentum, which consisted of blood-filled spaces surrounded by loose connective tissue walls. This was classified as an angiosarcoma.

4.4.4 Epithelial tumours in the peritoneal cavity

Routine sectioning of the pancreas, as one of the four tissue blocks always taken for histological examination, revealed that the majority of rats from all treatment groups, as well as the controls, developed very small pancreatic adenomas. Since none of these benign tumours was large enough to affect the overall health of the animal, and since similar tumours of other peritoneal organs, not subjected to routine sectioning, could not be ruled out, benign pancreatic adenomas are not further discussed.

In some animals, nodules large enough to be visible at autopsy, and therefore taken for routine histology, were found to be carcinomas; these are listed in Table 4.3. Most were pancreatic carcinomas but one animal from the PBS control group developed an adrenal carcinoma, one animal from the cellulose 10^7 group developed a carcinoma of the kidney and one of the 10^6 cellulose treatment group developed a carcinoma of the intestine.

4.4.5 Tumours in the pleural cavity

During the present study a few animals were discovered at autopsy to have tumour masses growing within the pleural cavity. With the injection techniques used, a mis-injection into this region is extremely unlikely and these pleural tumours are considered to be spontaneous. Two pleural tumours were found in the cellulose 10^6 group, one from the cellulose 10^7 group, one from the cellulose 10^8 group, one from the crocidolite 10^7 group and one from the crocidolite 10^8 group. None were found in the 10^9 groups, the crocidolite 10^6 group, or the saline control group. The histological pattern of these tumours was either clearly lymphoid or of a fibrosarcomatous pattern. It is considered that all may be of thymic origin. Similar tumours have been found occasionally in previous inhalation and injection studies from this laboratory.

4.5 SURVIVAL TIMES

4.5.1 Survival functions for all causes of death

Figure 4.2 shows the survival curves for the saline controls and for the crocidolite treatments. These curves are for mortality from all causes, including the animals with autolysis. Final sacrifices where mesotheliomas and sarcomas were found were treated as deaths with tumours, on the grounds that tumour-related deaths would have occurred before long. Final sacrifice animals showing no tumour were treated as censoring events. The two higher doses of crocidolite show greatly reduced survival, and the curves for even the two lower doses lie mostly to the left of that for the saline controls. The five survival curves differed highly

significantly ($p<0.0001$). Fitting a proportional hazards regression model to the logarithms of dose also gave a highly significant dose-response regression coefficient.

Figure 4.3 shows the curves for the cellulose treatments, again with the saline controls for comparison. Here, the separation is much less extreme, but it is noticeable that the curve for the highest dose of cellulose lies to the left, suggesting higher mortality in this high-dose group. It is also noticeable that all the treated groups lie to the left of the saline control group. However, the differences were not significant at the 5% level. Fitting a proportional hazards model to the logs of the doses gave a test statistic which reached the 0.055 significance level. Thus there was some evidence of a trend in mortality that was not due solely to chance variation.

Additional proportional hazards models fitted to both series included a term for cohort differences. In both series, the cohort term fell far short of statistical significance, and its inclusion left the log-dose coefficient essentially unaffected. Details of these models are not given here.

Table 4.4 summarises the median survival for each treatment, with approximate standard errors and 95% confidence intervals. In some cases, where the data are sparse, these quantities cannot be calculated reliably, and these cases are indicated as missing (*). The reduced survival in the higher crocidolite doses is very evident. The saline group had the longest survival, and for each fibre type the median survival decreased consistently with increasing dose. However, from the highest to lowest dose of cellulose, the range of values was only 56 days. And from the highest dose of cellulose to saline controls, the range was 91 days.

