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Durability of man-made fibres and their clearance from the lung: studies to complement the Colt Fibre Programme

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INSTITUTE OF OCCUPATIONAL MEDICINE
Research Avenue North, Riccarton, Edinburgh, EH14 4AP
Tel: +44 (0)870 850 5131 Fax: +44 (0)870 850 5132
e-mail publications@iomhq.org.uk

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Early results from the Colt Fibre Research Programme (CFRP) suggested that both the number of long thin fibres in the received dose and the ability of these fibres to persist within the lung (biopersistence) are important predictors of carcinogenicity in animal experiments. This report describes the results of some complementary studies, including completion of some biopersistence studies initiated during the CFRP and examination of a further fibre type, E glass.

The biopersistences of ten fibre types were investigated in an intratracheal injection experiment. The lung burden data are consistent with the hypothesis that short fibres are largely cleared by cellular processes whereas long fibres are cleared by the combined processes of dissolution and disintegration. The relative persistence of long fibres was similar to that in an *in vitro* dissolution assay. The biopersistence of four fibre types was also investigated following 12 months inhalation. The greater persistence of these fibres following inhalation is consistent with mild overloading of cellular clearance mechanisms. The composition of 100/475 fibres retained in lung over 12 months was substantially modified by differential leaching whereas the compositions of retained fibres of 104E and amosite were not.

The two microfibres tested, 104E and 100/475, had similar biopersistences and showed a similar level of activity in cell tests. Cell proliferation following short term exposure to the 104E was, however, substantially elevated relative to that seen with the 100/475. The microfibres showed very different carcinogenic potentials with 104E being as or more active than amosite asbestos in chronic inhalation and intraperitoneal experiments.

Statistical analyses of data from both the inhalation and injection studies suggested that important explanatory variables of the carcinogenic potential of fibres included the numbers or concentrations of long thin fibres, and measures of their ability to persist in the lung. However, the analyses showed that the available data could not predict all of the range of variability in carcinogenicity, failing in particular to explain the difference in the inhalation experiment between 104E and 100/475. This suggests a role for some other factor(s), possibly related to surface properties. It is suggested that modification of fibre surfaces by preferential leaching in the lung might have such an effect.

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INSTITUTE OF OCCUPATIONAL MEDICINE
8 Roxburgh Place, EDINBURGH, EH8 9SU, UK
Tel +44(0) 131 667 5131: Fax +44(0) 131 667 0136
e-mail publications@iomhq.org.uk

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SUMMARY

The Colt Fibre Research Programme included a range of short and long term assays that have been used to investigate fibre pathogenicity and related fibre properties. Early results suggested that both the number of long thin fibres in the received dose and the biopersistence of long fibres are important predictors of carcinogenicity in animal experiments. This report describes the results of some complementary studies, including examination of a further fibre type, E glass, that have important implications for the conclusions of the research programme.

The biopersistences of ten fibre types (size separated respirable samples of glasswool, rockwool and slagwool (MMVFs 10, 21, 22), refractory ceramic fibre (RCFs 1, 2), heat treated RCF (RCF4), two special purpose glass microfibres (100/475, 104E), amosite and silicon carbide whiskers) were investigated in an intratracheal injection experiment. Extra funding from another sponsor enabled us to undertake additional counting and sizing of fibres for five of the fibre types. Although the additional data did not lead to a substantial change in any of our original estimates of biopersistence, based on counts of 100 fibres/lung, it did tighten the confidence limits associated with these estimates. The main source of uncertainty associated with our biopersistence estimates remained in the form of interanimal differences in lung burden.

The lung burden data are consistent with the hypothesis that short fibres are largely cleared by cellular processes whereas long fibres are cleared by the combined processes of dissolution and disintegration. Short fibres of MMVF10, 104E, RCF4 and silicon carbide whiskers had longer clearance half times than short fibres of the other fibre types tested. Long fibres of MMVF10 and MMVF22 had relatively short clearance half times whereas long fibres of amosite, RCFs 2 and 4 and silicon carbide, all had relatively long to infinite clearance half times. The slow clearance of short fibres of MMVF10 is likely to reflect the creation of extra fibres through the disintegration of longer fibres. The slow clearance of short fibres of silicon carbide may be related to the cytotoxicity that they exhibit in short term assays. The rapid clearance of long fibres of MMVF10 and MMVF22 is likely to be due to the susceptibility of these fibre types to dissolution whereas amosite, RCFs 2 and 4 and silicon carbide are all relatively durable materials.

The relative persistence of long fibres of the test fibre types in rat lungs was similar to that in an *in vitro* dissolution assay. The *in vitro* dissolution assay was not a good predictor of the biopersistence of total lung burden, possibly because most of the injected fibres were within the size range that can be cleared by cellular processes.

The biopersistence of the two microfibres (104E and 100/475), amosite and silicon carbide was also investigated following 12 months inhalation. The lung burdens of animals exposed for 12 months to target concentrations of 1000f/ml were substantially greater than after injection of 1mg doses of each fibre type. There was no evidence of any significant clearance of silicon carbide over the 12 month period following cessation of inhalation. This was probably due to the toxicity of the silicon carbide, combined with a relatively large lung burden that would have overwhelmed the normal cellular clearance mechanisms. The other three fibre types all underwent substantial clearance over twelve months, but clearance rates were retarded relative to those in the injection experiment. This is consistent with mild overloading of cellular clearance mechanisms. Long fibres of 104E appeared to be less persistent over the 12 months following the cessation of inhalation than those of 100/475. The animals exposed to 104E, however, accumulated a greater burden of long fibres than those exposed to 100/475 despite similar exposure concentrations. The most important difference between the fibre types was that the composition of 100/475 fibres retained in lung over 12

months was substantially modified by differential leaching whereas the composition of retained fibres of 104E and amosite were not.

Cellular tests with the 104E showed it to have a similar potential to the other microfibre tested, 100/475, to stimulate production of the pro-inflammatary cytokine TNFα. Both microfibres were more active in this assay than the MMVFs and RCFs, but less active than asbestos or silicon carbide. The results of the epithelial detachment assay suggested that the microfibres had a similar activity to some of the MMVFs and RCFs but were less active than asbestos. In a short term inhalation experiment animals exposed to 104E showed evidence of an inflammatory reaction similar to that seen in animals exposed to 100/475, amosite or silicon carbide at the same target concentration. A significant difference, however, was that cell proliferation following exposure to the 104E was substantially elevated whereas a similar effect was not seen with the 100/475. In a chronic inhalation experiment, animals exposed to 104E, amosite or silicon carbide developed tumours whereas those exposed to 100/475 did not. The incidence of tumours was similar in animals exposed to 104E as in animals exposed to amosite. The lung burden of animals treated with 104E was very similar to that of animals treated with 100/475 such that the difference in toxicity can not attributed to differences in lung burden. In a long term intraperitoneal injection experiment, the median survival time of animals treated with 104E was shorter than for those treated with amosite and substantially shorter than for animals treated with 100/475. Overall, it appeared that the 104E had a similar or greater carcinogenic potential to amosite asbestos.

Statistical modelling was undertaken to investigate the relationship between fibre properties and pathogenic outcomes in the intraperitoneal injection and long term inhalation experiments. Data from chronic inhalation experiments performed at the RCC in Switzerland were included in the analysis to increase the number of fibres included in the inhalation model. The modelling was initially undertaken for nine fibres, excluding 104E. The derived models suggested that the main explanatory variables for the propensity of fibres to cause disease were the number of long thin fibres animals were exposed to, the biopersistence of long fibres and the susceptibility of fibres to dissolution.

The statistical model for the results from the intraperitoneal injection experiment was not substantially changed by the incorporation of the data for 104E. However, neither the original nine-fibre model nor the revised ten-fibre model sufficiently explained the variation between fibres, under-predicting for some fibres and over-predicting for others.

For the data from the inhalation experiment, the original statistical model failed to predict the carcinogenic potential of 104E. The model changed substantially when the data for 104E were added. However, the revised model over-predicted for 100/475, and under-predicted for 104E, suggesting that the differences in their carcinogenic potential can be explained only partly by the relatively small differences in their dimensions and persistence.

The failure of the data from these experiments to predict reliably the relative pathogenicity of the test fibres suggests that there must be some other factor(s) influencing fibre toxicity, most probably related to fibre surface properties. One possible factor may be modification of fibre surfaces by preferential leaching during retention in lung tissue, which in some fibres might lead to the development of a porous outer layer of silica gel, which might reduce toxicity.

1. INTRODUCTION AND AIMS

1.1 INTRODUCTION

Fibrous materials are used in a wide range of applications by modern society. They have excellent thermal and acoustic insulation properties and many are also heat and/or chemically resistant. Fibrous materials are incorporated into many composite materials such as cement and bitumen to improve their mechanical properties. The hazards associated with inhaled asbestos are well established and there is a continuing debate about the extent to which other fibrous materials may also be hazardous to health.

The Colt Fibre Research Programme (CFRP) was established to examine the relationship between fibre characteristics and their pathogenicity and to assess the effectiveness of a range of assays for the prediction of pathogenic effects. A total of ten fibre types have been included in the CFRP which has been funded by the Colt Foundation, HSE and industrial sponsors (EURISOL, ECFIA, Cape plc, T&N plc and BBA plc). This report describes work funded by the HSE between April 1997 and May 1999 and also incorporates relevant results from two related projects separately funded by EURIMA and HSE. It follows an earlier report to HSE (Jones *et al.*, 1998) which describes the work during earlier parts of the CFRP. The new work described in this report included the investigation of a tenth fibre using the same assays as used for the other nine fibres during earlier stages of the CFRP. It also included the completion of the analysis of lung burdens for an intratracheal injection experiment designed to investigate biopersistence and investigation of biopersistence of four of the ten fibres following long term inhalation. The tenth fibre was a special purpose glass microfibre (manufacturer's code 104E) with a relatively durable composition. It was of interest because it was expected to have a durability intermediate between fibre types such as amosite that are known to be carcinogenic and the more soluble glass wools that have not been shown to be carcinogenic in inhalation experiments.

The EURIMA funding allowed us to improve the precision of our lung burden analysis for five of the fibre types in the intratracheal injection experiment by undertaking additional fibre counts. The separate HSE project allowed us to improve estimates of the relative susceptibility of the fibre types to dissolution *in vitro* (Searl and Buchanan, 1999). The main outcome of the extra work described here has been to improve the parameters in our models linking fibre characteristics to disease outcome. Some of the work described here has already been published (Searl *et al.*, 1999, Miller *et al.*, 1999a and b). The new developments since publication include incorporation of the EURIMA funded fibre analysis, the tenth fibre type and a further improvement in our *in vitro* measures of dissolution.

This report initially summarises the background to and aims of the project, then describes the experimental methods and results and discusses the final outcome of the CFRP. The papers prepared during the course of this contract are appended. It is anticipated that a further short paper will be prepared to describe the final outcomes of the CFRP.

1.2 BACKGROUND

1.2.1 Relationship between fibre properties and hazard

The potential of inhaled fibres to damage health has been linked to fibre dose, dimensions and durability. The link between fibre dimensions and hazard is widely accepted. Fibre dimension governs the respirability of fibres and determines whether they are likely to be

deposited in the lungs. Once in the lung, fibres with lengths of more than about 20μm are believed to be too long to be readily cleared by macrophages and are therefore believed to be more hazardous than shorter fibres (Davis, 1994).

The link between biopersistence of inhaled fibres and risk of pathological response in the lung is also widely accepted (Davis, 1986), although reliable dose-response relationships have not yet been determined. One difficulty is that biopersistence is likely to involve several factors, such as chemical composition and structure, dissolution characteristics, fibre size and toxicity. Dissolution, in particular, has been shown to be important (Davis, 1994; Morris *et al.*, 1995; Eastes and Hadley, 1995; Bernstein *et al.*, 1996; Bellmann *et al.*, 1987; Morgan, 1994).

The early results of the Colt Fibre Research Programme (Jones *et al.*, 1998; Miller *et al.*, 1999a and b) have been consistent in associating each of these factors with the potential of fibres to cause disease in rats. In particular, the development of mesothelioma following injection of fibres into the peritoneal cavity is related to the number of long thin fibres injected and measured rates of biopersistence and *in vitro* dissolution. The development of cancers following inhalation exposure appears to be related to the rate at which they dissolve in *in vitro* experiments and the number of long, thin fibres within the aerosol.

The role of fibre surface properties in determining toxicity has been discussed by Fubini (1993), but as yet there is insufficient understanding of the role of surface properties to have allowed the development of relevant predictive assays. Surface properties of fibres might play an important role in determining their behaviour in *in vitro* cellular assays. None of the *in vitro* systems tested, however, have proved effective predictors of disease in the injection or inhalation experiments (Jones *et al.*, 1998).

1.2.2 Measurement of biopersistence

Ideally, pulmonary biopersistence is measured after inhalation, as this is the natural route of entry of fibres to the lung, and it ensures that fibres are well dispersed within the lung. Such studies are, however, resource intensive, and require relatively large amounts of material for aerosolisation. Some of the specially prepared fibre samples were made available to this research programme in quantities sufficient only for injection studies. The investigation of biopersistence after inhalation in this study was therefore restricted to four fibre types. Published studies of fibre biopersistence have used both short term and chronic inhalation protocols (eg Musselman *et al.*, 1994; Hesterberg *et al.*, 1995). Short term experiments have the advantage of being low cost and of being simpler to interpret. Chronic exposure was used in the CFRP in order that lung fibre burden could be related to disease outcome following chronic exposure to inhaled fibres. The main disadvantage of using chronic inhalation in biopersistence experiments is that there is no well defined starting point. The lung burden that is present after 12 months exposure will have been extensively modified by clearance processes that have operated throughout exposure.

Biopersistence has also been measured after intratracheal injection (Morris *et al.*, 1995; Bellmann *et al.*, 1994; Muhle and Bellman, 1995), which has the advantages of simplicity and a clearly defined lung burden at the start of the experiment. In spite of potential inhomogeneity of deposition and clumping of fibres (Bernstein *et al.*, 1997), other intratracheal injection studies have successfully shown differences in biopersistence between some of the fibre types we wished to study (Bellmann *et al.*, 1994; Muhle and Bellmann, 1995). Muhle and Bellmann (1995) had found rates of clearance following intratracheal injection to be similar to those following exposure by chronic inhalation. We therefore adopted a method of intratracheal injection to investigate biopersistence across ten fibre types.

1.2.3 Measurement of dissolution rates

Dissolution may play a key role in the removal of some fibre types from lung tissue (Davis, 1994; Eastes and Hadley, 1995; Searl, 1994), and therefore some information about the dissolution behaviour of the test fibre types was desirable. Although a number of published *in vitro* dissolution studies have included several of the test fibres, there are large discrepancies between the dissolution rates measured by different laboratories and no published study includes all the test fibres. Dissolution is a complex and dynamic process, and its quantification presents experimental difficulties. For an initial look at the effects of dissolution of all ten test fibre types in a single experiment, a simple *in vitro* dissolution assay was devised. Subsequently the dissolution of the eight vitreous fibres was investigated in a continuous-flow-through assay similar to that used by other laboratories (eg Potter and Mattson, 1991). Our measured dissolution rates are comparable with those from other laboratories (Searl and Buchanan,1999). Both our measurements and those reported by other laboratories, however, are associated with substantial error bars, typically ± 30% of reported values (Searl and Buchanan, 1999; EURIMA, 1998; Zoitos *et al.*, 1997). The dissolution rates of amosite and SiC were not measured in this assay because they were expected to be immeasurably small.

1.2.4 Assessment of carcinogenicity

There have been two main approaches to assessing the potential of fibrous materials to cause cancers in animals, usually rats. The method that is most widely accepted is to subject rats to chronic inhalation exposure to relatively high concentrations of airborne fibre. The other method is to inject fibres directly into the peritoneal cavity to ensure that they make contact with the target tissue of interest for mesotheliomia.

Inhalation studies mimic the human route of exposure, although exposure concentrations are several orders of magnitude greater than those likely to be encountered in the workplace. The disadvantages are that they are costly and some authors have questioned whether the assay is sufficiently sensitive enough to be a reliable method of screening for carcinogenicity (Pott, 1995). The advantages of injection studies are that they can be conducted with relatively small samples of fibre, they require less specialised equipment and facilities and they are highly sensitive. The disadvantages are that it is an unnatural route of exposure and the doses used are extremely high in comparison to likely fibre burdens following human exposure to fibrous aerosols. The test is only relevant to mesothelioma and not necessarily lung cancers.It has been accused of giving rise to false positives (McConnell, 1995).

In the intraperitoneal test, 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). However, peritoneal tests do allow ready comparison with published data for a wide range of fibre types (Pott 1993; Davis *et al.*, 1996; Roller *et al.*, 1996; Miller *et al.*, 1999). They 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. It has highlighted, for example, the roles of dose, fibre dimension and durability (Davis *et al.*, 1986, Miller *et al.* 1999a; Stanton *et al*, 1981). It has also been informative 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). The reproducibility of the intraperitoneal assay has been established by Pott's team, who have undertaken a large number of these experiments with a wide range of materials (Roller *et al.*, 1996).

In the CFRP, chronic inhalation experiments were performed with the four fibre types for which there was plenty of material available for aerosol generation. The protocol was based on that used in earlier IOM studies, mainly with asbestos, such as that reported by Davis *et al.* (1986). Injection studies were performed with all ten fibre types. Additional information about the fibre types not tested by inhalation at IOM was obtained from publications describing a series of chronic inhalation studies undertaken at the Research and Consulting Company in Switzerland (Bunn *et al*, 1993; Hesterberg *et al*, 1993; Hesterberg *et al*, 1995, Mast *et al*, 1995; McConnell *et al*, 1994) and from data provided by the Joint European Medical Research Board (JEMRB) on fibre concentrations and size distribution. The high cost of undertaking chronic inhalation studies has limited investigation of their reproducibility. The number of cancers observed in animals exposed to amosite by inhalation as part of the CFRP was, however, consistent with earlier experiments undertaken with the same fibre preparation but at a higher concentration (Davis *et al.*, 1986).

1.2.5 Short term predictive tests

Given the expense of performing long term assays to produce tumours in rats there has been considerable interest in the predictive value of short term assays with respect to carcinogenesis. Experiments undertaken during the earlier years of the CFRP showed that simple cellular assays including measurement of cytotoxicity to epithelial cells and the production of pro-inflammatory cytokine tumour necrosis factor are not good predictors of long term pathogenicity. Similarly the inflammatory response to inhaled fibres did not seem to discriminate clearly between fibres of very different pathogenicity. In contrast, the measurement of cell proliferation following short term inhalation appeared to be a more promising predictor of long term effects (Jones *et al.*, 1998; Miller *et al.*, 1999b).