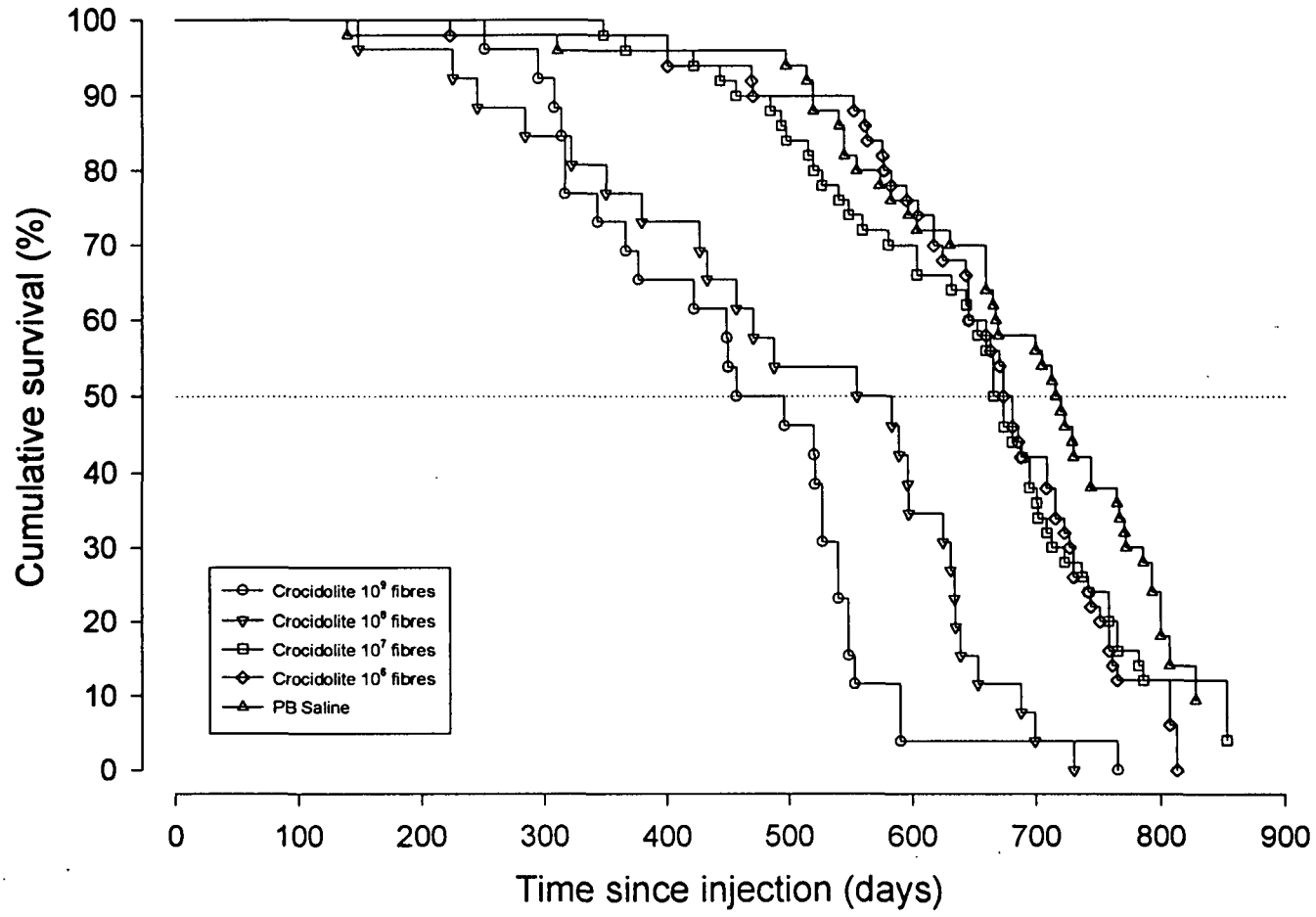

Figure 4.2
Estimated survival functions for saline control and crocidolite treatment groups: deaths from all causes