1.3 AIMS AND OBJECTIVES

The aims of the work described here were:

To test the hypothesis that *in vitro* solubility of vitreous fibres can be used to predict biopersistence in the lung;

To derive detailed time profiles of the clearance of 10 different fibres from the lung following intra-tracheal injection, to help identify the relative roles of dissolution and breakage;

To establish a more precise evaluation of the size distribution and clearance of 4 types of long fibres following inhalation exposure and to elucidate the possible clearance mechanisms in operation, including the possible identification of a size threshold above which macrophage mediated clearance is ineffective;

To compare the patterns of fibre clearance observed following inhalation with that following intra-tracheal injection;

To investigate the behaviour of a poorly soluble man-made vitreous fibre (104E) in short-term biological tests to elucidate the relative importance of solubility and fibre composition as predictors of biopersistence and to relate this information to fibre clearance profiles and the incidence of cancers in animals following exposure.

2. METHODS

2.1 OVERVIEW

A wide range of assays was conducted during the course of the CFRP (Table 2.1). The results of most of these have been previously reported to HSE (Jones *et al.*, 1998). The new information presented here is the biopersistence data from intermediate time points in the intratracheal injection experiment and from all time points in the chronic inhalation experiment. In addition, the data for E glass (code 104E) is presented for all the assays undertaken as this information was not previously available.

Table 2.1
Types of assay undertaken in the CFRP

Type of Assay	Measurement/ assessment
Chronic inhalation experiment (lifetime study in rats; 4 fibre types only)*	potential to cause tumours potential to cause fibrosis biopersistence
Intraperitoneal injection (lifetime study in rats)	potential to cause mesothelioma
Intratracheal injection (12 month study in rats)	Biopersistence
Short term inhalation (14 days, rats; 4 fibre types only)	Inflammation cell proliferation
Intraperitoneal injection (short term study in mice)	Inflammation
Cellular systems	Cytotoxicity cytokine (TNF) release
In vitro dissolution assay	fibre durability in simulated lung fluids

**results from chronic inhalation experiments performed at RCC were available for the other 6 fibres and were included in the statistical analysis*

2.2 TEST FIBRES

The test fibres investigated in the Colt Fibre Research Programme included eight man-made vitreous silicate fibres with a range of chemical compositions (Table 2.2). Six of these were specially size selected samples intended for research purposes B MMVF10 (insulation glass wool), MMVF21 (rock or stone wool), MMVF22 (slag wool), RCF1, RCF2, RCF4 (refractory ceramic fibres). The remaining two fibre types were commercially available special purpose glass microfibres code 100/475 and code 104E. RCF4 is a heat treated form of RCF1 that is composed of finely crystalline mullite, cristobalite and other minerals (Brown *et al.*, 1992). Other test fibres were the amphibole asbestos mineral, amosite ($(Fe,Mg)_7Si_8O_{22}(OH)_2$), and whiskers of silicon carbide (SiC). The silicon carbide whiskers are semi-crystalline and typically have small hooks at one end and/or buds along their length. About 40% of the particles comprise two or more fully developed, bonded, whiskers. The four fibre types used in the inhalation experiments were the two glass microfibres, amosite and silicon carbide. All ten fibre types were used in the other assays.

The glass microfibres 100/475 and 104E (Manville Insulation) was obtained from the Mountain Technical Centre, Schuller International Inc., Littleton, Colorado, USA. The other man made vitreous fibres (MMVFs10, 21, 22, RCFs 1, 2 and 4) were obtained from the Thermal Insulation Manufacturers Association (TIMA) repository of size selected fibres. The amosite was from a consignment of commercial amosite that produces a dust aerosol containing a significant proportion of fibres longer than 20μm (Davis *et al.*, 1986). The

silicon carbide whiskers were obtained from the Advanced Composite Materials Corporation, Greer, S. Carolina, USA.

Table 2.2
Chemical compositions, expressed as weight percentages of component major element oxides, of the man-made vitreous fibres tested. Data supplied by TIMA for the TIMA repository samples. Cells are blank where no data were available.

Fibre type	SiO_2	Al_2O_3	Fe_2O_3	TiO_2	CaO	MgO	Na_2O	K_2O	ZnO	ZrO_2	B_2O_3	BaO
100/475	57.5	5.5	0.05	0.05	2.5	0.05	10.5	2.5	4		10.5	5
104E	54.0	14.0	0.25	0.04	20.5	2.5	1.0	1.0			7.5	
MMVF10	57.5	5.1	0.07	0.01	7.5	4.13	14.95	1.06		0.03	8.75	
MMVF11	63.4	3.88	0.25	0.06	7.45	2.82	15.45	1.32			4.45	
MMVF21	46.2	13.0	7.00	2.95	16.9	9.25	2.64	1.25		0.03		0.04
MMVF22	38.4	10.6	0.30	0.45	37.5	9.9	0.38	0.45		0.06		0.04
RCF1	47.7	48.0	0.97	2.05	0.01	0.08	0.54	0.16		0.11		
RCF2	50.0	35.0	<0.05	0.04	0.05	0.01	<0.3	<0.01		15.0		
RCF3	50.8	48.5	0.16	0.02	0.04	<0.01	0.19	<0.01		0.23		
RCF4*	47.7	48.0	0.97	2.05	0.01	0.08	0.54	0.16		0.11		

**composition may have been modified by heat treatment at 1300EC*

The test fibres had slightly different size characteristics and specific surface areas (Table 2.3).

Table 2.3
Mean dimensions of test fibres as determined by SEM and specific surface areas as determined by BET nitrogen gas absorption (Searl and Buchanan, 1999).

Fibre	Geometric Mean Length (μm)	Geometric Mean Diameter (μm)	Specific surface area m^2g^{-1}
100/475	2.9	0.22	5.13
104 E	3.5	0.25	3.43
MMVF10	23.9	1.13	1.07
MMVF11	14.2	0.57	1.08
MMVF21	15.7	0.81	1.38
MMVF22	13.7	0.89	1.14
RCF1	10.4	0.79	1.46
RCF2	12.4	0.84	1.12
RCF4	6.8	0.94	1.08

Several additional fibres were used in some of the *in vitro* assays. These included crocidolite asbestos from the TIMA repository, silicon carbide from another supplier (Third Millennium), a glass fibre type – MMVF11 and a further kaolin ceramic fibre – RCF3. The crocidolite contained a lower proportion of long fibres than the amosite asbestos.

The test fibres are representative of those used in a wide variety of applications. Glass wools, rock wools and slag wools are used for commercial and residential insulation, acoustic panels and as a horticultural growing medium. Glass wools are also used in ceiling panels and in air handling ducts and rock and slag wool are used for fire protection. The refractory ceramic fibres are used for high temperature insulation, gaskets and joints and filtration. The microfibres are used for battery separators and filtration. E glass (104E) has a range of specialist and military uses arising from its electrical insulation properties. Silicon carbide has a specialised use in high temperature composites. The glass, rock and slag wools tested are single size selected examples of commercial fibre types, and a wide range of similar products are available, but with varying chemical compositions.

2.3 FIBRE CHARACTERISATION

The dimensions of the injected fibres and those subsequently recovered from lung digests (following administration by both inhalation and injection) were determined using scanning electron microscopy (SEM). The SEM fibre counting and measurement was undertaken by fully trained and experienced analysts following validated procedures documented in the IOM's United Kingdom Accreditation Service (UKAS) quality system (EN45001). Regular sample exchanges and replicate analyses were undertaken to ensure comparability between different analysts and through time. The precision of fibre measurements by SEM (at the IOM) has previously been determined during validation procedures undertaken for UKAS. The uncertainty of repeat measurement was found to be ± 13% and ± 0.09 μm for fibre length and diameter respectively, based upon 95% confidence intervals.

For the initial characterisation of fibres, known masses of each of the test fibres were deposited from aqueous suspension on to polycarbonate filters. It was necessary to add a small amount of Teepol to the suspensions of RCF2 and RCF4 in order to disperse these fibres in water prior to filtration. Fibre measurements were made with two scanning electron microscopes (SEMs), a Hitachi S520 SEM and a Cambridge S250 MkII SEM at a magnification of x5000, following a modified version of the WHO-EURO method of SEM fibre analysis (World Health Organisation, 1985). The magnification of the SEMs was calibrated against a grid calibrated by the National Physical Laboratory. Fibres were defined as having a length:diameter (aspect) ratio >3:1 and a length >0.4μm. Fibre dimensions were measured to the nearest 0.1μm. The data recorded for each fibre were field number, length, diameter (maximum), and number of ends in the field. The length of complex silicon carbide whiskers was defined as the total length of the entity, parallel to the longest component whisker (as viewed in 2 dimensions), while the diameter was that of the thickest component whisker. It was thought that these parameters were of most relevance to the respirability of silicon carbide particles. Repeat counts were made for at least 1 in ten samples and sample exchanges were undertaken to ensure comparability between analysts. The fibre size data was summarised in bivariate tables (see Data analysis).

Semiquantitative compositional analyses were made for amosite, code 104E, code 100/475 using the energy dispersive X-ray analysis system (EDX) attached to the transmission electron microscope. This involved measuring the relative intensity of X-rays generated at the specific wavelength for each element. No (ZAF) corrections were made to this intensity data to allow for the effects of atomic number (relative response increases with atomic number) and effects such as X-ray fluorescence within the sample, and thus the results are semi-quantitative. Analysis was performed for untreated fibres and for the same fibre types recovered from lung digests at the 12+12 time point. For each sample, fifty randomly selected fibres were analysed. The data was entered into a spread sheet and arithmetic means calculated for each element analysed for each sample. No compositional analyses were made

of silicon carbide because all that could be detected is silicon. It is also very unlikely, on mineralogical grounds, that silicon carbide would undergo incongruent dissolution (i.e. selective leaching of one element from its composition).

2.4 MEASUREMENT OF BIOPERSISTENCE AND DURABILITY

2.4.1 Experimental design

Injection experiments

In the intratracheal injection biopersistence experiments a known amount of the test material was injected into the lung via the trachea. The fate of this material was then investigated through measurement of the lung burden of groups of animals that were sacrificed at predetermined time points. The injected dose was 1mg for all ten fibre types, which was expected to be less than the quantity required to cause severe overloading of the normal cellular processes by which particulate is cleared from rat lungs. For each fibre type, groups of four animals were sacrificed at each of four time points: 3 days, 1 month, 6 months and 12 months after injection as long-term biopersistence was expected to be an important determinant of biological risk.

Long term inhalation

The long term inhalation experiments were primarily undertaken to investigate tumour risk in relation to inhaled lung burden. Groups of 40 rats (AF/HAN strain) were exposed in whole body chambers for 7 hours per day for 238 working days (i.e. almost one year) at a target concentration of 1000 fibres/ml. Follow-up of exposed animals was continued for a further 12 months. Lung burden was determined for groups of six animals following 6, 9 and 12 months exposure and 3 days recovery and then at 1 month, 3 or 6 months and 12 months recovery after 12 months exposure. The 3 month recovery point was used for the 100/475 microfibres as these were expected to clear relatively rapidly and the 6 month recovery point used for the other 3 fibre types.

2.4.2 Fibre preparation

The MMVFs and RCFs were supplied from the TIMA repository as respirable fibres and were not subjected to any additional processing. The silicon carbide was also supplied as a fine powder comprising whiskers that were entirely within the respirable size range. Respirable samples of the 100/475 microfibre, 104E and amosite were collected from aerosols for use in injection experiments (Beckett, 1975). The aerosols were generated using Timbrell fibrous dust dispensers fitted with glass linings. Both types of glass microfibre were supplied in lengths that were too long to allow direct aerosolisation. The 100/475 was processed for approximately 30 minutes in a Waring blender before lofting. The 104E used for testing was supplied as a bale of fibres. The raw material was chopped in a Manesty rotary chopper and then milled in a Retsch pin mill (type ZM1) fitted with a 1 mm mesh.

2.4.3 Administration of test fibres

Intracheal injection

Samples of respirable fibre were weighed and suspended in sterile saline to give a concentration of 2mg per ml. These were ultrasonicated for about 1 minute to break up any clumps of fibre and, immediately prior to injection, the suspensions were mixed by rapidly

inverting the tube three or four times by hand. For each of the nine fibre types, 16 specific pathogen free male Wistar rats were randomly selected. Under fluothane anaesthesia, a small area of the trachea was exposed by cutting the overlying skin and muscle. A small incision was made in the trachea and the test fibres, suspended in 0.5ml saline, were injected into the deep lung using a syringe connected to a blunt-ended cannula.

Inhalation

Groups of 40 animals were exposed for 7 hours a day, 5 days a week for 12 months to a target concentration of 1000 fibres/ml as determined by optical microscopy (MDHS 39/4, Health and Safety Executive 1994; MDHS 59, Health and Safety Executive 1988). Dust clouds were generated in 1.3 m^3 chambers using a Timbrell Dust Feeder (fitted with glass linings) as described by Beckett (1975). The respirable dust concentrations in the exposure chambers were determined gravimetrically using a Casella MRE 113A. Fibre concentrations were monitored by hourly counts (PCOM) of short period (1 minute) 0.2 litre samples (8 per monitoring day) following the procedures in MDHS 59 (Health and Safety Executive, 1988). A modified procedure was developed to count complex silicon carbide fibres (Jones *et al*, 1998). The respirable dust mass concentrations were measured daily and a fibre number to mass ratio determined using PCOM every two weeks. The bivariate size distribution of fibres in the dust cloud was determined by scanning electron microscopy (SEM) performed on short period samples collected on polycarbonate filters.

2.4.4 Measurement of lung fibre burden

The animals were sacrificed by CO_2 suffocation. The lungs were dissected free of surrounding tissue including lymph nodes, finely chopped and treated with a series of ether:ethanol mixes to remove excess fat. The lung fibre burdens were recovered by either bleach digestion (5% NaOCl: Sebastien *et al.*, 1989) for fibres which survived a 24 hour treatment in bleach with minimal change in mass and mean fibre dimensions (amosite, code 100/475 glass fibre, silicon carbide, MMVF21, RCF1 and RCF2) or by plasma ashing (Bolton *et al.*, 1983) (MMVFs 10 and 22, RCF4) using an Applied Vacuum Technology solid state plasma chemistry unit. The bleach used was the UK Co-operative Society's own label retail bleach. Filters for EM analysis were prepared from measured aliquots of the bleach digests or from measured aliquots of the ashed lung suspended in filtered water. The bleach digests in the injection experiment were ultrasonicated for 10 minutes which was sufficient to disaggregate all but a few small clumps of fibres. The ash suspensions required a longer ultrasonication treatment and 20 minutes was found to be sufficient to disaggregate most clumps. Most of the lung digests for the inhalation experiment were ultrasonicated for 10 minutes before aliquots were taken for SEM analysis, but the 6+0, 9+0 for the code 100/475 and amosite and the 12+1 lungs for the code 100/475 in the inhalation experiment were only ultrasonicated for two minutes. The two minute ultrasonication had been initially adopted to minimise damage to recovered fibres. The ten minute ultrasonication was later adopted to ensure that clumps of fibres within the digests were fully disaggregated.

The numbers and dimensions of recovered fibres for each lung sample were measured using the SEM, as described above for the original fibre samples. At least one hundred fibres were sized for each animal, and pooled size distributions for groups of animals in the injection experiment were initially based on measuring the dimensions of between 300 and 650 fibres. Subsequently EURIMA funded additional counts of fibres in samples prepared from lung digests of amosite, code 100/475, code 104E, MMVF10 and MMVF21. These counts made up the total number of fibres counted to the minimum required by the draft EU protocol for the assessment of biopersistence following intratracheal injection (ECB/TM/27 rev 6). This protocol requires that 400 WHO fibres (length greater than 5μm and diameter less than 3μm)

are sized, or 1000 fibres of all lengths, or at least 1mm^2 of the filter is scanned. The protocol also stipulates that a minimum area of 0.15mm^2 is scanned. However, given that the lung digest samples had been prepared before the protocol existed, it was not possible to meet this minimum area specification for all fibre types because of the high densities of fibres on some filters. The final numbers of fibres sized for each fibre type at each time point are shown in Table 2.4. The groups included four animals each, except for amosite and RCF2 at 3 days, MMVF21 at 1 month and RCF 1 at 12 months, which included only three animals each.

The original CFRP lung burden estimates were generally based on a single count (minimum of 100 fields or 100 fibres) of a single SEM stub. Occasionally these had been supplemented by an additional count of either the same or a different stub. Lung burden estimates for these animals were therefore arithmetical means of the burdens corresponding to these replicate counts. The additional fibre counts funded by EURIMA were spread across all available and valid stubs for each lung. The new burden estimates were generated by pooling the numbers of fibres and fields counted across all examined stubs to produce a single estimate per lung.

Measured fibre dimensions were scaled using a calibration factor determined during separate exercises carried out at regular intervals.

Table 2.4
Number of fibres sized for each time point in the injection experiment (number of animals given in text)

	3 days	1 month	6 months	12 months
Amosite	3015	3287	2516	2323
100/475	4707	5953	4594	4121
104E	3858	4096	3998	4056
SiC	604	501	611	503
MMVF10	1798	1969	1969	1566
MMVF21	1750	1352	1735	1248
MMVF22	403	404	500	498
RCF1	500	399	400	499
RCF2	405	399	436	500
RCF4	500	495	498	603

The pooled size distributions in the inhalation experiment were based on measurements of between 507 and 1359 fibres. The number of animals available for each time point is shown in Table 2.5.

Table 2.5
Number of animals for which lung burden was determined at each time point in the inhalation experiment.

Exposure	6 months	9 months	12 months				
Recovery	3 days	3 days	3 days	1 month	3 months	6 months	12 months
Amosite	6	6	6	6	0	4	4
100/475	6	6	6	6	5	0	3
104E	6	6	6	6	0	4	6
SiC	6	6	6	6	0	4	6

2.4.5 *In vitro* measurement of dissolution

In the early stages of the Colt Fibre Research Programme a simple closed cell *in vitro* assay was designed to compare the dissolution rates of the test fibre types. Sodium oxalate was chosen as a simple salt system to approximate lung fluid, as both sodium salts and organic acids are important components of the fluids present in the lung. Subsequently, under a separate contract to HSE, the dissolution rate of most of the test fibres has also been measured in six week continuous flow through (CFT) experiments using experimental apparatus similar to that described by Potter and Mattson (1991). Measurements were made using a modified version of the EURIMA (1998) protocol for continuous flow through dissolution experiments with simulated extracellular fluids. The main modifications to the EURIMA protocol were that experiments were run over 6 weeks rather than 3 weeks and the specific surface area of samples was determined by gas absorption. These new measurements of dissolution in the continuous flow assay have been incorporated into the data analysis described here. Each measurement was made in triplicate. Full details of the experimental methods and the results are given by Searl and Buchanan (1999).

2.5 E GLASS

2.5.1 Overview

A range of experiments were undertaken with code 104E in order to provide a similar range of data to that available for the other test fibre types tested within the CFRP as described by Jones *et al* .(1998).

The assays included two *in vitro* assays undertaken to assess the potential of 104E to stimulate production of the pro-inflammatory cytokine, tumour necrosis factor alpha (TNFα), and the toxicity of the 104E to epithelial cells. TNFα was of interest because of its pleiotropic effects and its stimulation of the release of other cytokines involved in the inflammatory process. Its key role in particle-induced lung disease has been shown by Piguet *et al* (1990), Bissonette and Rola-Pleszczynski (1989), Driscoll *et al* (1990) and Simeonova and Luster (1995). Alveolar epithelial cells are primary targets for any toxic effects of fibres depositing in the lung and loss of epithelial integrity is an important event leading to inflammation.

A short term inhalation study was undertaken to examine the potential of 104E to cause inflammation of the lungs and its effect on cell proliferation. Bronchoalveolar lavage (BAL) following short term inhalation has been used extensively to explore the biological activity and mechanism of fibre pathology (reviewed in Donaldson and Brown 1993). Inhalation of asbestos fibres, for example, is characterised by an influx of inflammatory neutrophils to the bronchoalveolar space with evidence of macrophage activation and cell injury (e.g. Donaldson *et al.* 1988). The recruitment of inflammatory cells may have the ability to produce injury to the epithelium which eventually could lead to Type 2 cell hyperplasia, and the starting point for pathological change. An early indicator of cellular damage is the elevation of protein levels in the bronchoalveolar fluid. Alveolar proteinosis and type 2 cell hyperplasia have been described after quartz dust exposure (Brown *et al.*, 1989). The inflammatory response following inhalation of 104E was assessed from total and differential cell counts in lavage fluids and the protein content of lavage fluids was measured as a marker of cell injury.

Proliferation is an important part of the normal maintenance of tissue structure as ageing cells are replaced. Increases in the proliferative activity of cells in the lungs of rats exposed to chrysotile asbestos fibres have been described in detail by Brody and his associates

(McGavran and Brody 1989; Brody and Overby 1989). Warheit *et al.* (1994a and b) have also utilised measurement of proliferation to assess the toxic response to aramid and carbon fibre. These studies generally embrace the hypothesis that any fibre that stimulates proliferation has the potential to cause pathological change (Chang *et al.*, 1988). In addition, when cell injury occurs there is an increase in proliferation to replace these cells. Cell proliferation may therefore be an important marker for the progression of disease in the lung. Proliferation was assessed following short term inhalation using the BrdU assay.

Long term inhalation and injection studies were undertaken to investigate the carcinogenic potential of 104E in relation to that of the other test fibres.

2.5.2 *In vitro* assays

In vitro TNFα production and assay

Alveolar macrophages were harvested from rat lungs by bronchoalveolar lavage (BAL) and cultured in F-10 medium containing 0.2% bovine serum albumin at $1x10^6$/well in 24-well plates. An aliquots of $8.2x10^6$ fibres(>5 μm in length) of test fibre in aqueous suspension was added to each well. After 24 hours, supernatants were harvested, centrifuged free of cells and fibres, and stored at -70°C until required for assay. TNFα is toxic to some cell lines and this is the basis for a bioassay of the supernatants using L929 cells (Flick and Gifford 1984). In brief, L929 cells were cultured in MEM medium containing 5% foetal calf serum and 1 μg/ml actinomycin D in 96-well plates. Serial dilutions of the macrophage supernatants were made and aliquots of these added to the L929 cells. Dilutions of a recombinant human TNFα standard were used to produce a standard curve. The following day the remaining cells were stained with crystal violet and the amount of stain per well quantified at 540 nm using a microplate reader (Dybnatech, Billingshurst, UK). Each supernatant dilution was measured in triplicate and the results were expressed in international units per 10^6 macrophages. The experiment was repeated three times.

Detachment injury to A549 epithelial cells

Cells of the alveolar epithelial A549 line in 96-well plates were labelled with radioactive [^{51}Cr]chromium and seeded in 96-well plates at $5x10^4$/well. Fibres in suspension at concentrations of 10, 25, 50 and 100 μg/ml were added to wells. Wells containing medium and cells alone were used as a negative control. After 4 hours incubation, the amount of cell detachment from the plastic wells was determined by measuring free and cell associated radioactivity in the supernatants of each well as described by Donaldson *et al.* (1988).

2.5.3 Short term inhalation experiment

Inhalation exposure

Rats were exposed by whole body inhalation to the fibre types at a concentration of 1000 (WHO) fibres/ml for 7 hours per day using the methods described above for the long term inhalation experiment (section 2.4.3). Untreated control animals were kept in normal room air. Four dosing regimes were used: 1, 3, 8 and 14 days of actual exposure over a 3 week calendar period. The 8-day and 14-day regimen included one and two non-inhalation weekend breaks respectively. Six rats per time point were used for the SiC whiskers group and between 6 and 12 rats were used per time point for the amosite and code 100/475 groups. Animals were sacrificed with a single intraperitoneal injection of Nembutal 18 hours following the completion of the final day's exposure.

Bronchoalveolar lavage

Following sacrifice, the thoracic cavity was opened, the trachea were cannulated and removed and sequentially lavaged with four 8 ml aliquots of saline at 37°C. Cells were recovered from lavage fluid by centrifugation, pooled from the four aliquots, and resuspended in F-10 medium (Gibco, Paisley) containing 0.2% bovine serum albumin (Sigma, Poole, Dorset). Total cell counts were made, and cytocentrifuge smears were prepared and stained with Diffquick (Merz Dade, Switzerland) to obtain differential cell counts.

Protein measurement in bronchoalveolar lavage (BAL) fluid

The cell-free fluid from the first 8ml lavage aliquot from each animal was used to determine protein levels in lavage using a standard spectrophotometric assay (Biorad Laboratories, Munich, Germany). Bovine serum albumin was used to provide a standard curve. Standards and test samples were measured in triplicate at 595nm. Protein concentrations in the samples were calculated from the standard curve by regression and expressed in mg/l.

Cell proliferation using BrdU DNA labelling

Additional groups of 3 rats (4 for long amosite) were exposed by inhalation for 1 day and were given an intraperitoneal injection of 5'-bromo-2'-deoxyuridine (BrdU) (20 mg/kg) 2 hours before sacrifice. BrdU is incorporated into the nuclei of dividing cells and can be detected by immunohistochemical staining in 5 μm sections cut from 6 levels through the lung (apex to base), as described in more detail by Donaldson *et al.* (1995). Results were expressed as the number of positive staining cells per mm length of terminal bronchiolar/alveolar duct perimeter determined with an image analysis system (Donaldson *et al.*, 1995).

2.5.4 Long term inhalation experiment

Study design

The exposure regime was as described above for all four fibres tested by inhalation (section 2.4.3). A total of eighty three male Wistar (AF/HAN) strain rats were exposed for up to 12 months (5 day weeks, 7 hour days) to a target dose of 1000 fibres/ml and allowed to recover for up to a further 12 months. The lung burdens of groups of six animals were measured after 6, 9 and 12 months of exposure and after 1, 6 and 12 months of recovery following 12 months exposure. Forty seven rats that had been exposed for 12 months were assigned for pathological investigations. The 12 month exposure had previously been demonstrated to be sufficient to produce a carcinogenic response with asbestos (Davis *et al.*, 1986).

During the course of these experiments, a control group of 38 animals was maintained in conventional cages for their natural lifespan in order to provide information on spontaneous pulmonary tumour development and fibrosis.

Pathology

At the end of the 12 months inhalation period, 4 rats were killed to examine levels of tissue damage and fibrosis. The remaining 43 rats were allowed to live out their full life span, to allow tumour development, until only six survived at which point this part of the study was terminated. The estimates of advanced alveolar interstitial fibrosis in the oldest animals were made for the last nine dying within 2 months of the final kill date. Lungs from all the animals

not used in the lung burden study were examined histologically for the presence of neoplasms.

Tissue used for histological examination was fixed with buffered formol saline and embedded in paraffin wax. Lungs were fixed by inflation at a standard pressure of 30 cm of fixative. Subsequently, the tracheas were ligated and the lungs excised and immersed in fixative. Sections were cut in the coronal plane at 1 mm intervals and were stained by either haematoxylin and eosin, Van Giesen's method for collagen or Gordon and Sweet's stain for reticulin.

Classification of levels of fibrosis in animals at the end of the inhalation period was undertaken using the Wagner scale (McConnell *et al.*, 1984). Fibrosis in the older animals was estimated by similar methods to those previously published by Davis *et al.* (1996) in which an electronic image analyser was used in conjunction with light microscopy. Single lung sections were examined with the section selected to contain the maximum area of lung parenchyma. Areas of fibrosis were estimated as a percentage of total lung tissue area.

2.5.5 Intraperitoneal injection experiment

Known masses of respirable samples of 104E were deposited from aqueous suspension on to Millipore filters, and fibres (defined as objects with diameter <3 μm, length >5 μm, aspect ratio >3:1) were counted using optical microscopy following the MDHS 59 protocol (Health and Safety Executive, 1988). The mass of fibres required to give a dose of 10^9 fibres (length>5μm) was then calculated.

The appropriate mass of 104E was then administered in phosphate buffered physiological saline to a group of 24 male SPF Wistar rats (Charles River UK Ltd, Margate, UK) approximately 12 weeks old, as a single intraperitoneal injection of 2 ml. The elapsed time in days from injection treatment to death was recorded for each animal. All animals were autopsied. Diagnosis of peritoneal mesothelioma was usually obvious at autopsy, but where no apparent tumour was present the diagnosis was based on histological examination of tissue sections.

2.6 DATA ANALYSIS

2.6.1 Biopersistence

For each SEM filter, the number of fields and the number and size of fibres counted were used, together with the known aliquot fraction, to produce estimates per lung of fibre volume burdens and fibre number burdens in each of five fibre length classes: < 5, 5X10, 10X15, 15X20 and > 20 μm. Fibre persistence rates during the 12 months post-injection were estimated by fitting first-order exponential decay functions to fibre number burdens in each length class separately. These functions were fitted using simple linear regression of the log-transformed burdens, weighted by the number of fibres counted per lung. The estimated rate coefficients and standard errors were transformed to provide estimates and 95% confidence intervals of the persistence of fibres in each length class in the lungs of rats after injection. For the analyses relating fibre characteristics to their *in vivo* toxicity, it was required to calculate additional biopersistence rate coefficients for cumulative length classes (Miller *et al.*, 1999 a,b). At each time point, the fibre numbers were totalled in cumulative length classes and exponential regressions were fitted as described above.

2.6.2 Dissolution assay

In vitro dissolution rates were estimated from the silicon concentrations in samples collected over 42 days from test cells in the CFT dissolution assay. This analysis assumed that the rate of dissolution was proportional to the reaction surface area:

$$dM/dt = -k_{dis}A$$

where M is the mass of fibre remaining at time t, A is the reaction surface area, and K_{dis} is the dissolution rate constant, expressed in units of $ng.cm^{-2}.h^{-1}$. Dissolution rate constants were estimated for each test cell using linear regression, assuming uniform initial fibre diameter. The dissolution constants used in the data analysis for this project are the mean of three determinations. The precision of the dissolution assay at this level of replication is of the order of 20 to 30% of estimated values of K_{dis}

To account for differences in reaction surface area among samples of equal mass of each fibre type, the dissolution constants, K_{dis}, were re-expressed in terms of mass lost per initial sample mass per hour, using the estimates of specific surface area provided by BET, and labelled in what follows as adjusted K_{dis}.

2.6.3 Data from short term tests with 104E

The TNFα data were analysed by analysis of variance on the log scale, to investigate the significance of fibre effects and to estimate means and standard errors. Data from all the other assays required some adaptation of this approach: to deal with imbalance through unequal replication (epithelial cell detachment); to adjust for random fluctuations between experimental runs (BAL parameters); or to allow for Poisson variation in counts (BrdU) or binomial variation in proportions (% granulocytes). Since individual means were often based on few replications, standard errors have been calculated from pooled estimates of residual variation. The doses for epithelial cell detachment were not equal in fibre number, and an additional analysis estimated adjusted means for the fibre types by taking account of fibre number as a continuous covariate. All analyses were carried out in Genstat (Genstat 5 Committee, 1993).

2.6.4 Statistical analysis

Survival data from intraperitoneal injection study

The mortality data available from the intraperitoneal injection study comprised the date on which each animal died and the diagnosis of whether a mesothelioma was present at death. Dates were converted to times (in days) from the date of injection. The data were summarised as survival functions estimated by the Kaplan-Meier method (Collett, 1994), which can show important distinctions between the pathogenicity presented by the different fibres, even among groups where there is maximal response in terms of numbers of animals developing a mesothelioma (Bolton *et al.*, 1982). Calculations were performed using the BMDP package (Dixon, 1992), which also provided estimates of the median survival time and its standard error, for each of the fibre types. Graphs of survival functions were prepared using the package Sigmaplot (Kuo and Fox, 1993).

The relationship between median survival and the characteristics of individual fibres was investigated by standard methods of multiple linear regression (Draper and Smith, 1981), using the statistical software package Genstat (Genstat 5 Committee, 1993). The explanatory variables available included mass dose injected and the durability variables from Chapter 3

i.e. length-specific clearance after intratracheal injection and silicon dissolution *in vitro*. Also available were the data on the size distributions of the fibre samples. 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 in cumulative length categories and in two diameter classes, according to whether the fibre diameters (which were measured to the nearest 0.1 μm) were greater or smaller than 0.95μm. This threshold was selected because evidence suggests that fibre diameters less than about 1μm 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 0.95μm gave adequate numbers of fibres for statistical power and likely reliability.

Data on cancer incidence in long-term inhalation study

The incidence of tumours was treated as a binomial response variable. Its relationship with characteristics of individual fibre types was investigated by methods of multiple logistic regression (Collett, 1991), using the statistical software package Genstat (Genstat 5 Committee, 1993), which was also used for tabulations. The explanatory variables available were the durability variables, i.e. length-specific clearance after intratracheal injection and silicon dissolution *in vitro*; plus the size distributions of the samples of airborne fibres gathered in the inhalation chambers. These were summarised in the same length and diameter categories as described above.

Standard techniques of logistic regression were used to investigate the evidence for a relationship between cancer incidence and each of the potential predictor variables. One aim of regression modelling is to explain as much as possible of the variation in the response. In logistic regression the unexplained portion is quantified by the residual mean deviance, an analogue of the residual mean square in linear regression.

3. BIOPERSISTENCE DATA

3.1 INJECTION EXPERIMENTS

3.1.1 Initial lung burden

The mean lung burden across animals sacrificed at 3 days (Table 3.1) ranged from $3.8x10^6$ (RCF2) to $1.8x10^9$ (code 100/475 glass microfibre). The injected amosite and 100/475 microfibre contained greater proportions of short fibres (Table 3.2) than the other fibre types. These fibres were also thinner in diameter (Table 3.3). The bivariate size distributions of fibres recovered from lungs 3 days after injection and fibres from the administered aqueous suspensions were not significantly different. These size distributions of fibres sampled from the TIMA repository were broadly similar to those reported in other studies (Hesterberg *et al.*, 1993).

Table 3.1
Estimated burden per lung of all fibres longer than 0.4µm recovered after 3 days and 12 months following injection. Shown are the inter-animal means and standard errors (s.e.) of lung fibre burden in terms of millions of fibres per lung. n: number of animal lungs evaluated

	3 days			12 months			ratio
	mean	se	n	Mean	se	n	%
Amosite	310	50.9	3	48.3	4.0	4	2
100/475	1848	162	4	306	55.0	4	17
104E	381	97.5	4	222	25.2	4	58
SiC	80.9	14.0	4	29.3	6.6	4	36
MMVF10	4.14	0.61	4	0.79	0.22	4	19
MMVF21	4.80	0.32	4	1.69	0.36	4	35
MMVF22	7.09	0.85	4	1.50	0.04	4	21
RCF1	7.39	0.97	4	4.53	0.88	3	61
RCF2	3.81	0.62	3	1.62	0.24	4	43
RCF4	8.90	0.86	4	7.08	0.75	4	80

The estimated retained lung burdens for each batch of four animals for any fibre type showed a large degree of inter-animal variation after 3 days recovery (greater than 100% of the mean burden for some fibres). In comparison, the differences between repeat counts on single filters by individual or different analysts or between counts on replicate filters from the same digest were largely within 20% of the mean of the analyses for a single lung. All were within 40% of the mean (the normal criteria for acceptability for occupational hygiene measurements of fibres in workplace air). Therefore most of the variation in lung burdens at any specific time post injection was due to inter-animal differences.

3.1.2 Persistence over 12 months

Comparison of mean lung burdens at each of the four time points (all fibres longer than 0.4µm) showed large differences between fibre types in the persistence of fibres after injection (Table 3.2). The ratio of fibre number burdens at 12 months relative to those at 3 days ranged from 7% for amosite, to 80% for RCF4. The two fibre types with the highest

proportion of short fibres administered, amosite and Code 100/475 showed the greatest relative reduction in fibre numbers over 12 months.

The trends in persistence rates with fibre length differed among the fibre types. Figure 3.1 shows the estimated length-specific persistences over 12 months of each fibre type based on the fitted first-order exponential decay models. For amosite and RCF2, persistence increased with increasing fibre length, and there was no evidence for any clearance of fibres within the longest length class (> 20 μm). The same was true for RCF4, but, in comparison, this fibre type exhibited greater persistence of short fibres. For MMVF10 and MMVF22, persistence decreased with increasing length class, and the persistence of fibres > 20 μm, estimated to be less than 5%, was significantly lower than for the other fibre types. For Code 100/475, 104E, SiC, MMVF21 and RCF1, there was less evidence of a difference in length-specific persistence rates. Among these five fibre types, persistence was lowest for Code 100/475 (weighted average 17%), and greatest for SiC (weighted average 53%). Although none of these five fibre types show any statistically significant trends in persistence relative to length, the graphs in Figure 3.1 suggest that for Code 100/475, 104E, SiC and MMVF21, fibres of between 5-10μm and greater than 20μm in length were more persistent than fibres of other lengths.