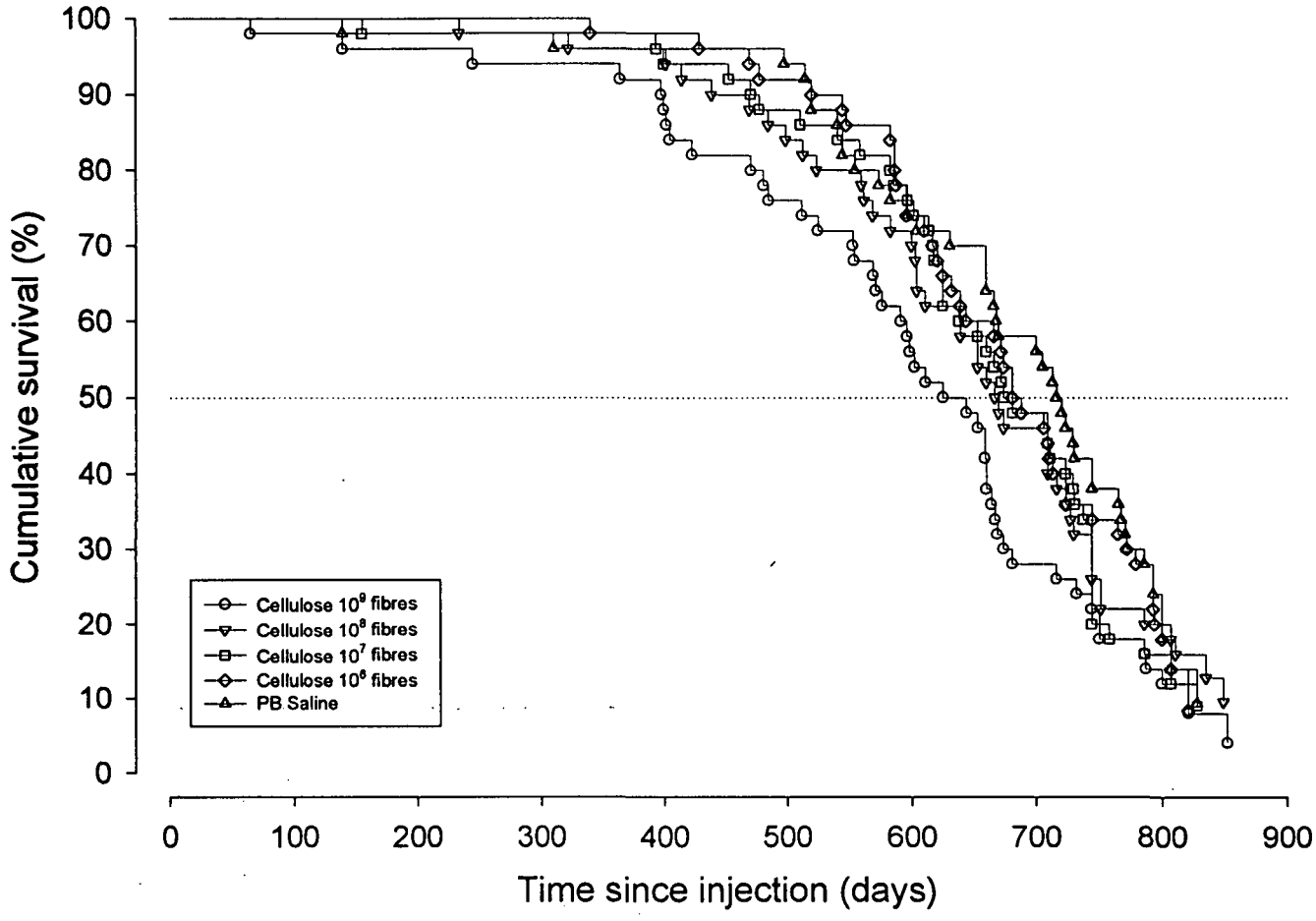

Figure 4.3
Estimated survival functions for saline control and cellulose treatment groups: deaths from all causes

Table 4.4
Estimates of median (50%) survival (days) following intraperitoneal injection, by treatment: deaths from all causes

Injection treatment	Survival (days)			
	Median	SE	*95% CI*	
Crocidolite 10^9 fibres	457	45	*377*	*527*
Crocidolite 10^8 fibres	555	75	*433*	*625*
Crocidolite 10^7 fibres	666	12	*644*	*701*
Crocidolite 10^6 fibres	674	13	*646*	*716*
Cellulose 10^9 fibres	625	31	*576*	*664*
Cellulose 10^8 fibres	667	27	*611*	*723*
Cellulose 10^7 fibres	674	29	*625*	*730*
Cellulose 10^6 fibres	681	22	*639*	*723*
PB Saline controls	716	17	*666*	*765*

Table 4.5
Estimates of median (50%) survival (days) following intraperitoneal injection, by treatment: all deaths with mesothelioma or sarcoma

Injection treatment	Survival (days)			
	Median	SE	*95% CI*	
Crocidolite 10^9 fibres	487	57	*377*	*540*
Crocidolite 10^8 fibres	595	10	*433*	*634*
Crocidolite 10^7 fibres	788	26	*742*	*853*
Crocidolite 10^6 fibres	*[a]	*	*	*
Cellulose 10^9 fibres	*	*	*	*
Cellulose 10^8 fibres	*	*	*	*
Cellulose 10^7 fibres	*	*	*	*
Cellulose 10^6 fibres	*	*	*	*
PB Saline controls	*	*	*	*

a) The survival functions for death with tumours for the cellulose and saline treatments did not fall to 50%, and so the medians are not estimable for these treatments

4.5.2 Survival functions for deaths with tumours

The analyses in the previous section were for survival from all causes. Further analyses were performed of the times to deaths associated with tumours (mesotheliomas and sarcomas), treating other causes and deaths with autolysis as censoring events. Figure 4.4 shows the estimated survival functions for the saline and crocidolite treatments, showing an even clearer separation between the treatments than in Figure 4.2. Differences between the treatment

groups were again highly significant, as was the regression coefficient from a proportional hazards analysis.

Figure 4.5 shows the deaths from mesotheliomas and sarcomas in the cellulose treatments. The 10^9 dose shows clearly poorer survival here. Formal significance tests of the differences between treatments were highly significant ($p<0.001$), and the proportional hazards regression analysis also gave a highly significant regression coefficient ($p<0.005$).

Table 4.5 shows the median survival estimates for all deaths with mesotheliomas and sarcomas. The survival functions for the cellulose and saline treatments did not fall to 50%, and so the medians are not estimable for these treatments.

4.5.3 Survival functions for non-tumour deaths

Figures 4.6 and 4.7 show the survival functions for deaths definitely not associated with mesothelioma or sarcoma (treating tumour and autolysis deaths as censoring events). These showed no significant treatment differences, nor significant dependence on dose. Their median survival times are summarised in Table 4.6. Note that for the 10^8 crocidolite treatment the non-tumour animals did not survive long enough to enable a median to be calculated.

Table 4.6
Estimates of median (50%) survival (days) following intraperitoneal injection, by treatment: all deaths with neither mesothelioma nor sarcoma

Injection treatment	Survival (days)			
	Median	SE	*95% CI*	
Crocidolite 10^9 fibres	598	62	*548*	*
Crocidolite 10^8 fibres	*[a]	*	*688*	*
Crocidolite 10^7 fibres	696	23	*666*	*758*
Crocidolite 10^6 fibres	681	21	*664*	*730*
Cellulose 10^9 fibres	660	6	*625*	*716*
Cellulose 10^8 fibres	671	18	*639*	*730*
Cellulose 10^7 fibres	678	30	*638*	*737*
Cellulose 10^6 fibres	687	22	*644*	*772*
PB Saline controls	720	14	*668*	*786*

a) The survival functions for the 10^8 crocidolite treatment did not fall to 50%, and so the median was not estimable for this treatment

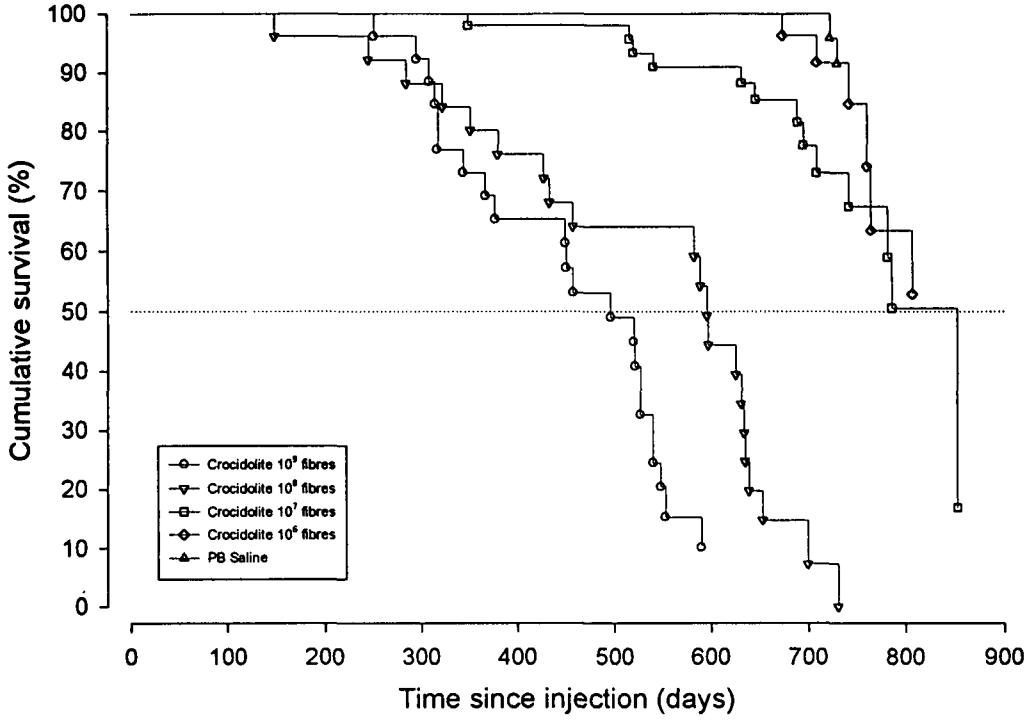

Figure 4.4
Estimated survival functions for saline control and crocidolite treatment groups: deaths with mesothelioma and sarcoma only

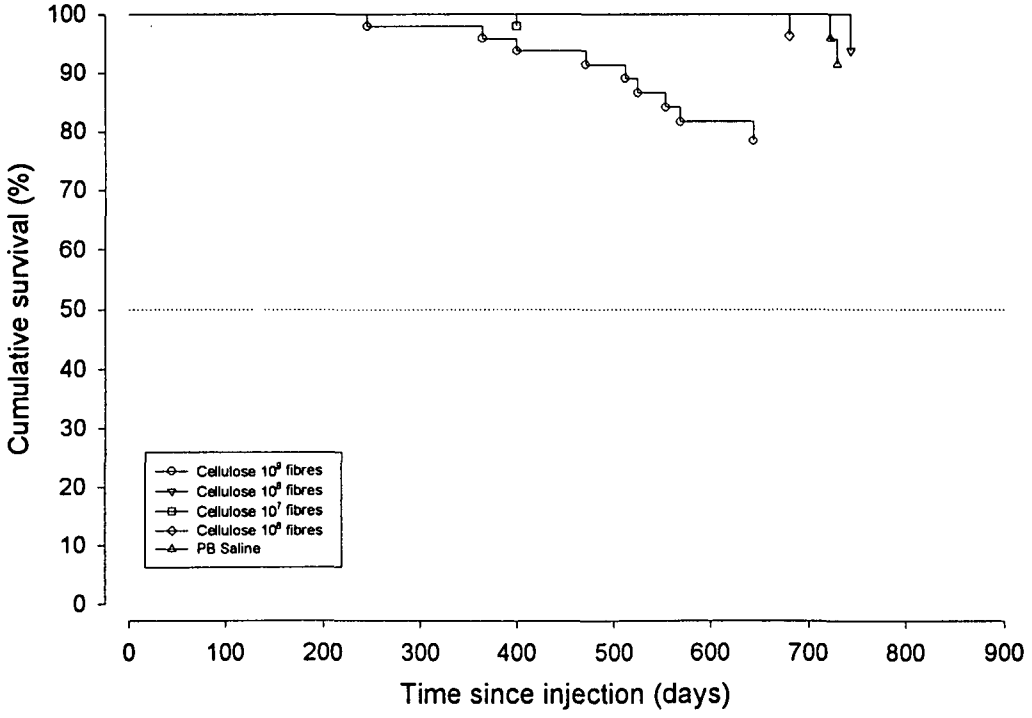

Figure 4.5
Estimated survival functions for saline control and cellulose treatment groups: deaths with mesothelioma and sarcoma only