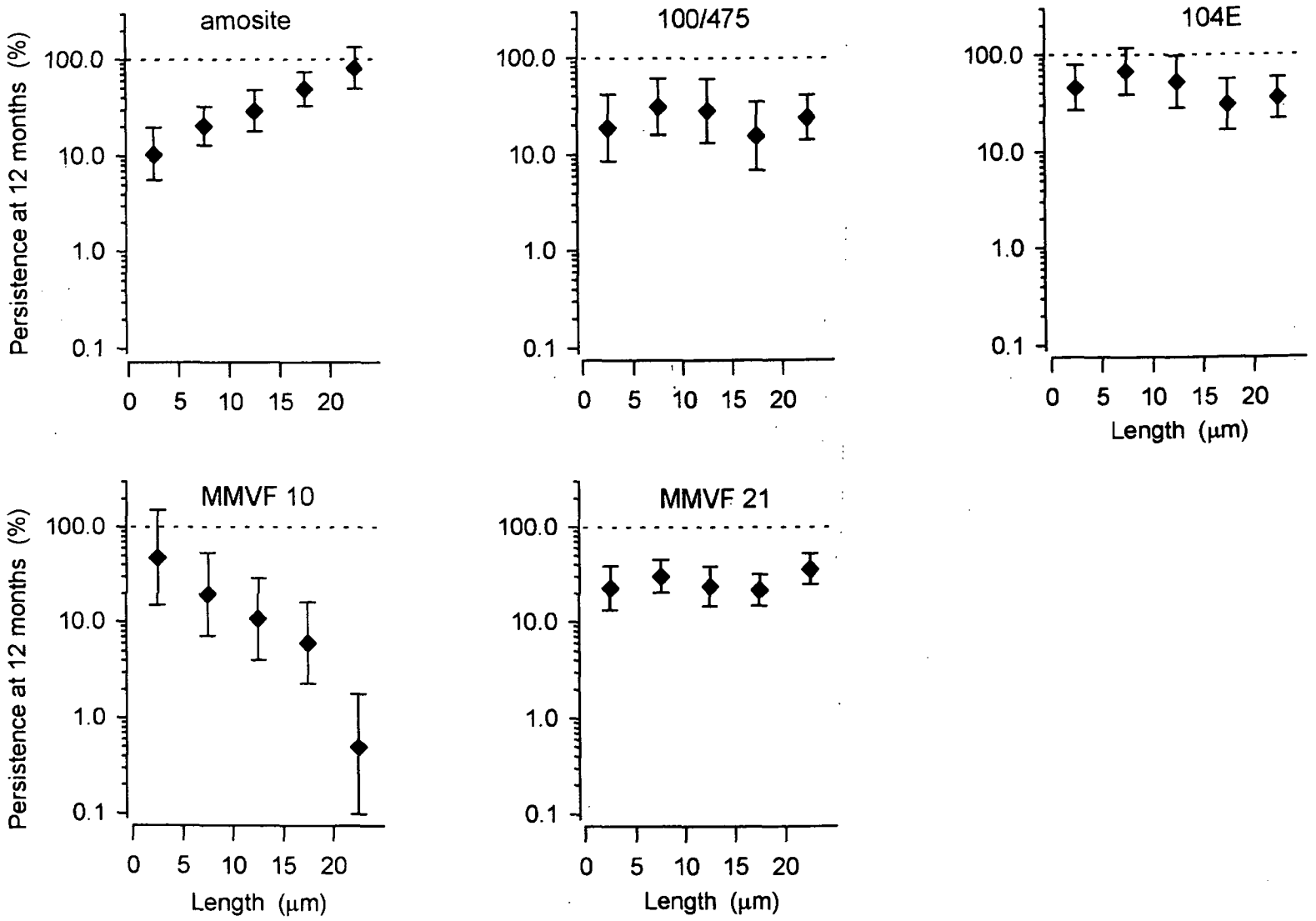

Figure 3.1 Estimated persistence of fibres by length category 12 months after intratracheal injection. Error bars represent 95% confidence intervals. The longest length category consists of all fibres longer than 20 μm.

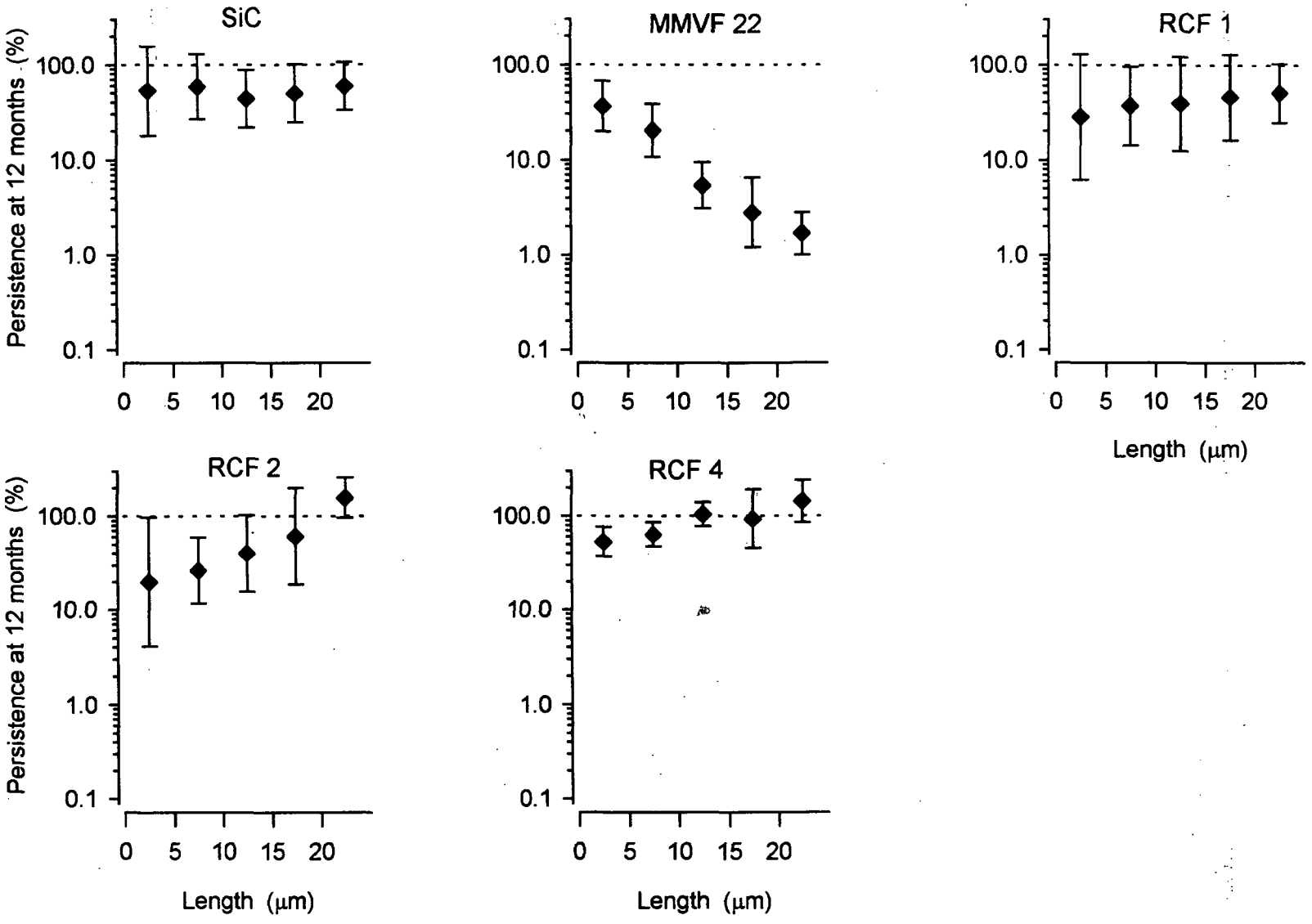

Figure 3.1 continued

Table 3.2
Length distribution of fibres recovered at 3 days, 1 month, 6 months and 12 months after injection, expressed as mean number of fibres per lung (millions).

Fibre type	Timepoint	Length category (μm)				
		0.4-5	5-10	10-15	15-20	>20
Amosite	3 days	219	61.2	18.3	6.26	5.44
	1 month	106	54.9	18.8	8.81	9.62
	6 months	24.6	18.1	6.10	3.87	5.48
	12 months	18.7	13.2	6.18	3.92	6.32
100/475	3 days	1593	196	44.5	9.34	5.73
	1 month	644	140	26.5	8.37	3.79
	6 months	512	106	16.6	4.16	1.30
	12 months	232	60.2	10.8	1.64	1.31
104E	3 days	275	78.1	19.2	5.68	3.24
	1 month	418	120	27.0	8.65	4.12
	6 months	217	87.6	18.1	3.97	2.29
	12 months	148	58.7	10.8	2.24	1.39
SiC	3 days	30.6	24.2	13.3	6.2	6.5
	1 month	5.8	8.7	5.0	2.5	4.2
	6 months	15.1	19.8	6.1	4.0	4.9
	12 months	9.0	9.2	5.1	2.6	3.3
MMVF10	3 days	0.564	0.969	0.645	0.464	1.50
	1 month	1.59	1.95	1.55	0.965	1.61
	6 months	1.78	1.88	0.876	0.444	0.34
	12 months	0.390	0.24	0.104	0.036	0.014
MMVF21	3 days	0.690	1.06	0.786	0.621	1.65
	1 month	0.797	1.46	1.044	0.706	2.43
	6 months	0.537	0.885	0.499	0.265	1.36
	12 months	0.189	0.384	0.249	0.157	0.710
MMVF22	3 days	1.75	2.02	1.41	0.75	1.16
	1 month	2.62	3.59	1.91	1.04	1.16
	6 months	0.91	0.72	0.31	0.10	0.16
	12 months	0.74	0.58	0.10	0.04	0.03
RCF1	3 days	1.52	2.20	1.25	0.78	1.64
	1 month	4.57	5.92	2.80	1.99	3.34
	6 months	0.37	0.62	0.26	0.22	1.51
	12 months	0.80	1.33	0.79	0.59	1.02
RCF2	3 days	0.93	0.92	0.66	0.42	0.88
	1 month	0.99	1.69	0.75	0.50	1.10
	6 months	0.32	0.55	0.44	0.27	0.97
	12 months	0.21	0.38	0.35	0.27	1.41
RCF4	3 days	3.01	3.57	1.26	0.61	0.44
	1 month	2.72	4.08	1.55	0.60	0.48
	6 months	1.96	2.90	1.18	0.47	0.46
	12 months	1.59	2.64	1.50	0.62	0.73

3.1.3 Changes in fibre diameter

The diameter distributions of injected fibres were modified to varying degrees over 12 months residence in lungs. The mean diameters of MMVF10 and MMVF22 fibres of all lengths (> 0.4 μm) recovered from lungs after 12 months were reduced relative to those measured after 3

days (Table 3.3). This reflected a large reduction in the number of thick fibres recovered, coupled with a slight increase in the number of fine fibres recovered (i.e. diameter < 0.25μm). No fibres with diameters less than 0.15μm were observed for these two fibre types at any recovery time. There were little or no changes in mean diameter among the other fibre types. Of fibres longer than 20μm, there was a substantial decrease in mean diameter for MMVF10, and smaller decreases for 100/475 and RCF2. There was a statistically significant increase in the mean diameter of long fibres of MMVF21, presumably due to the disappearance of thinner fibres from this length category.

Table 3.3
Mean diameters (μm) of fibres recovered from lungs at 3 days and 12 months after injection. se: standard error

Fibre type	All fibres (>0.4μm)				Long fibres (>20μm)			
	3 days		12 months		3 days		12 months	
	mean	se	mean	Se	mean	Se	mean	se
Amosite	0.33	0.01	0.35	0.01	0.51	0.02	0.49	0.01
100/475	0.20	0.01	0.22	0.01	0.54	0.03	0.48	0.04
104E	0.24	0.01	0.24	0.01	0.47	0.02	0.44	0.03
SiC	0.48	0.01	0.50	0.01	0.53	0.02	0.55	0.02
MMVF10	1.27	0.02	0.54	0.02	1.58	0.02	0.99	0.11
MMVF21	0.95	0.01	1.15	0.02	1.10	0.02	1.34	0.03
MMVF22	0.89	0.02	0.47	0.01	1.11	0.05	1.08	0.16
RCF1	0.95	0.03	0.93	0.03	1.18	0.06	1.10	0.06
RCF2	1.11	0.04	1.30	0.03	1.64	0.08	1.46	0.04
RCF4	1.21	0.02	1.29	0.02	2.10	0.09	1.96	0.07

The ratio of complex silicon carbide whiskers, i.e. those composed of more than one fibrous component, to single silicon carbide whiskers, did not appear to change after 12 months in the lung (data not shown).

3.1.4 Estimated half times of clearance

Estimated length-specific half times of clearance calculated using a simple exponential decay model are shown in Table 3.4. The exponential decay model was not a good fit to all length categories of amosite or to the length categories shorter than 20μm for MMVF10. For amosite, average persistence was lower at 1 and 6 months than predicted by the model whereas for MMVF10, average persistence was greater at 1 and 6 months than predicted by the model.

Table 3.4

Estimated half time of persistence in months (95% confidence intervals) for different length categories in the injection experiment. ne: not estimable (essentially infinite)

Fibre type	Length category (μm)				
	0.4-5	5-10	10-15	15-20	>20
Amosite	3.7 (2.9-5.0)	5.2 (4.1-7.3)	6.8 (4.9-11.1)	11.6 (7.5-25.9)	39.6 (11.9-ne)
100/475	5.0 (3.4-9.3)	7.2 (4.6-16.5)	6.6 (4.2-16.4)	4.5 (3.1-7.8)	5.8 (4.3-9.2)
104E	10.6 (6.3-32.5)	20.9 (8.8-ne)	12.8 (6.6-217)	7.0 (4.7-13.9)	8.0 (5.5-15.0)
SiC	13.2 (4.9-ne)	15.6 (6.3-ne)	10.0 (5.5-54.7)	11.9 (6.0-1194)	ne (7.6-ne)
MMVF10	11.1 (4.4-ne)	5.1 (3.2-12.7)	3.7 (2.6-6.6)	3.0 (2.2-4.5)	1.6 (1.3-2.0)
MMVF21	5.6 (4.2-8.8)	7.0 (5.2-10.4)	5.8 (2.6-6.6)	5.4 (4.4-7.2)	8.1 (6.0-12.7)
MMVF22	8.3 (5.3-20.3)	5.2 (3.8-8.5)	2.9 (2.4-3.5)	2.3 (1.9-3.0)	2.0 (1.8-2.3)
RCF1	6.6 (3.0-ne)	8.3 (4.3-133)	8.9 (4.1-ne)	10.7 (4.6-ne)	12.2 (6.0-ne)
RCF2	5.2 (2.6-150)	6.2 (3.9-15.5)	9.2 (4.5-ne)	16.9 (5.0-ne)	ne (305-ne)
RCF4	13.3 (8.4-31.3)	18.5 (11.2-53.2)	ne (34.0-ne)	119.2 (10.8-ne)	ne (54.4-ne)

The half times of clearance of short and intermediate length fibres (those <15μm) of most of the ten fibre types were between 4 and 7 months. The clearance half times of short fibres of MMVF10 (<5mm) and of short and intermediate length fibres of 104E, SiC and RCF4 were somewhat longer than those for the other fibre types. The half times of clearance of long fibres were much more variable. The clearance half times for long fibres of MMVF10 and MMVF22 were only a couple of months whereas those for long fibres of amosite, RCF2 and RCF4 and silicon carbide were of the order of years or possibly infinite.

3.1.5 Differences in estimates of biopersistence following additional counts funded by EURIMA

The effect of the extra counts on estimated biopersistences for amosite, the two microfibres and MMVFs 10 and 21 was relatively small as the major source of uncertainty in the estimates arises from interanimal differences. Estimated mean persistences for each size category for each of these fibre types generally showed little change (Fig. 3.2). The greatest differences between the old and new counts were in the longest size categories for amosite, the two microfibres and MMVF21, where relatively few fibres were counted. The main effect of the extra data was to reduce the width of the confidence limits associated with the estimates of persistence over 12 months for each length category for most of the fibre types, apart from MMVF10. The confidence intervals associated with the estimates of persistence for MMVF10 remained similar to those based on the original counts.

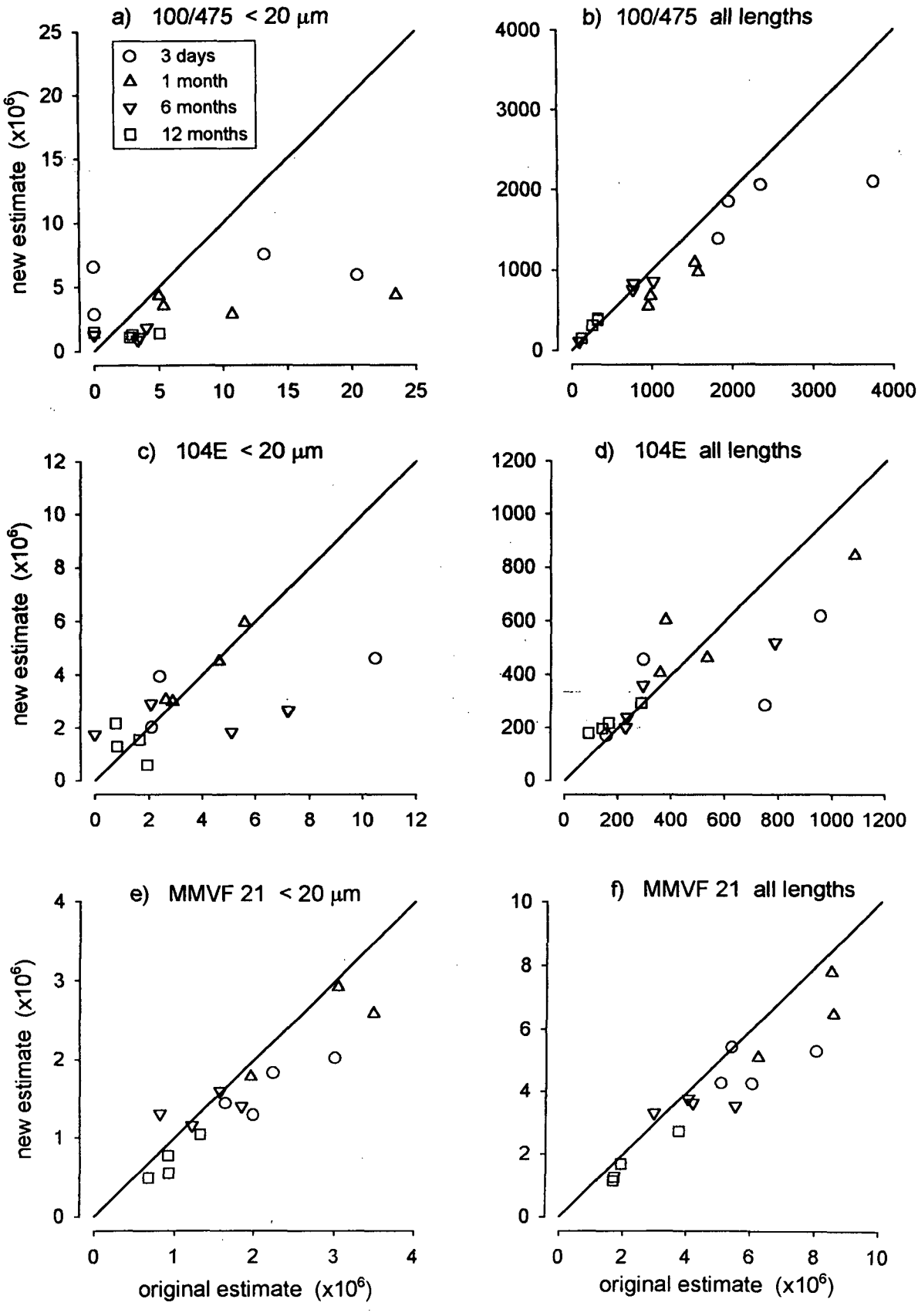

Figure 3.2 Comparison between original and new lung fibre number burden estimates.