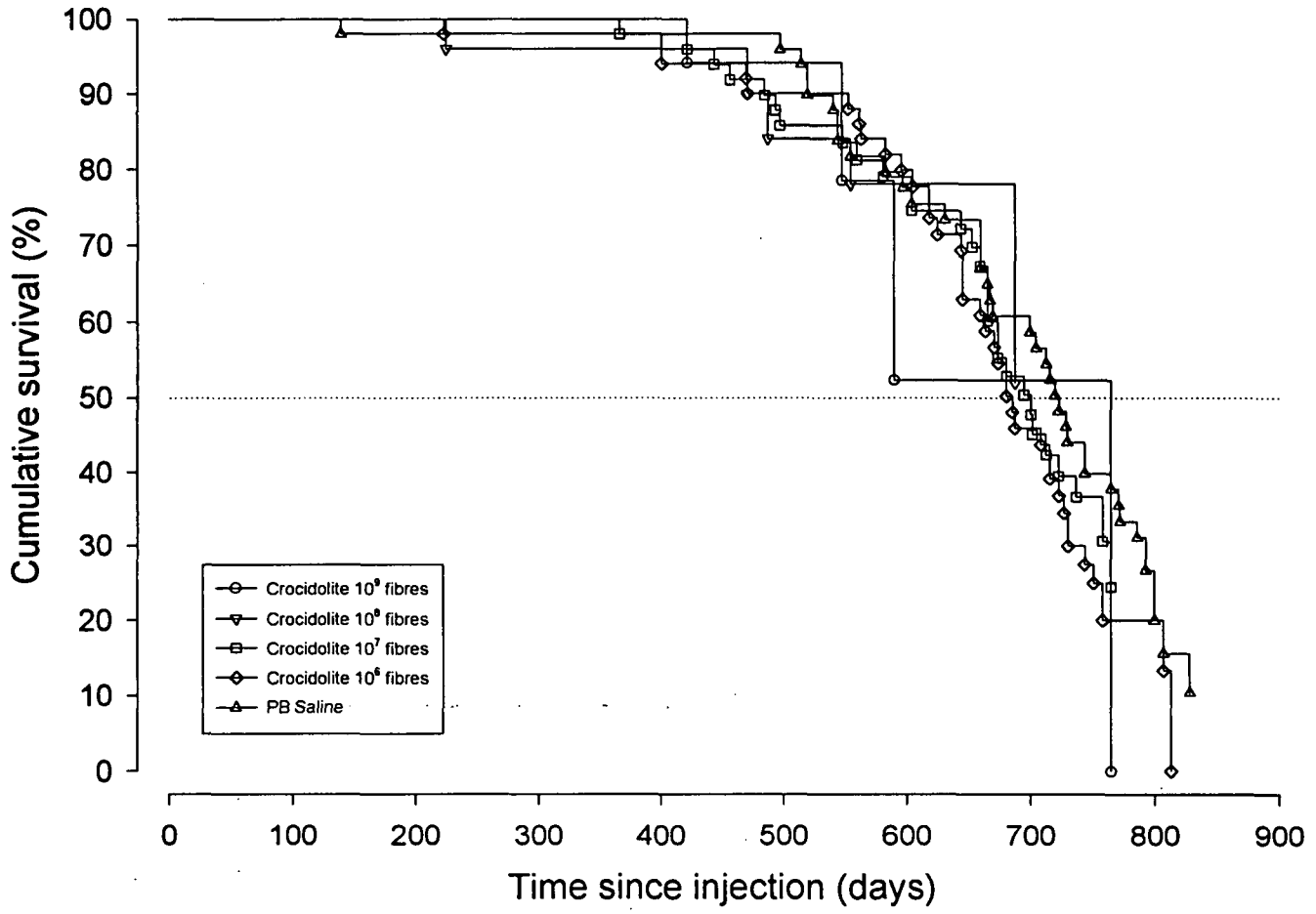

Figure 4.6
Estimated survival functions for saline control and crocidolite treatment groups: all deaths with neither mesothelioma nor sarcoma

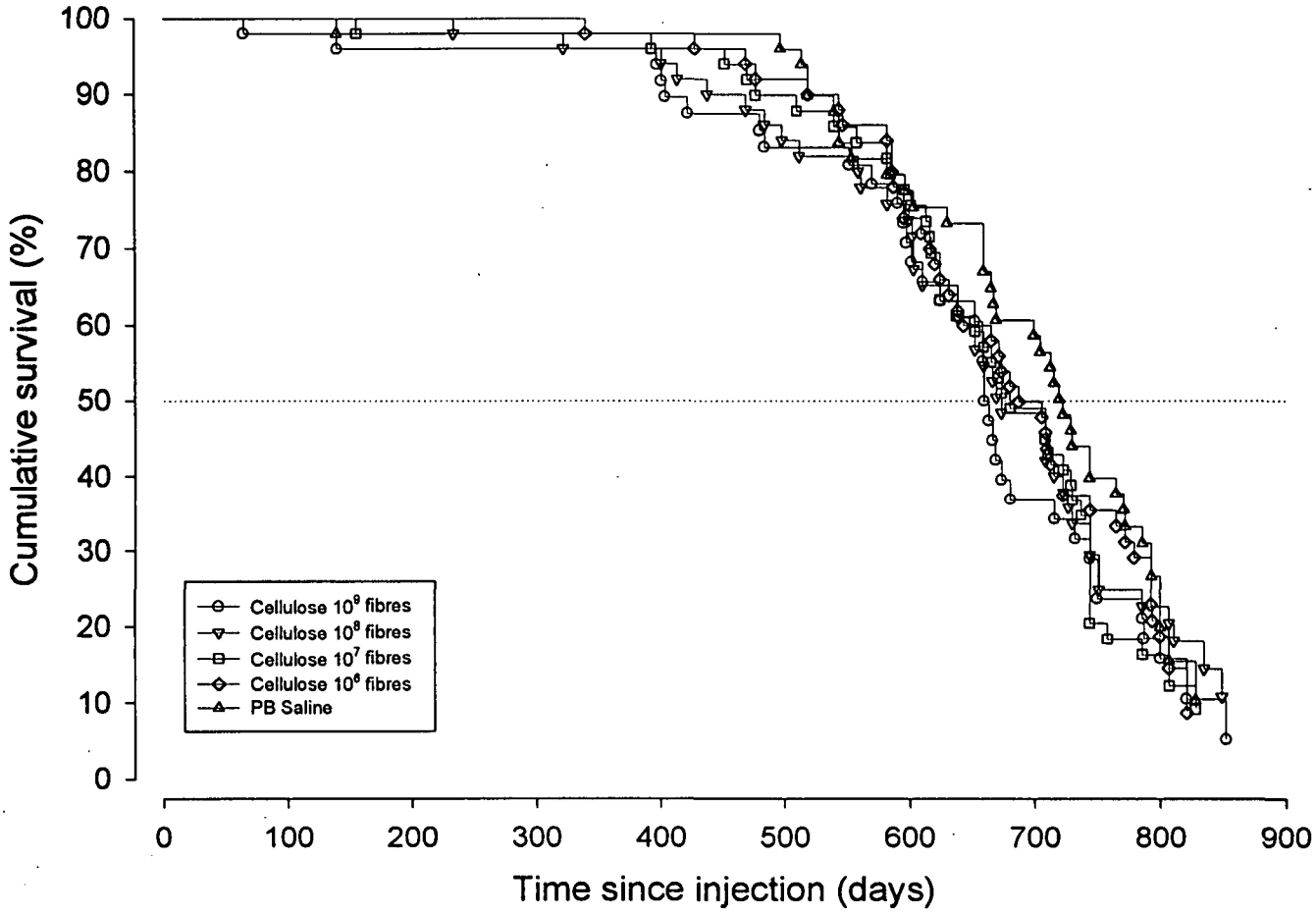

Figure 4.7
Estimated survival functions for saline control and cellulose treatment groups: all deaths with neither mesothelioma nor sarcoma

5. DISCUSSION

5.1 MAIN FINDINGS

Using intraperitoneal injection in rats we have presented evidence of a dose-related increase of risk of tumour production for the sample of cellulose fibres used in the present study. This rests on treating the sarcomas and mesotheliomas as a combined group of tumours. Cellulose also caused widespread tissue reaction with granulomas and fibrosis.

5.2 CELLULOSE TOXICITY

That cellulose fibres are biologically active has already been demonstrated in a number of short-term studies. For example, cellulose fibres have been shown to be toxic *in vitro* to mouse peritoneal macrophages and cause them to release greater amounts of inflammatory mediators than asbestos or some man-made vitreous fibres (Godelaine and Beaufay, 1989). Several studies have shown pathological changes (granuloma, alveolitis, epithelial hyperplasia, fibrosis) in the lung following the instillation or inhalation of cellulose fibres (Milton *et al.*, 1990; Hadley *et al.*, 1992; Tatrai *et al.*, 1995,1996; Muhle, 1997; Adamis *et al.*, 1997).

The same preparation of respirable cellulose fibres used for our intraperitoneal injection study in rats was also studied in our laboratory for short-term toxicity assays. These compared both injection in the peritoneal cavity of mice and inhalation by rats for three weeks (Cullen *et al.*, 2000). Cellulose fibres caused marked dose-dependent recruitment of inflammatory cells to the mouse peritoneal cavity although this effect persisted for only a few days. Evidence of later fibrous or granuloma formation was not sought in that study. Similar results were found when rats were exposed by inhalation for three weeks (5 days per week) to a concentration of 1000 cellulose fibres.ml^{-1}. This inhalation exposure induced an early inflammatory response in rat lungs, as determined by the number of neutrophils recovered by bronchoalveolar lavage, which peaked after the first day of inhalation and thereafter declined. The function of lavaged macrophages became more affected towards the end of exposure. Thus, by the end of the short exposure period, *in vitro* production of the pro-inflammatory cytokine TNFα by lavaged macrophages was markedly depressed in cellulose-exposed animals, compared to untreated controls. This effect was still present for rats which had been allowed to recover for 28 days beyond the end of exposure.

5.3 LIMITATIONS AND VALIDITY OF THE INTRAPERITONEAL TEST

The intraperitoneal injection assay as a test for the hazard of inhalable fibres is limited in that large fibre doses are administered non-physiologically to an enclosed site with no clearance mechanism other than by dissolution (see, for example, Rossiter, 1991; Johnson, 1994). Some glass fibre types may persist within the peritoneal cavity longer than in lung tissue (Collier *et al.*, 1994,1995). However, these peritoneal tests do allow ready comparison with published data for a wide range of fibre types (Pott, 1993; Davis *et al.*, 1996a; Roller *et al.*, 1996; Miller *et al.*, 1999b) and are currently accepted as part of testing protocols for fibre carcinogenicity within the European Community (CEC, 1997). Direct injection into the peritoneal cavity has also provided valuable data on the mechanisms of fibre-induced pathology and carcinogenesis, for example with regard to the roles of dose, fibre dimension and durability (Davis *et al.*, 1986,

Miller *et al.*, 1999b; Stanton and Wrench 1972; Pott and Friedrichs, 1972), and also on the cellular and molecular mechanisms leading to mesothelioma (Moalli *et al*, 1987; Friemann *et al*, 1990; Kane and Macdonald, 1993; Unfried *et al.*, 1997).