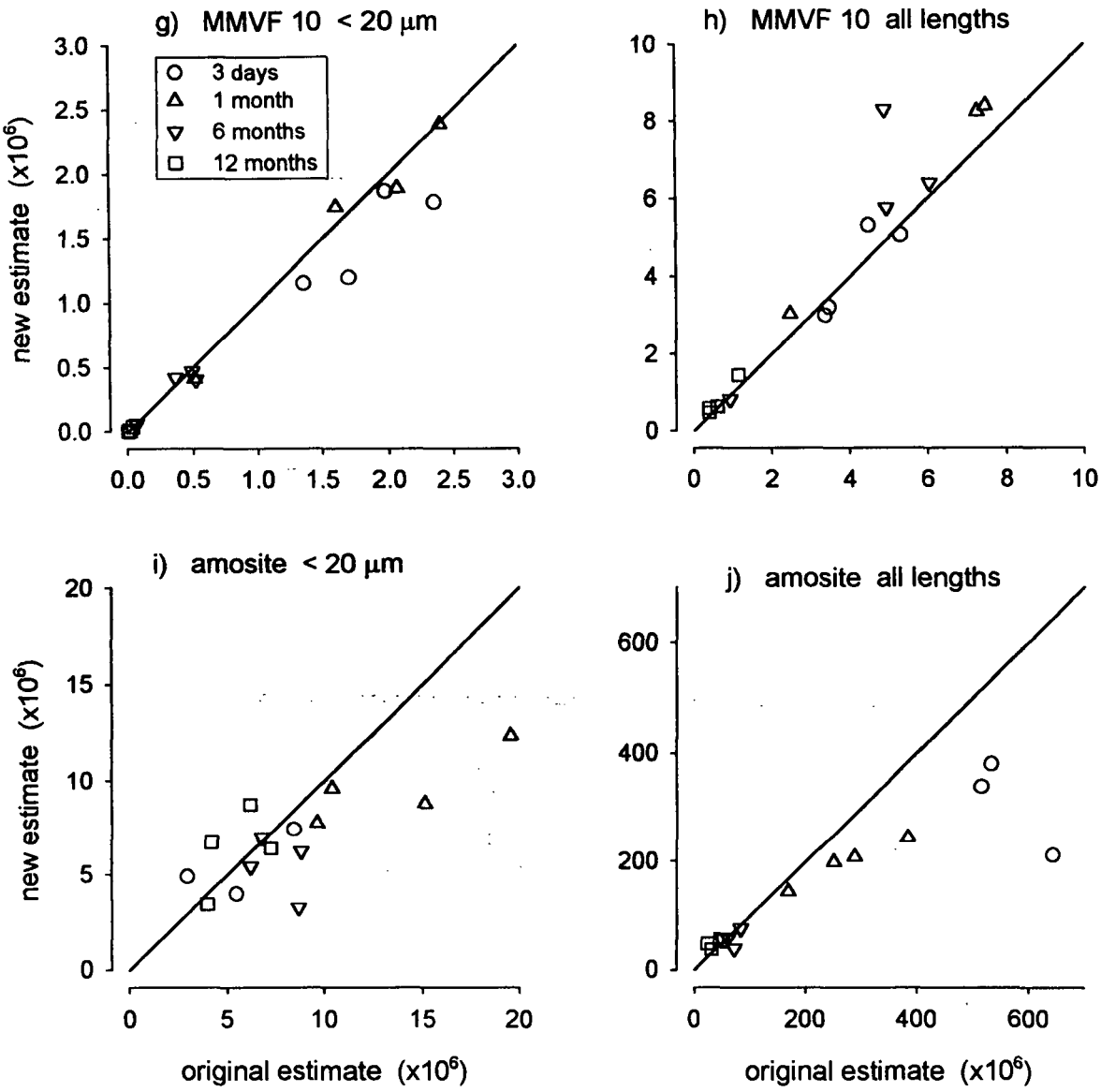

Figure 3.2 continued

3.2 INHALATION EXPERIMENTS

3.2.1 Exposure concentrations achieved

The four dust clouds were matched in terms of optical fibre counts with a target concentration of 1000f/ml as WHO fibres. The total fibre concentrations (in terms all fibres longer than 0.4μm) achieved as determined through SEM analysis therefore varied with fibre type (Table 3.5). Fibre concentrations were greatest for the code 100/475 and amosite and least for the SiC. This reflects the differences in size distribution between fibres (Fig. 3.3). The dust cloud for code 100/475 contained the greatest number of short fibres (<5μm) and the least number of long fibres (>20μm). In terms of diameter, 104E contained a greater proportion of the finest fibres (<0.1mm) and much fewer thicker fibres (>0.45μm) than the other fibre types (Fig. 3.3).

Table 3.5
Mean aerosol concentrations of fibres for each fibre type

Fibre type	Mean fibre concentrations for different length classes (f/ml)		
	all fibres >0.4μm	(WHO) fibres >5μm	fibres >20μm
Amosite	3850	981	89
code 100/475	3977	1066	38
code 104E	2354	974	72
SiC	1978	965	53

3.2.2 Lung burden data

The data for all four fibre types showed a wide spread of estimated total lung burden for individual animals at each time point relative to the differences between time points (Fig. 3.4). The retained mean total fibre burden of the animals exposed to code 100/475 was much greater in terms of fibre numbers than for the other 3 fibre types (Table 3.6). The estimated volumes of retained fibre were, however, more similar. The number of retained long fibres following 12 months was less for code 100/475 (>15, >20μm) than for the other fibre types. It should be noted, however, that the numbers of long fibres counted of all fibre types were extremely small, giving rise to relatively large errors in estimated lung burden. The relative proportion of very short (<1.5μm) fibres retained of code 100/475 was much greater than for the other 3 fibre types. In general the size distribution of fibres retained in lung tissue was similar to that in the aerosols (Figs. 3.5, 3.6). The proportion of very fine fibres of 100/475 (<0.2μm) recovered from rat lungs was, however, small in comparison to that present in the dust cloud.

Table 3.6
Retained mean lung burden after 12 months exposure (3 days recovery point) by inhalation in terms of millions of fibres per lung

Fibre type	length category (μm)					all fibres
	<5	5-10	10-15	15-20	>20	
Amosite	3009	569	273	131	123	4106
100/475	12741	1723	412	95	11	14982
104E	8673	1720	386	167	83	11028
SiC	1526	895	277	148	130	2975

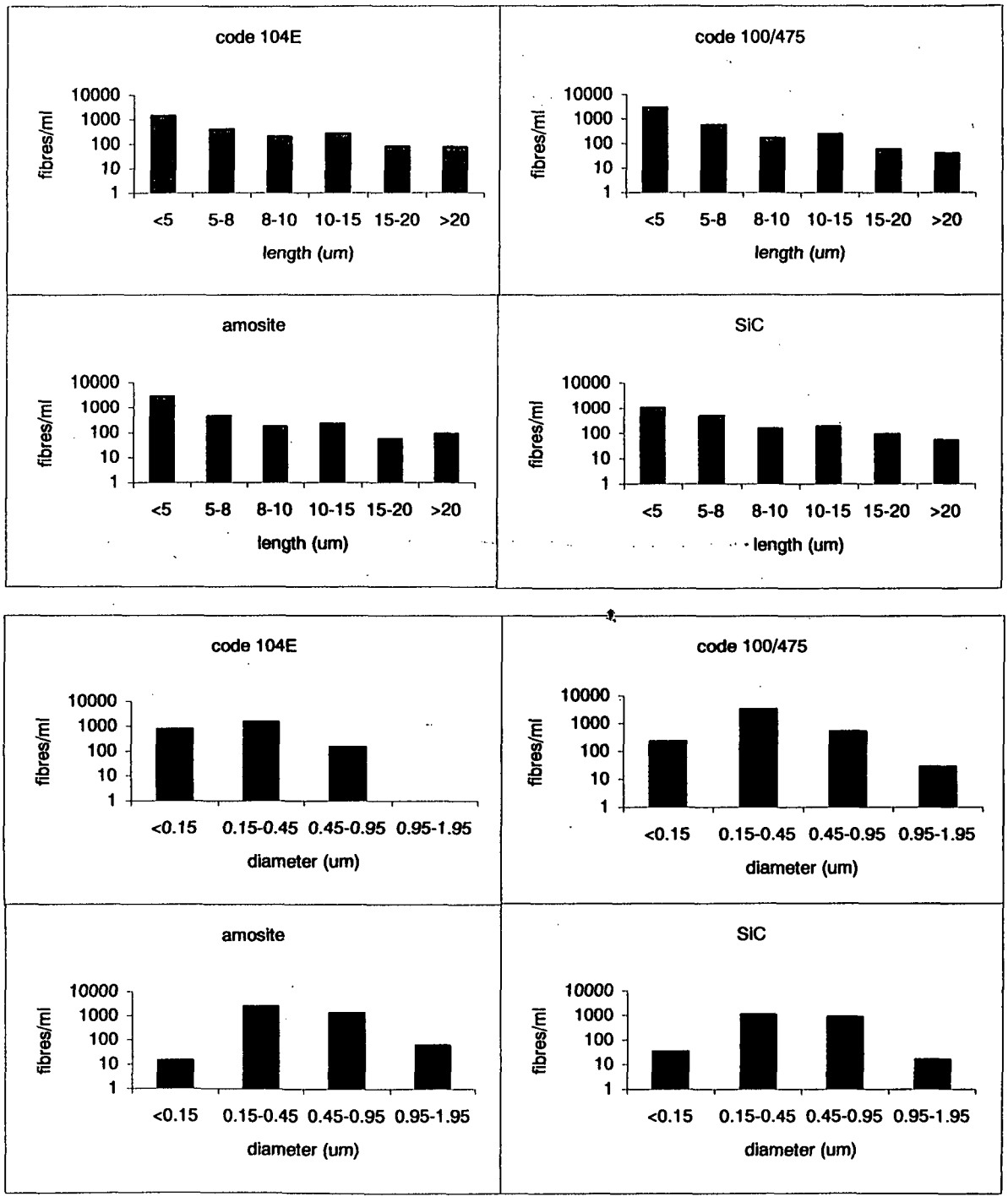

Figure 3.3: Size distributions of fibres in the aerosols in the inhalation experiment

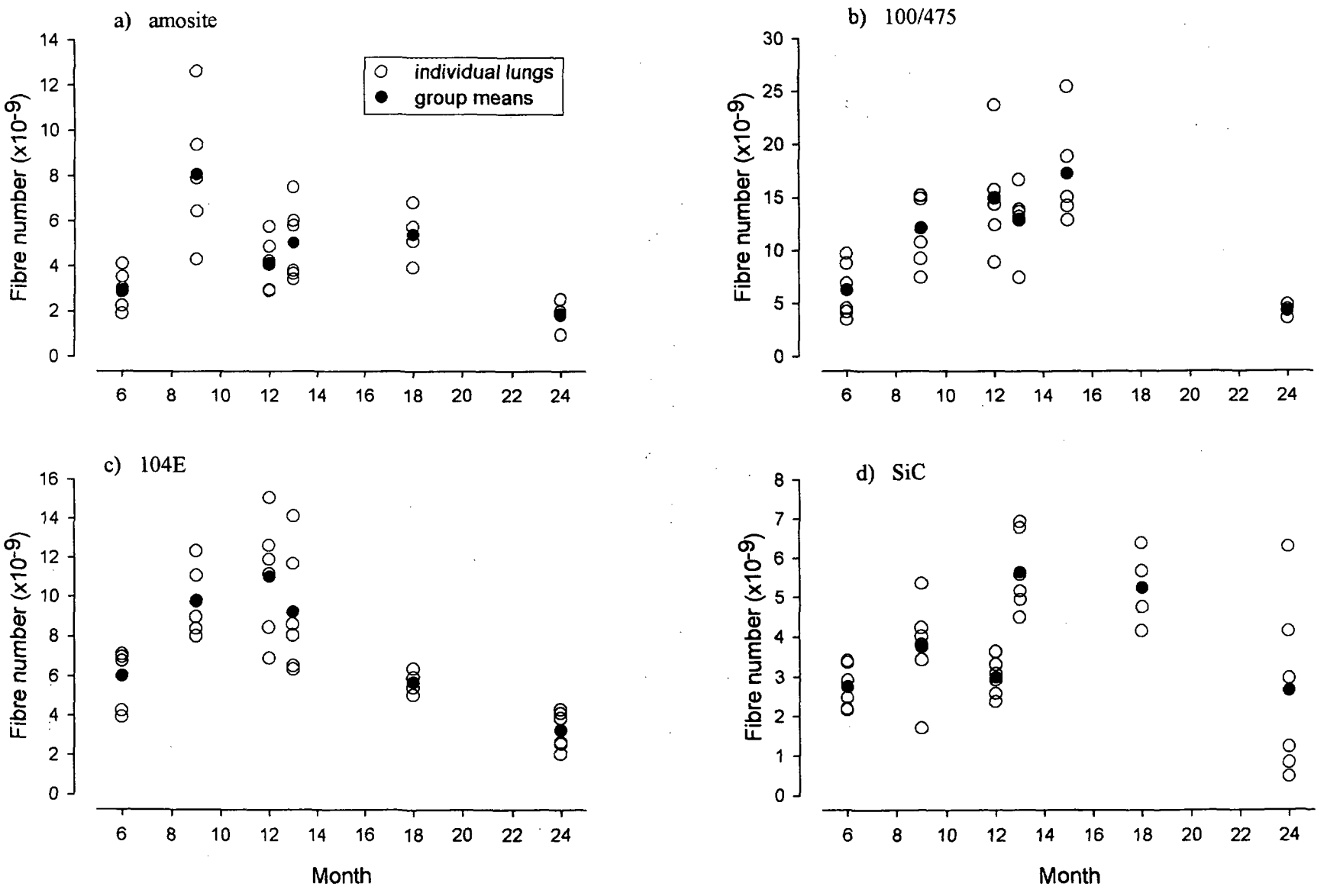

Figure 3.4 Fibre number burdens recovered from lungs exposed by inhalation for 12 months

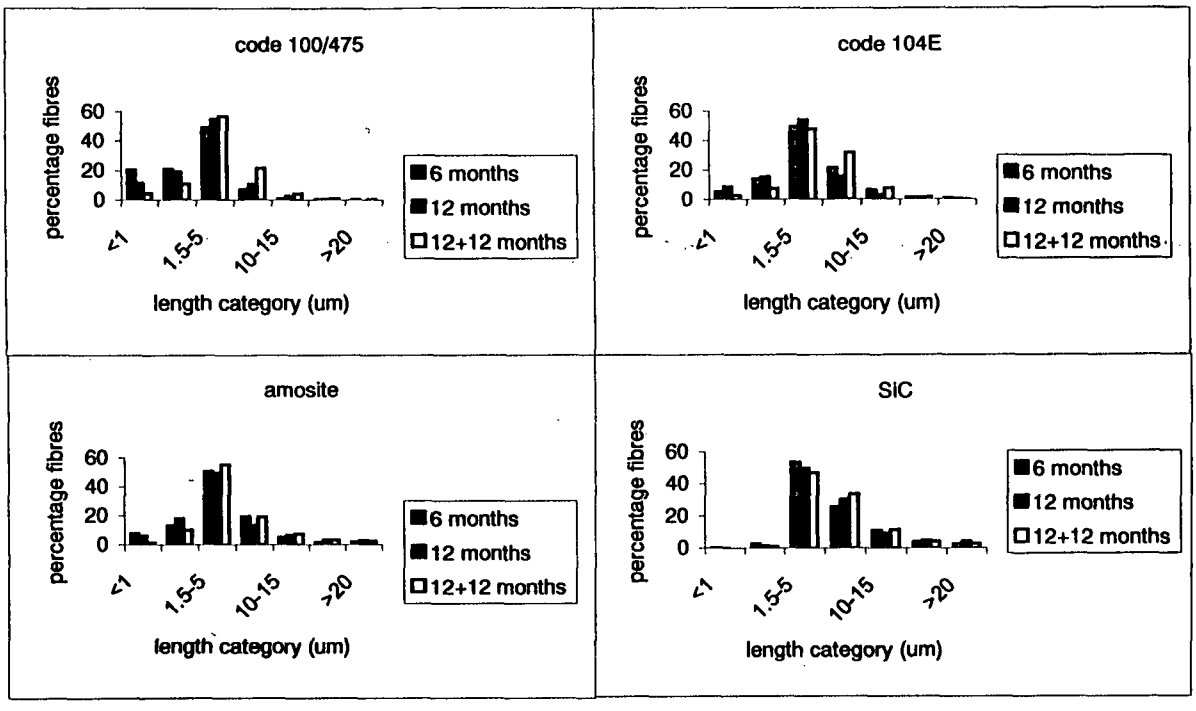

Figure 3.5: Length distributions of fibres recovered from rat lungs in the chronic inhalation experiment following 6 months and 12 months exposure by inhalation and 3 days recovery and following 12 months exposure and 12 months recovery.