5.4 TUMOUR PRODUCTION

In the present study the intraperitoneal test has proved positive with the highest dose of cellulose producing a significantly increased number of peritoneal tumours compared to the negative control (saline) group. The pattern of tumour development was, however, unusual. Firstly the tumours in cellulose treated animals showed only the histological pattern of sarcomas, without apparent involvement of mesothelial tissue, and secondly these tumours developed relatively early in the study, possibly suggesting that the potency of the fibres had reduced with time following injection. With inhalation studies however, fresh fibres would be deposited in the lung day by day during the whole lifespan of the rat and any carcinogenic potential might well be retained into advanced age.

It has been demonstrated in a number of studies using asbestos and man-made vitreous fibres (MMVFs) injected into the rat peritoneal cavity that risk of tumour development is dose-related. At lower doses of injected material, the pattern of tumour induction is slower with the last "positive" doses producing only a few tumours at the end of the rat lifespan (Davis *et al.*, 1991, 1996a; Miller *et al.*, 1999b). This pattern has been produced once more in the positive control groups from the present study. With the highest dose of cellulose fibres, however, peritoneal tumours were produced relatively early in the study with none developing during the last seven months of the experimental period. This is in contrast to mesothelioma where these tumours are found right up to the end of a study as long as animals survive.

While lesions classifiable as sarcomas have been present in most studies involving the IP injection of fibres, the present study is, to our knowledge, almost unique in that a significant number of such tumours has been found in one experimental group without any classical mesotheliomas and with the sarcomas presenting as single lesions with no blood-stained ascites present. This group of animals had received a massive dose of cellulose fibres which produced widespread fibrosis. It may be that such a massive dose produces tumours by a different mechanism to most intraperitoneal production of classical mesotheliomas and that this mechanism affects connective tissue to produce sarcomas rather than mesotheliomas. This possibility is suggested by the low tumour induction period for cellulose-related sarcomas and the lack of these tumours in the oldest animals. With cellulose, the response appears to be modified as the animals age and although granulomas and adhesions remain in the oldest animals, no peritoneal sarcomas were found during the last seven months of the study. The reason for this loss of response is uncertain although fibre disappearance is not the cause as Muhle *et al.*, (1997) reported that cellulose fibres persist for long periods in tissues and, in the present study, cellulose fibres were still visible in granulomas from the oldest animals. It may be that the cellulose surface is modified or occluded after long periods in tissue resulting in a reduced tissue response that is less likely to lead to tumour production. Since the cellulose dose that produced tumours also produced widespread fibrosis, tumour induction may be related to the vigorous inflammatory response produced by such a dose (see discussion below in Section 5.6).

5.5 TUMOUR CLASSIFICATION AND MISDIAGNOSIS

The exact diagnosis of tumours produced following the intraperitoneal injection of fibres has always been problematic due to the various histological patterns involved. Most studies have produced variations on three patterns. One pattern involves papillary growths covering much of the peritoneal surface where supporting fronds of connective tissue are covered with cells of mesothelial type. A second pattern involves multiple small pedunculated nodules, with the central regions showing cells of sarcomatous type and with a few layers of rounded "mesothelial" cells on the surface. The third pattern involves tumours growing as sheets covering the peritoneal surface and made up of cells of sarcomatous type although any free surfaces to be found are covered with several layers of more rounded cells. With all these patterns, the peritoneal cavities are found to be filled with blood-stained ascites fluid if the tumours are relatively advanced although in the early stages, diagnosed only by histology, this fluid is not present. Since mesotheliomas are characterised by showing histological patterns of both epithelial and connective tissue type, this whole range of tumours has been classified as mesothelioma in many publications (Davis *et al.*, 1991; 1996a,b; Miller *et al.*, 1999b). In some of the early studies undertaken by intraperitoneal injection in Germany, 'sarcomas' were classified separately from classical mesotheliomas. In one paper (Scheuer *et al.*, 1973) one out of ten groups of rats injected with asbestos had all tumours classified as sarcomas. Here, however, they were described as multinodular lesions associated with blood-stained ascites and this pattern was different to the sarcoma tumours reported here from the injection of cellulose.

Where many tumours have been recorded in an experimental group, the occasional misdiagnosis will not have changed the overall results. The possibility of misdiagnosis exists, particularly where female rats have been used and spontaneous uterine tumours will have occurred in some animals (the present study used male rats). However, the use of the intraperitoneal test to evaluate the carcinogenic potential of new types of mineral or fibre of high solubility has increased the importance of occasional misdiagnosis. Since these materials produce at most a few tumours in each experimental group, any incorrect classification is more likely to affect the overall evaluation of any study. One possibility of overcoming this difficulty has been suggested by Lambré *et al.* (1998) who proposed that an overall classification of "Intra-abdominal tumours with serosal spread" (IATSS) might be used.

The diagnosis problem was recognised in the only intraperitoneal study using cellulose fibres that we are aware of (Rosenbruch *et al.*, 1992). In that study, intraperitoneal tumours were classified as either sarcomas or mesotheliomas although all four of the tumours from the 28 rats in the cellulose injection group were classified as mesotheliomas. This finding does not necessarily conflict with the results of the present study in which one peritoneal mesothelioma was recorded in an animal that had received 10^8 cellulose fibres (approximately 10 mg), and one in an animal that had received 10^7 cellulose fibres. The dose of cellulose used by Rosenbruch to produce four mesotheliomas was 20 mg. They did not use a dose as high as the 115 mg of the present study.