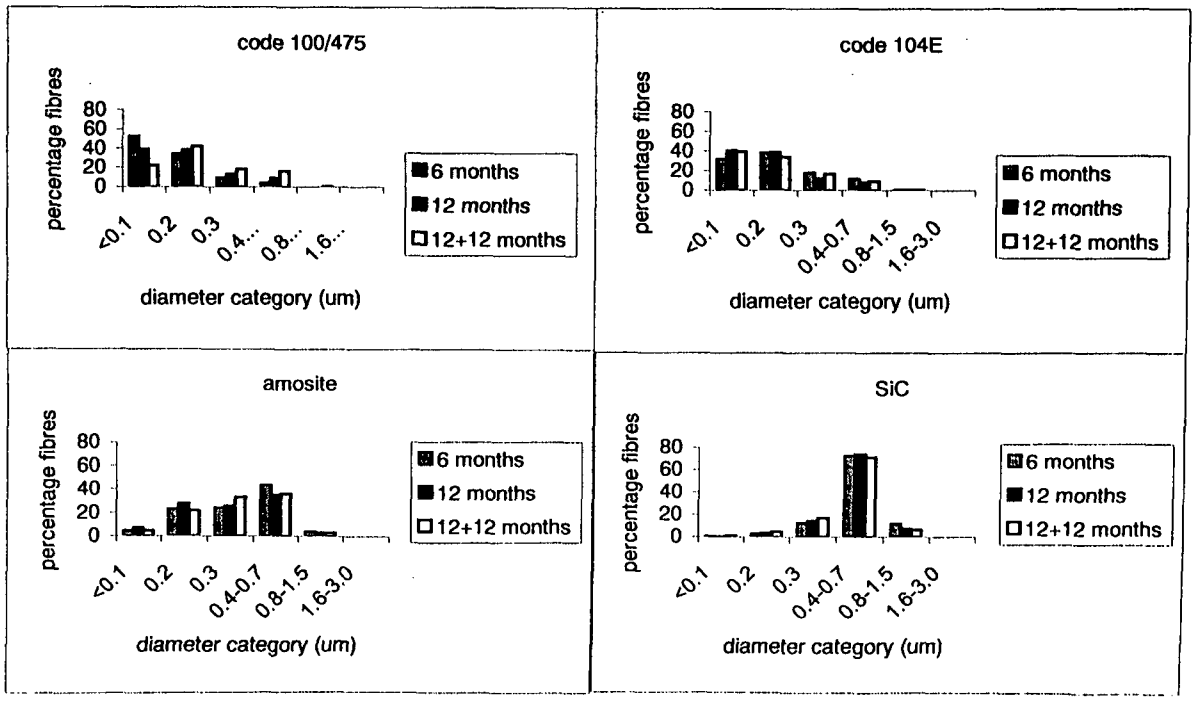

Figure 3.6: Diameter distributions of fibres recovered from rat lungs in the chronic inhalation experiment following 6 months and 12 months exposure by inhalation and 3 days recovery and following 12 months exposure and 12 months recovery.

The data for amosite, code 100/475 and code 104E suggest that there was a progressive accumulation of lung fibre burden during exposure followed by a substantial reduction in lung burden over the 12 months following cessation of exposure. In terms of total fibre numbers (Fig 3.4), fibre volumes and summed lengths, the rates of clearance of these three fibres appear to have been fairly similar. The silicon carbide data show progressive accumulation during exposure but no significant clearance following cessation of exposure (Fig 3.4).

For amosite and the two glass microfibres, the length distribution of recovered fibres shifted slightly towards longer length classes over the 12 months following cessation of exposure (Fig. 3.5). This would be consistent with the preferential removal of short fibres. The diameter data for 100/475 show a slight increase in the percentage of thicker fibres present through time. The diameter distribution of the other three fibre types underwent little modification over 12 months (Fig.3.6).

Retained fibres of amosite and 104E did not undergo any substantial modification in composition during 12 months following the cessation of inhalation, whereas fibres of code 100/475 appeared to have undergone differential leaching with preferential removal of sodium, potassium, calcium and barium (Table 3.7).

Table 3.7
Mean (standard deviation) apparent compositions of fibres in terms of percent element oxide as determined using EDX for untreated fibres (code 100/475) or fibres recovered from lung tissue 3 days after injection (E glass) and at 12+12 months in the inhalation experiment (nd= not detected)

Element oxide	Amosite		Code 104E		code 100/475	
	Untreated	12+12 months	Untreated	12+12 months	untreated	12+12 months
Na	Nd	nd	Nd	nd	0.8 (1.1)	Nd
Mg	1.2	1.2	0.5 (2.8)	1.1 (0.6)	nd	Nd
Al			11.7 (2.5)	12.3 (1.9)	1.9 (1.0)	2.0 (0.8)
Si	31.9	30.9	59.7 (3.2)	59.9 (5.6)	74.5 (11.1)	87.2 (6.1)
K			Nd	nd	8.4 (3.8)	2.7 (1.1)
Ca	0.6	1.3	28.0 (3.5)	26.7 (4.3)	6.8 (1.6)	1.7 (1.7)
Ba			Nd	nd	6.9 (2.6)	0.1 (1.2)
Fe	61.4	62.6	Nd	nd	0.6 (1.4)	6.5 (6.4)

3.2.3 Comparison of lung burden and changes in lung burden following inhalation and injection

The estimated numbers of retained fibres following 12 months inhalation were much greater than following injection of 1mg of each fibre type into rat lungs (Table 3.8).

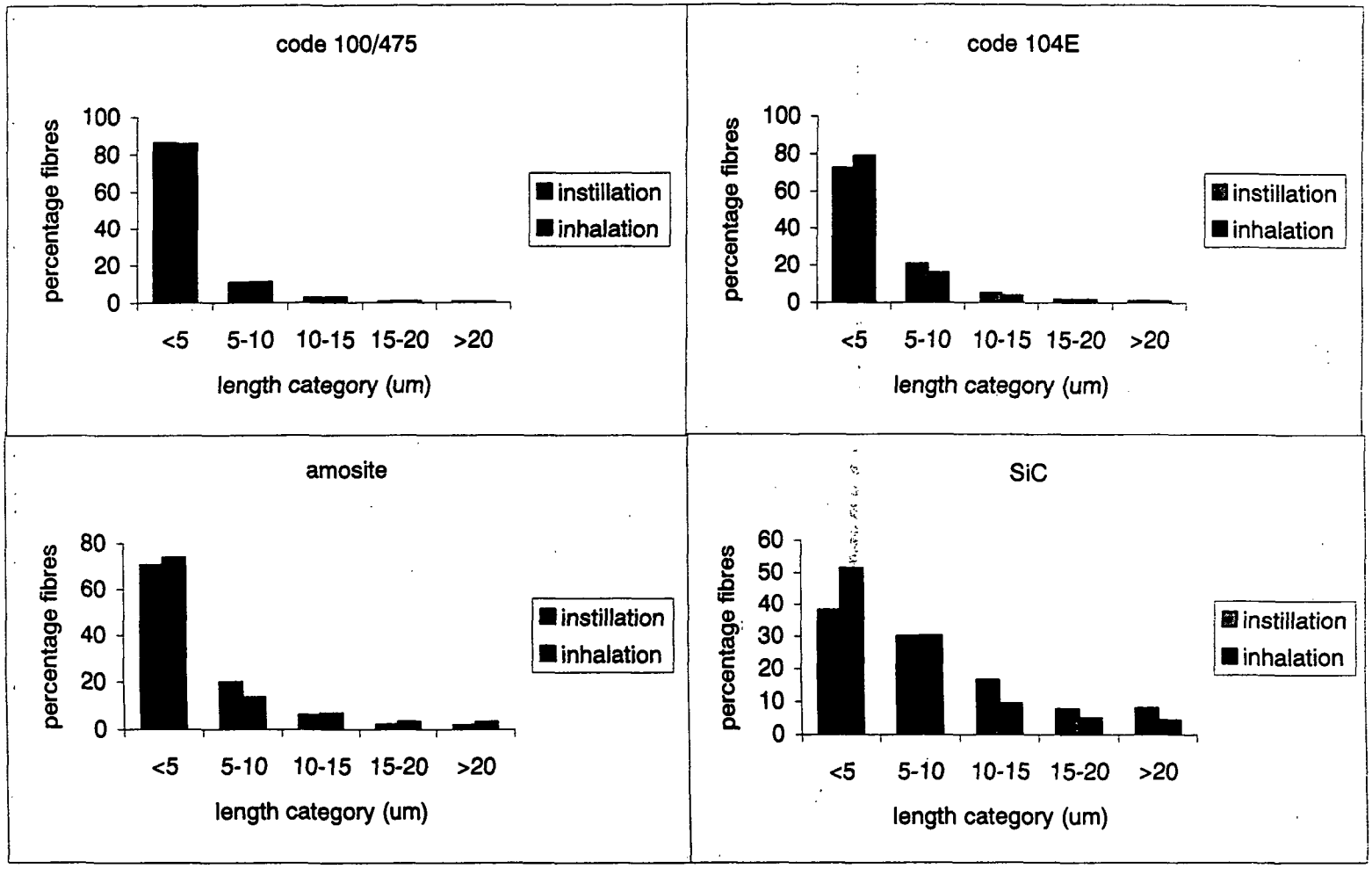

Figure 3.7: Comparison of the length distributions of fibres recovered from rat lungs following 12 months exposure by inhalation with those of fibres recovered from rat lungs 3 days after instillation of a 1mg dose of fibre.

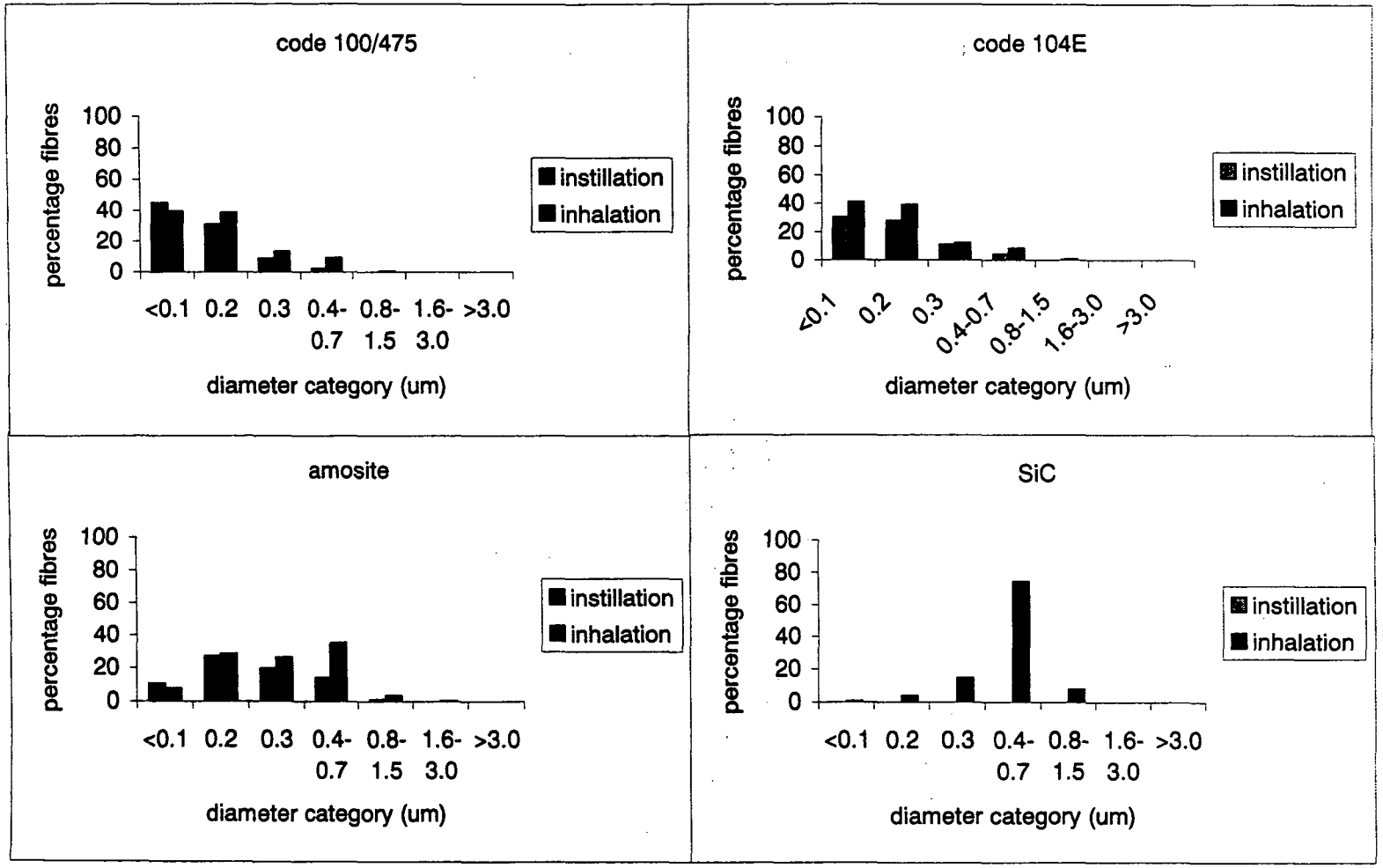

Figure 3.8: Comparison of the diameter distributions of fibres recovered from rat lungs following 12 months exposure by inhalation with those of fibres recovered from rat lungs 3 days after instillation of a 1mg dose of fibre.

Table 3.8
Comparison of the mean numbers of retained fibres (in terms of millions of fibre per lung) for each fibre type following 12 months inhalation and after injection of 1mg of fibre

Fibre type	fibres longer than 0.4μm		WHO fibres		fibres longer than 20μm	
	injection	inhalation	Injection	inhalation	injection	Inhalation
Amosite	310	4106	91	1078	5.4	123
100/475	1848	14982	225	2156	5.7	11
104E	381	11028	106	2355	3.2	82
SiC	81	2975	50	1449	6.5	130

The size distribution of fibres retained after 12 months inhalation was similar to that of fibres recovered after injection (Figs 3.7, 3.8).

With the exception of the 104E, the percentage clearance of fibres in terms of total fibre numbers generally appeared to be less over the 12 months following the cessation of inhalation than over the 12 months after injection (Table 3.9). The relationship between fibre length and persistence seen with amosite in the injection experiment was less clear from the inhalation data. In contrast, both the glass microfibres showed the same suggestion of slightly preferential retention of fibres in the 5-10μm length category relative to fibres of other lengths.

Table 3.9
Estimated retention after 12 months of recovery of fibres of all lengths (>0.4μm) relative to the lung burden after 3 days (injection experiment) and 12 months inhalation

Fibre type	Injection	Inhalation
Amosite	16%	44%
100/475	17%	29%
104E	58%	30%
SiC	36%	89%

3.3 DISSOLUTION DATA

The *in vitro* dissolution data are fully described in a separate report to HSE (Searl and Buchanan, 1999). Table 3.10 lists the estimated dissolution constant for each fibre type and the estimated time required for half the fibres in a sample to be completely dissolved in the dissolution assay. The estimated half times of persistence were much greater than those observed in the injection experiment.

Table 3.10
Estimates of mean (range) K_{dis} and half time of persistence *in vitro* dissolution experiments run following a modified version of the EURIMA protocol over 6 weeks

Fibre type	K_{dis} ($ngcm^{-2}hr^{-1}$)	Half time of persistence (days)
100/475	22 (11-28)	28
104E	10 (3.1-21)	62
MMVF10	135 (98-185)	28
MMVF21	14 (12-15)	162
MMVF22	73 (66-91)	31
RCF1	10 (3.1-21)	221
RCF2	9.8 (6.4-12)	232
RCF4	1.0 (0.0-1.8)	-

There is a general correlation between the dissolution rates measured *in vitro* and the observed biopersistence of long fibres in the injection experiment (Fig. 3.9). Both assays clearly discriminate between fibres that disappear relatively rapidly and those that disappear relatively slowly. The detailed rankings of individual fibre types in the *in vitro* and *in vivo* assays of persistence are, however, of limited statistical significance.

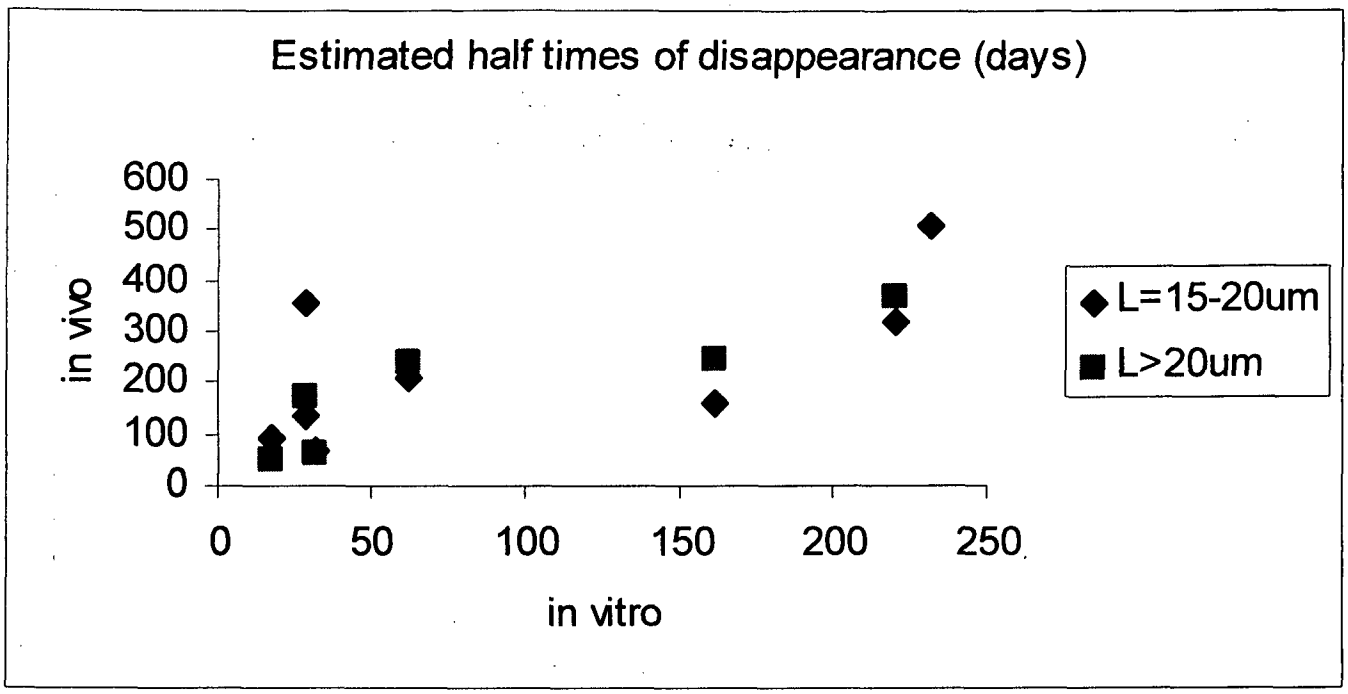

Figure 3.9: Comparison of estimated half times of persistence for fibre samples in the in vitro dissolution assay with estimated half times of persistence of long fibres in lung tissue in the instillation experiment

4. TOXICOLOGY OF E-GLASS

4.1 *IN-VITRO* EXPERIMENTS

4.1.1 *In vitro* TNFα production

TNFα production by alveolar macrophages in vitro is shown in Table 4.1 as the geometric means of data pooled over three experiments. The fibre types have been ranked according to increasing TNFα production. The 104E glass microfibres have an activity intermediate between the most active fibres - silicon carbide whiskers and asbestos – and the relatively inactive MMVFs and RCFs. The 104E appears to have a similar activity in this assay to the other microfibre, 100/475, that was tested.

Table 4.1
TNFα production by alveolar macrophages exposed in vitro to equal fibre numbers. Table contains means with estimated standard errors in *italics*.

Fibre type	TNF Units/ 10^6 cells	
No fibre	37	*10*
MMVF 10	34	*9*
RCF 4	39	*10*
MMVF 21	39	*11*
RCF 2	45	*12*
RCF 1	46	*12*
MMVF 22	50	*14*
100/475	60	*16*
104 E	71	*19*
Amosite	91	*24*
SiC (3rd Millennium)	125	*34*
SiC (ACMC)	1527	*409*

4.1.2 Detachment injury to A549 cells

The experiments were conducted in two series with 104E included only in the second series. The first series is included here to allow comparison across all the fibre types tested. The first series, using 10 fibre types, consisted of six experimental runs and the second series, using 7 fibre types, consisted of two experiments. Analysis of variance was carried out on log-transformed data. Results are displayed in Table 4.2 as smoothed geometric means of radioactive counts per minute. For both series of experiments, there was a greater level of variation between the experiments than within experiments. Within experiments, there were significant effects of dose, and of fibre; but there was no evidence of any fibre × dose interaction. The data are a little difficult to interpret because in the second series of experiments, the MMVF 21 treatments all produced less detachment than the no fibre control, a result that was quite different from Series 1.

The 104E showed a relatively low level of activity in this assay, being much less active than amosite and by inference crocidolite and MMVF22. The 104E also appeared to be less active than the 100/475. By inference the 104E appears to have a similar activity to the RCFs and have a greater activity than the glasswools, MMVFs 10 and 11. Overall, however, the activity

of fibres in this assay is not clearly related to pathogenicity as crocidolite caused no more detachment than many of the man-made fibres that are believed to be much less pathogenic.

Table 4.2
A549 epithelial cell detachment following *in vitro* exposure to fibres at mass doses of 10, 25, 50, and 100 μg/well. These doses are equivalent to approximately 31, 78, 156, and 312 μg per cm^2 of cell monolayer. The table contains smoothed geometric mean counts per minute.

			Fibre dose (μg/ml)			
Experiment series	**Fibre**	**None**	**10**	**25**	**50**	**100**
	No fibre	2414				
	RCF 1		3082	3183	3729	4017
	RCF 2		3021	3120	3655	3937
	RCF 3		3102	3203	3753	4042
Series 1	RCF 4		2617	2702	3166	3410
	MMVF 10		2995	3093	3623	3903
	MMVF 11		2734	2823	3307	3562
	MMVF 21		3500	3614	4233	4560
	MMVF 22		3270	3376	3955	4261
	Crocidolite TIMA)		3298	3405	3989	4297
	Amosite		4169	4305	5043	5432
	No fibre	2592				
	100/475		3343	3468	3862	4831
	104E		2582	2678	2983	3731
Series 2	SiC (ACMC)		2566	2662	2965	3708
	SiC (3^{rd}) Millennium)		2704	2805	3124	3908
	RCF 4		2699	2800	3119	3901
	MMVF 21		1565	1623	1808	2261
	Amosite		3650	3786	4217	5274

4.2 SHORT TERM INHALATION EXPERIMENT

4.2.1 Bronchoalveolar lavage studies following short term inhalation

Table 4.3 summarises the data for total cells, % granulocytes, and protein in lavage fluid recovered after exposure by inhalation to four fibre types for 1, 3, 8 or 14 days. Overall, the total numbers of cells recovered were increased when compared with the unexposed controls, but there were no clear differences between fibre types, nor any clear trend with length of inhalation exposure. The 104E did not give rise to very different effects from those previously observed for the other three fibre types. Cell numbers in the amosite and silicon carbide groups appeared to return to control levels after longer exposures but a similar effect was not seen with either of the microfibres.

Each of the fibre types caused some inflammation as shown by the significantly increased proportions of granulocytes (principally neutrophils) among the recovered cells. There were, however, no consistent differences between the fibres, nor any convincing trends with length of exposure. The early response of animals exposed to either microfibre appeared to be much less than that of animals exposed to amosite or silicon carbide. By the end of the inhalation experiment, however, the silicon carbide appeared to produce the greatest response, the 100/475 the least response and both the 104E and amosite had produced intermediate responses.

A similar lack of pattern was observed in the levels of protein in the lavage fluid. Although exposed animals had higher average levels than the controls, again there was little evidence of trends with length of exposure, nor consistent differences between fibre types.

Table 4.3
Results of assessments from bronchoalveolar lavage: total cell numbers, % granulocytes, and protein concentrations as means and estimated standard errors in *italics*.

	Length of inhalation (days)									
Fibre inhaled	**0**		**1**		**3**		**8**		**14**	
	Total cells in BAL ($x10^6$)									
No fibre	6.2	*0.5*								
100/475			7.2	*1.4*	6.8	*1.0*	10.0	*1.9*	9.5	*1.8*
104 E			7.1	*1.8*	5.5	*1.4*	7.8	*1.9*	11.9	*3.0*
SiC			5.9	*1.5*	8.0	*2.0*	6.2	*1.5*	6.2	*1.5*
Amosite			9.0	*1.8*	7.4	*1.0*	6.5	*1.2*	4.5	*0.8*
	% granulocytes in BAL									
No fibre	0.4	*0.2*								
100/475			1.9	*1.1*	3.6	*1.3*	0.8	*0.7*	4.0	*1.7*
104 E			2.2	*1.7*	5.1	*3.4*	6.0	*3.9*	8.9	*5.4*
SiC			5.0	*3.3*	5.8	*3.8*	3.5	*2.4*	12.1	*7.0*
Amosite			6.5	*2.8*	4.4	*1.5*	2.9	*1.6*	6.2	*2.5*
	Protein in BAL (Units/ml)									
No fibre	144	*12*								
100/475					442	*98*	157	*22*	251	*34*
104 E			162	*29*	175	*31*	258	*46*	253	*45*
SiC			148	*26*	234	*42*	277	*49*	262	*47*
Amosite					258	*57*	203	*28*	235	*31*

4.2.2 Cell proliferation in terminal bronchiolar/alveolar ducts following short term inhalation

Table 4.4 summarises the numbers of cells staining positive for BrdU per mm of terminal bronchiolar/alveolar ducts at the 6 lung levels for 4 fibre types. The effects were most marked in the upper part of the lung and decreased towards the base of the lung. Exposure to 104E resulted in increased proliferation but the effect was smaller than that seen with amosite or silicon carbide. In contrast, however, exposure to the other microfibre tested, code 100/475 appeared to have no significant effect on cell proliferation and the average proliferation index was similar to that of the control animals. There were substantial differences between animals treated with the same fibres, that are reflected in the standard errors (Table 4.4). However, despite this between animal variation, there were consistent and significant differences between fibre types in the ability to stimulate cell proliferation ($p<0.05$). Analysis showed that the principal patterns of the means from Table 4.4 could be adequately summarised as multiplicative effects of the estimated fibre type and lung level. This summary is shown graphically in Figure 4.1, which displays both the fibre type and lung level effects as constant differences on a logarithmic scale.

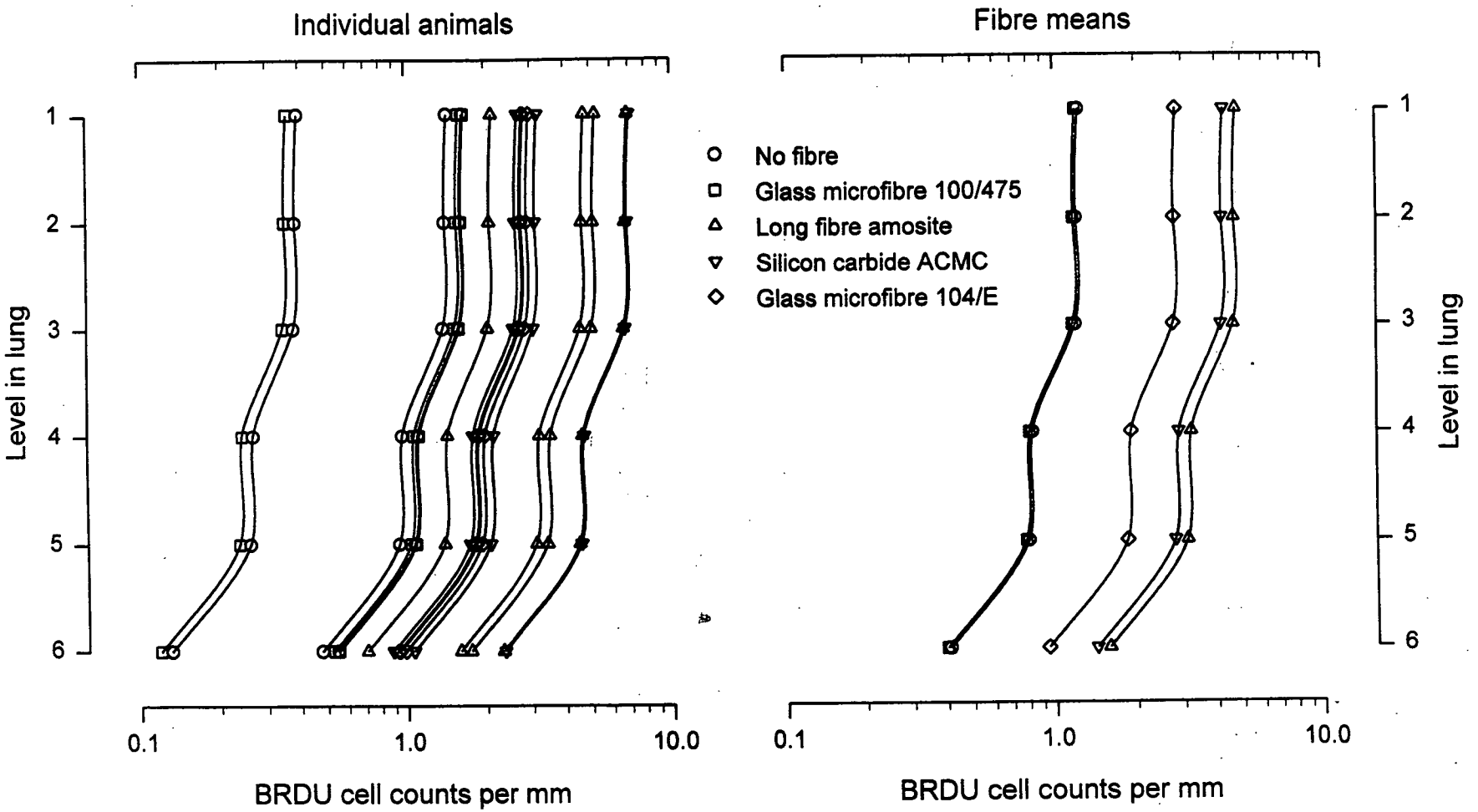

Figure 4.1: Cell proliferation (as numbers of BrdU-positive cells per mm of duct perimeter) at six lung levels (from apex to base). Estimated differences between fibre types and lung levels are shown. Results are averaged across animals within each treatment group.

Table 4.4
Numbers of BrdU-positive cells at six levels (from base to apex) in the lungs of rats after inhaling fibres. Table shows means over animals, with estimated standard errors in *italics*.

Fibre type	Level in lung (apex = 1, base = 6)													
	1		2		3		4		5		6		Mean	
	Counts per mm duct													
No fibre	0.81	*0.35*	0.70	*0.28*	1.04	*0.32*	1.04	*0.29*	0.46	*0.16*	0.57	0.26	0.74	*0.13*
100/475	1.47	*0.49*	1.09	*0.35*	1.02	*0.37*	0.29	*0.17*	0.42	*0.19*	0.51	0.27	0.71	*0.13*
104 E	2.63	*0.68*	3.20	*0.66*	3.42	*0.57*	1.12	*0.34*	1.77	*0.46*	0.57	0.40	2.04	*0.21*
SiC	2.99	*0.73*	4.58	*0.86*	3.49	*0.58*	3.32	*0.66*	2.53	*0.73*	0.01	0.05	2.80	*0.28*
Amosite	4.78	*0.68*	3.94	*0.46*	3.98	*0.45*	2.99	*0.35*	3.20	*0.40*	1.41	0.36	3.14	*0.19*

4.3 LONG TERM INHALATION EXPERIMENT

4.3.1 Aerosol characteristics

The 12 month average exposure concentration was 1022 $f.ml^{-1}$, close to the planned 1000 $f.ml^{-1}$ for fibres longer than 5 μm, as measured by PCOM. The achieved exposure concentration was similar to those attained in the earlier experiments with amosite and code 100/475 fibres (Jones *et al*, 1998; Davis *et al.*, 1996).

The SEM bivariate size distribution data (for all fibres longer than 0.4 μm) were converted to fibre number concentrations by scaling in proportion to the concentrations measured for fibres longer than 5 μm by PCOM. The PCOM concentrations being based on approximately 200 short period samples provided a reliable estimate of the average concentration over the exposure time.

The bivariate size distribution of the 104E aerosol was similar to that for the other fibres tested by inhalation (Section 3.2.1). The 104E, however, contained approximately half as many short fibres (lengths less than 5 μm) as the 100/475 or the amosite. The number of long (> 20 μm) fibres in the E-glass and the amosite aerosols appeared to be double that in the 100/475 aerosol. This may not be significant, however, given that very few long fibres were counted of any fibre type. Comparing across fibre types within length categories, 104E had the greatest number of very fine fibres (≤ 0.1 μm).

4.3.2 Biopersistence

The lung burden data and biopersistence information from the long term inhalation experiment are described fully in section 3.2.2. The persistence of short and intermediate length fibres (<15μm) over 12 months following inhalation appears to be similar for the 104E, 100/475 and amosite. The persistence of long fibres (>20μm) of 104E over 12 months in comparison to the lung burden after 12 months inhalation appears to be less than for the other three fibre types tested by inhalation. The 104E does not appear to undergo any significant change in composition during retention in rat lungs over a 12 month period (section 3.2.2).

4.3.3 Histopathology

At completion of the twelve months of inhalation exposure to 104E microfibre, four rats were

killed to provide lungs for evaluation of the development of fibrosis. The rat lungs already showed considerable pathological change, most of which was centred around the terminal and respiratory bronchioles. In these areas there was a marked macrophage reaction and the walls of adjacent alveoli were thickened, mainly by the rounding of alveolar epithelial cells, but also with the production of some new connective tissue fibres. Fibrosis was particularly marked at the bifurcations of the small airways themselves where small nodular lesions developed. These lesions were classified as Wagner grade 4, but showed little extension to include the surrounding alveoli. Two of the animals showed single small patches where alveolar wall fibrosis had spread away from the terminal bronchioles. Area estimates of these small lesions of the four animals from the twelve month kill showed a mean area of only 0.3% of the lung parenchyma (data not shown). Comparison with animals which survived until later in the study indicated that this more advanced fibrosis became progressively more widespread with time.

By two years after the start of treatment (12 months inhalation + 12 months recovery), significant areas of advanced alveolar fibrosis and bronchoalveolar hyperplasia (BAH) had developed (Table 4.5). In those nine animals that survived until within two months of the termination of the study, the mean area of these lesions was 8.0% of lung parenchyma. This value was similar to that for amosite but much higher than for glass microfibre Code 100/475. Occasional areas of fibrosis/BAH do occur in old control animals but the mean area of fibrosis for fifteen control animals was only 0.08%. (Table 4.5).

Table 4.5
Summary of pathological findings from inhalation studies: minimum, maximum and mean level of advanced fibrosis (% of lung area)

Fibre Type	Animals For Fibrosis	Level of advanced fibrosis (% of lung area)		
	No.	Min	Mean	Max
104E	9	1.1	8.0	12.5
100/475	11	0.0	0.2	0.7
Amosite	9	3.5	7.6	14.8
Controls	15	0.0	0.08	0.6

4.3.4 Tumour development

Table 4.6 shows the numbers of rats exposed to the different fibre types by inhalation developing pulmonary tumours. Among the forty three long-term survivors exposed to 104E, seven rats had carcinomas and three had benign adenomas. One of the two rats with mesotheliomas also had a carcinoma. The percentages of animals with carcinoma and mesothelioma were not significantly different to those obtained for amosite asbestos. In comparison, none of the animals exposed to 100/475 had carcinoma or mesothelioma, the difference in carcinoma incidence rate being statistically significant using Fisher's exact test ($p=0.02$). Two of 38 control rats of similar ages to the treated rats developed pulmonary tumours, one adenoma and one adenocarcinoma. There was a statistically significant higher tumour incidence rate among rats exposed to 104E compared to control rats ($p=0.026$).

Table 4.6
Summary of pathological findings from inhalation studies: numbers of animals with tumours (percentage in italics)

Fibre Type	Animals for pathology	Carcinoma		Adenoma		Mesothelioma	
	No.	No.	%	No.	%	No.	%
104E	43	7	*16.3*	3	*7.0*	2	*4.7*
100/475	38	0	*0.0*	4	*10.5*	0	*0.0*
Amosite	42	7	*16.7*	9	*21.4*	2	*4.8*
Controls	38	1	*2.6*	1	*2.6*	0	*0.0*

4.4 LIFE TIME INTRAPERITONEAL INJECTION EXPERIMENT

In the intraperitoneal study with injection of 10^9 WHO fibres (as counted by PCOM), survival time and the incidence of peritoneal tumours were recorded. Figure 4.2 shows estimated Kaplan-Meier survival functions for deaths from mesothelioma (adjusted for depletion by natural mortality) against time since injection for all three fibre types. It is clear that tumour-associated death occurred more quickly in the 104E group than in the other groups. At a survival probability of 50%, the survival functions for the three fibres are separated by about 100 days, with median survival becoming progressively shorter from 100/475 (median survival of 642 days) to amosite to 104E. Almost all of the rats (21 out of 24) in the 104E and amosite groups had mesothelioma compared to only 8 of 24 rats in the 100/475 group.