It is not certain whether the two mesotheliomas found in the 10^8 and 10^7 fibres cellulose groups were spontaneous or treatment-related. No mesotheliomas were found in the 47 control animals in this study. In most previous IOM studies using this rat strain, the peritoneal contents were examined at autopsy but only tissue with macroscopic abnormalities was taken for histology. With this approach mesotheliomas, usually originating in the *tunica vaginalis*, were found, but in less than one percent of animals. In the present study, histological examination of macroscopically normal tissues was undertaken for a large part of the peritoneal contents of treated and control animals. This approach resulted in the discovery of the two very early

mesotheliomas (in the cellulose groups) that would certainly have been missed in the previous IOM work. However, the supplier of the rats used in this study (Charles River) have reported on a long term observation of 200 control male rats of this strain in which detailed histological examination of all organs was undertaken. In this group a single mesothelioma was found originating in the epididymis (Vandenberghe 1990). Thus the incidence of mesothelioma in each of these two cellulose groups appears much higher than in the controls, but the comparison relies on only very few animals with mesothelioma.

5.6 POSSIBLE MECHANISMS LEADING TO FIBRE-INDUCED TUMOURS

Although the present study examined tumour incidence, it was not intended to examine the mechanisms of the initiation and development of cancer. Nevertheless, this section summarises the likely mechanisms which probably lead from the early stages of the response to fibres, as examined in many short term studies (Cullen *et al.*, 1997, 2000), to the production of fibrosis and tumours. The development of fibre-induced cancer is likely to be a progressive process requiring the promotion of conditions favouring proliferative regeneration but which also allow the survival, replication and further genetic change of genetically compromised cells. It is thought that the actions and products of macrophages and other inflammatory cells contribute to the necessary conditions required both for this process and for the development of fibrosis. It has been hypothesised that the pathogenic changes are initiated and sustained in response to particles through the production of reactive oxygen radicals at the surface of particles and/or within cells associated with those particles. The release of oxidant radicals might induce genetic damage to proliferating epithelial cells which is then clonally expanded. This might lead to an increased probability that the genetic changes necessary for neoplastic transformation will occur (Driscoll *et al.*, 1995). The release of reactive oxygen species also triggers a cascade of chemokine, cytokine and growth regulatory molecules (Petruska *et al.*, 1991; Brody, 1993). There is, however, not yet a complete picture of the complex sequence of events within the cytokine network, which must occur to produce fibrosis and cancer. This scenario is based mainly upon studies of inorganic fibres and there is insufficient information to say whether such processes will also occur similarly for organic fibres. The very marked fibrotic response to cellulose might indicate that some mechanistic differences exist between organic and inorganic fibres although this could simply be due to the large mass dose of cellulose injected (see Section 5.7).

5.7 FIBROSIS AND GRANULOMA FORMATION

Multiple large granulomas with fibrous adhesions were found in all animals treated with the highest cellulose dose (10^9 fibres) but in the 10^8 cellulose group the reaction was less vigorous with mostly small granulomas and slight adhesions. Cellulose fibres were found within the granulomas, including those animals sacrificed at the termination of the study, thus indicating the durable nature of the fibres.

The finding that fewer cellulose fibres were visible in granulomas than previously found with crocidolite may at first seem surprising since the cellulose fibres are much thicker. However, this observation may be due to the greater inflammogenicity of the cellulose. While both crocidolite and cellulose granulomas are qualitatively similar in their cellular makeup, it may be that with a greater inflammatory response the ratio of cells to fibres is higher in the cellulose-treated animals.

Formation of widespread large granulomas was also found in other studies where very large mass doses of man-made vitreous fibre (in excess of 100 mg to give 10^9 fibres) have been injected (Davis *et al.*, 1996a). However, with man-made vitreous fibres, granuloma formation was not accompanied by widespread fibrosis, which appears to result from a specific toxicity of cellulose.

In the crocidolite groups in the present study, granulomas were not seen, in contrast to our previous IP injection studies, using higher doses of crocidolite (10-25mg), that produced typical granulomas consisting of macrophages, fibroblasts and foreign body giant cells among which crocidolite fibres were clearly visible (Bolton *et al.*, 1982; Davis *et al.*, 1991). These granulomas remained cellular throughout the rat lifespan but were not associated with the production of significant fibrous adhesions. It would be expected that this pattern of granuloma formation occurred in the present study with at least the highest dose (10^9 crocidolite fibres) but that the lesions were obscured by multiple tumour masses and blood stained ascites fluid. However, Collier *et al.*, (1994, 1995) have reported that with very low doses of glass fibres, granuloma formation does not take place and all the injected fibres remain distributed on the mesothelial surfaces. This suggests that, in our study, granulomas may not have formed with crocidolite.

6. CONCLUSIONS

This study has demonstrated that high doses of cellulose fibres are carcinogenic when injected into the peritoneal cavity of rats. However, at the highest dose of cellulose (10^9 WHO fibres) the tumours produced were recorded as sarcomas, rather than mesotheliomas. There were only two mesotheliomas, from dosing at 10^7 and 10^8 WHO fibres, reported. The cellulose preparation used in the present study does not readily cause classical mesotheliomas even in the extremely sensitive rat model.

The implications for the ability of cellulose fibres to cause pulmonary carcinomas following inhalation remains unknown since long-term inhalation studies have not been undertaken. The present study re-emphasises the need for such work.

7. ACKNOWLEDGEMENTS

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