4.5 COMPARISON OF E GLASS WITH OTHER FIBRE TYPES TESTED

Both the microfibres tested (104E and 100/475) have borosilicate glass compositions intended for special purposes. The two microfibres contain similar proportions by mass of silica and boron, but E glass has a higher aluminium content, much higher calcium content and lower sodium content than 100/475 (Table 2.2).

In terms of durability, the *in vitro* rate of dissolution of the two microfibres was similar and not significantly different from that of the RCFs or MMVF21 (stonewool). Both microfibres dissolved much more slowly than the traditional glasswool formulations represented by MMVFs 10 and 11. The microfibres would be expected to dissolve more rapidly than amphibole asbestos types, including amosite.

In terms of fibre size distribution both the microfibres (104E, 100/475) have much thinner mean diameters and shorter mean lengths than the MMVFs and RCFs (Table 2.3). This reflects the high proportion of short fibres (<5μm) and relatively small proportion of long fibres (>20μm) within these two fibre samples. The fibre size distributions of both microfibres were, however, fairly well matched with each other and with that of the amosite asbestos (Table 4.7). The 104E contained a smaller proportion of short fibres than the 100/475 and a slightly greater proportion of long fibres (Table 4.7). It also contained a slightly smaller proportion of very thin fibres in the 0.1 and 0.2μm diameter categories than the 100/475.

Figure 4.2: Survival functions for mesothelioma, adjusted for deaths from other causes, in the intraperitoneal injection study.

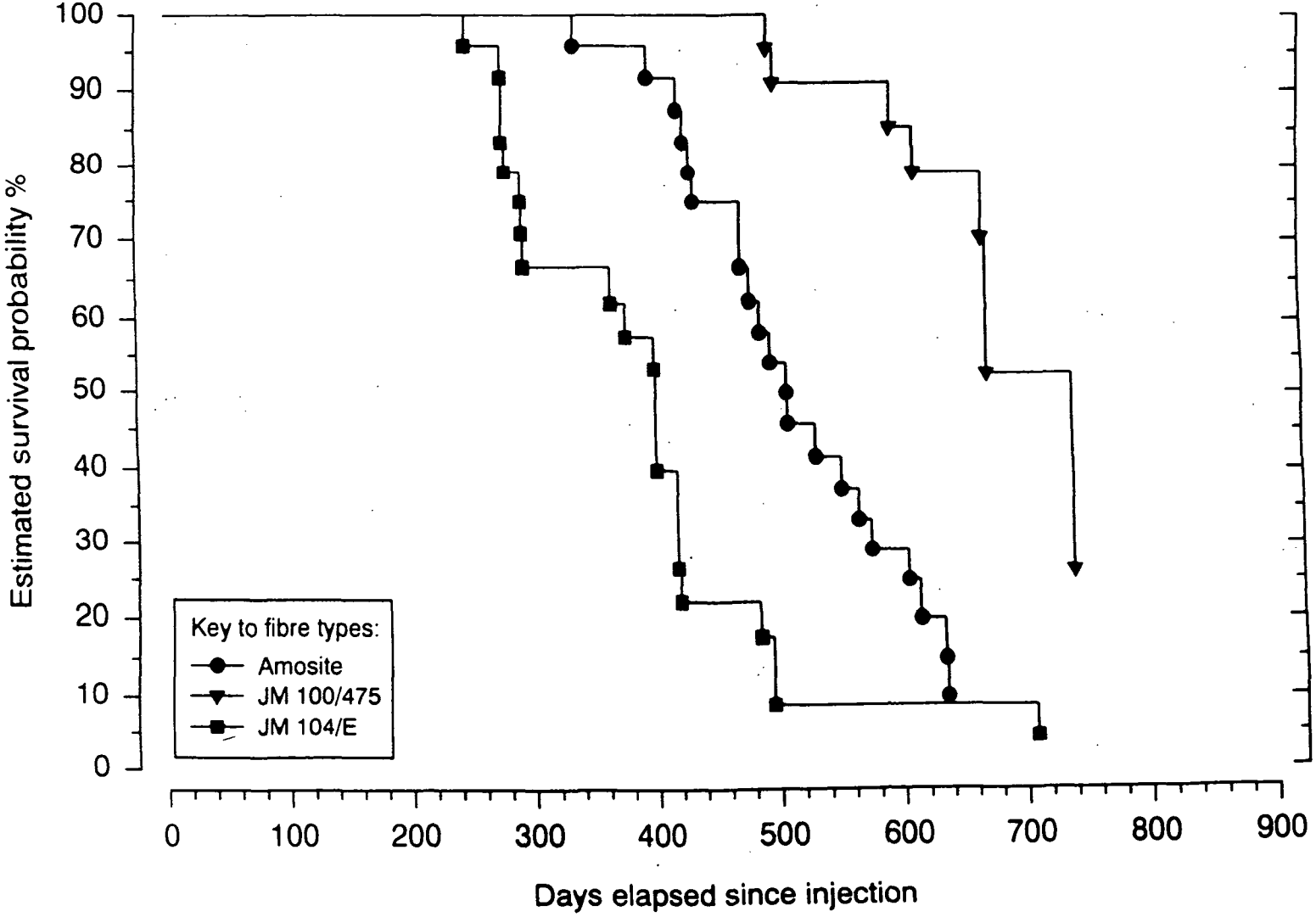

Table 4.7
Summary of size characteristics and comparative doses of fibre samples used in the intratracheal and intraperitoneal injection experiments

Fibre type		100/475	104E	amosite
%diameter distribution	0.1 μm	18.6	13.8	17.8
	0.2 μm	52.0	45.6	33.6
	0.3 μm	17.6	21.0	23.0
	0.4-0.7 μm	10.4	19.2	28.2
	0.8-1.5 μm	1.2	0.6	1.6
	>1.5 μm	0.0	0.0	0.2
%length distribution	<1.0 μm	8.2	7.0	13.0
	1-1.4 μm	11.6	12.0	17.8
	1.5-5 μm	60.4	47.6	44.8
	5-10 μm	13.8	21.6	14.4
	10-15 μm	3.2	6.6	5.0
	15-20 μm	1.2	2.8	3.2
	>20 μm	1.6	2.6	6.4
total number of injected fibres	intraperitoneal	11046×10^6	12292×10^6	1801×10^6
	intratracheal	1331×10^6	976×10^6	295×10^6
Number of WHO fibres injected	intraperitoneal	1880×10^6	3678×10^6	410×10^6
	intratracheal	315×10^6	284×10^6	65×10^6
Number of long (>20μm) fibres	intraperitoneal	99×10^6	209×10^6	71×10^6
	intratracheal	12×10^6	16×10^6	12×10^6
mass of injected fibres	intraperitoneal	8.3mg	12.6mg	6.1mg
	intratracheal	1mg	1mg	1mg

The slight differences in size distribution between the two microfibres are reflected in slight differences in the injected dose used for each fibre type in the injection experiments. In the intraperitoneal carcinogenicity experiment, the total number of injected fibres was similar for both microfibres, although the mass dose of the 104E was greater than that of 100/475. The numbers of WHO and long (>20μm) fibres in the injected dose of 104E were, however, double those for the 100/475. Long fibres, however, only constituted a very small part of the total injected dose for both microfibres. The total number of amosite fibres in the injected dose was only about 20% of that in the microfibres. In the intratracheal biopersistence experiment, the total number of injected fibres of 104E was less than for 100/475 but much greater than for the amosite. The number of WHO fibres and long fibres injected was similar for both microfibres.

The biopersistence of long fibres of both microfibres in the injection experiment was similar (Table 3.4). It was less than that of long fibres of amosite, silicon carbide and the RCFs but significantly greater than that of long fibres of MMVF10 (glasswool) and MMVF22 (slagwool). Although there were no statistically significant differences in the clearance of the two fibre types, the data suggest that 104E fibres in the 5-10μm length category cleared more slowly than those of 100/475 (Table 3.4).

In contrast to the findings from the intratracheal injection experiment, the biopersistence of long fibres of 104E in the inhalation experiment appears to have been less than that of long

fibres of amosite, 100/475 or silicon carbide. The biopersistence of short and intermediate length fibres of the two microfibres was similar in the inhalation experiment. Analysis of the composition of fibres recovered from lung tissue from the inhalation experiment suggests that the prolonged retention of 104E within lung tissue did not lead to any substantial change in composition. In contrast fibres of 100/475 had undergone considerable modification of composition with the loss of sodium, potassium, calcium and barium (Table 3.7).

In the short term *in vitro* assays, the 104E appeared to have a similar activity to the 100/475 and both microfibres had activities intermediate between amosite asbestos and most of the other man-made vitreous fibre types tested. In the short term inhalation experiment, exposure to 104E gave rise to a smaller proliferative response than seen with amosite or silicon carbide, but much greater than that seen with 100/475.

In the two long term carcinogenicity assays, the 104E appeared to have a similar or greater carcinogenic potential to that of amosite and a considerably greater carcinogenic potential than 100/475.

5. PREDICTIVE MODELS OF FIBRE CARCINOGENICITY

5.1 INTRAPERITONEAL INJECTION STUDY

5.1.1 Strategy

The intraperitoneal injection experiment with nine fibre types is described by Miller *et al* (1999a) and the results for the tenth fibre, 104E, are described in Chapter 4 above. Miller *et al.* (1999a) used standard multiple linear regression techniques to investigate whether mortality, as characterised by the median survival time, could be related to fibre characteristics. The data now available differ in two ways: they include improved estimates of *in vitro* dissolution rates and (for some fibres) of biopersistence in the lung following intratracheal injection; and data are now available for the glass microfibre 104E. The new estimates of biopersistence are not substantially different from those used in the analysis reported by Miller *et al.* (1999a and b; see chapter 3). The new estimates of *in vitro* dissolution rate were also not substantially different from those used earlier, although the relative ranking of the fibre types was slightly different. It is of prime interest to know to what extent the carcinogenicity of a fibre can be predicted from the available measurements. We first report analyses, similar to those of Miller *et al.* (1999a), with the improved data, but excluding 104E. For comparison, we then report analyses that included 104E.

As before, since the rankings of all-cause survival and mesothelioma-specific survival were very similar, the former was taken as the response variable, so that the comparisons could include RCF 4 (where no mesotheliomas were produced). The linear regression facilities of Genstat (Genstat 5 Committee, 1993) were used to compute, for each of the potential predictor variables, the contribution that it would make to the regression model if added on its own. One aim of the regression modelling was to explain as much as possible of the variation in the response. The amount of variation left unexplained by the chosen predictor variables is quantified by the residual mean square.

The main sources of uncertainty with respect to the conclusions of the statistical analysis arise from the relatively small number of fibre types used in the model and the lack of multiple doses for individual fibre type. Given that the experiment had been specifically designed to compare the effects of fibres at nominally identical doses in terms of fibre numbers, it had limited power to determine the specific effects of fibre number on disease outcome. Although there are substantial uncertainties associated with the estimation of the some of the predictor variables including biopersistence and dissolution rates, these are not thought to significantly affect the outcome of the statistical analysis.

5.1.2 Input variables used in regression modelling

Table 5.1 summarises the mortality experience in each of the experimental groups, including the data for 104E. The analyses sought to relate mortality experience to the numbers of fibres injected, in various size classes, which are shown along with the injected mass dose in Table 5.2; and to the revised estimates of dissolution and persistence in the lung, which are shown in Table 5.3. For the very poorly soluble silicon carbide and amosite fibres, the value substituted was not measured, but adopted to represent very low dissolution.

Table 5.1
Summary of mortality experience for each fibre type

Fibre label	Animals in group	Number with mesothelioma	%	Median all-cause survival (days)	*Estimated standard error*	Median mesothelioma survival (days)	*Estimated standard error*
100/475	24	8	*33*	642	*	679	*24*
104E	24	21	*88*	359	*	359	*
SiC 1	24	22	*92*	250	*45*	257	*52*
Amosite	24	21	*88*	509	*27*	509	*27*
MMVF 10	22	13	*59*	643	*87*	676	*43*
MMVF 21	20	19	*95*	281	*	284	*
MMVF 22	24	13	*54*	658	*	695	*
RCF 1	24	21	*88*	337	*17*	337	*17*
RCF 2	18	13	*72*	376	*25*	391	*25*
RCF 4	22	0	*0*	725	*	†	†

**Sparse data – no reliable estimate*
† No deaths – function not defined

Table 5.2
Distribution injected fibre dose characterised by mass and by numbers of fibres ($\times 10^6$) in two diameter classes and six cumulative length classes

Fibre label	Mass dose (mg)	Diam. Class (µm)	Length class (µm)					
			> 0.4	> 5	> 8	> 10	> 15	> 20
100/475	8.3	< 0.95	11034	1868	680	421	186	99
		> 0.95	12	12	12	12	0	0
104E	12.6	< 0.95	12292	3678	1853	1203	470	207
		> 0.95	0	0	0	0	0	0
SiC 1	14.2	< 0.95	821	577	387	307	185	121
		> 0.95	4	3	3	3	3	1
Amosite	6.1	< 0.95	1791	402	225	164	103	63
		> 0.95	10	8	8	8	8	8
MMVF 10	144.4	< 0.95	376	314	264	236	155	119
		> 0.95	665	659	598	567	506	436
MMVF 21	183.1	< 0.95	1349	1012	744	628	439	344
		> 0.95	701	644	558	514	411	335
MMVF 22	129.6	< 0.95	898	671	492	402	263	142
		> 0.95	570	544	466	388	291	207
RCF 1	110.9	< 0.95	713	394	280	228	129	85
		> 0.95	399	374	302	260	194	140
RCF 2	188.8	< 0.95	958	619	392	320	201	111
		> 0.95	565	550	480	455	340	231
RCF 4	90.4	< 0.95	648	264	134	81	15	6
		> 0.95	548	466	311	230	111	36

Table 5.3
Measures of fibre durability: *in vitro* dissolution from continuous flow through experiments; and estimated persistence in the lung over twelve months after intratracheal injection, in selected cumulative length classes

Fibre label	k_{dis} (ng cm^{-2} hr^{-1})	Adjusted k_{dis} (ng cm^{-2} hr^{-1})	Persistence % of injected fibres at 12 months					
			Length class (µm)					
			> 0.4	> 5	> 8	> 10	> 15	> 20
100/475	21.8	1.118	20.7	30.0	29.4	25.9	19.7	24.1
104E	9.5	0.326	50.2	61.8	53.4	45.5	31.8	35.5
SiC 1	0.2*	0.002*	52.6	53.7	47.7	49.2	54.5	59.2
Amosite	0.2*	0.002*	16.9	28.8	38.7	44.2	64.5	81.0
MMVF 10	134.8	1.442	14.6	8.8	5.7	4.7	2.2	0.5
MMVF 21	13.9	0.192	29.5	30.5	30.6	30.2	32.3	35.9
MMVF 22	72.7	0.829	16.6	10.6	6.1	3.8	2.2	1.7
RCF 1	9.8	0.143	40.3	42.8	44.9	46.6	49.6	50.5
RCF 2	10.2	0.114	59.1	70.8	83.9	99.9	130.0	157.3
RCF 4	0.9	0.010	72.2	81.2	95.7	108.4	113.1	142.7

**Substituted values*

5.1.3 Results excluding 104E

In general, the results from the revised data set were very similar to those reported by Miller *et al.* (1999a). Table 5.4 shows, in the section labelled Step 1, the residual mean squares achieved by models containing each of the potential predictors alone. Generally, smaller residual mean squares were achieved after logarithms were taken of the fibre numbers and of solubility, and all the results shown here use the logarithms of all the predictor variables. Zero counts of longer thicker fibres for 100/475 microfibre were replaced by the small value 0.2 before logs were taken.

Table 5.4

Results of three steps of regression modelling. Response variable is median lifetime from IP injection study. All predictors are on logarithmic scale. Glass microfibre 104E not included in analysis

Step 1		Step 2		Step 3	
Candidate model change	**Residual mean square**	**Candidate model change**	**Residual mean square**	**Candidate model change**	**Residual mean square**
Add Thinner Fibres (>20 μm)	26948	Add Biopersistence (>15 μm)	9161	Add Thinner Fibres (> 0.4 μm)	4718
Add Thinner Fibres (>15 μm)	28402	Add Biopersistence (>5 μm)	9163	Add Thinner Fibres (>5 μm)	6074
Add Biopersistence (>15 μm)	31037	Add Biopersistence (>10 μm)	9471	Add Thinner Fibres (>15 μm)	7634
Add Thinner Fibres (>10 μm)	31097	Add Biopersistence (>8 μm)	10001	Add Thinner Fibres (>8 μm)	7778
Add Biopersistence (>20 μm)	31535	Add Biopersistence (> 0.4 μm)	10742	Add Biopersistence (> 0.4 μm)	7848
Add Biopersistence (>10 μm)	32694	Add Biopersistence (>20 μm)	11808	Add Dissolution K_{dis} (adjusted)	8692
Add Biopersistence (>5 μm)	32851	Add Dissolution K_{dis} (adjusted)	15287	Add Thinner Fibres (>10 μm)	8853
Add Biopersistence (>8 μm)	33135	Add Dissolution K_{dis}	15966	*No change*	9161
Add Biopersistence (> 0.4 μm)	33219	*No change*	26948	Add Biopersistence (>20 μm)	9488
No change	33499	Add Thinner Fibres (>15 μm)	28984	Add Dissolution K_{dis}	9725
Add Thinner Fibres (>8 μm)	33632	Add Thicker Fibres (>10 μm)	29687	Add Biopersistence (>5 μm)	10301
Add Dissolution K_{dis} (adjusted)	34115	Add Thicker Fibres (>8 μm)	29723	Add Thicker Fibres (>15 μm)	10462
Add Dissolution K_{dis}	34585	Add Thinner Fibres (> 0.4 μm)	29834	Add Thicker Fibres (>20 μm)	10743
Add Thicker Fibres (>5 μm)	36827	Add Thicker Fibres (>5 μm)	29908	Add Biopersistence (>8 μm)	10931
Add Thicker Fibres (>8 μm)	36969	Add Thinner Fibres (>8 μm)	29989	Add Thicker Fibres (> 0.4 μm)	10935
Add Thicker Fibres (> 0.4 μm)	37063	Add Thinner Fibres (>5 μm)	30073	Add Mass Dose	10935
Add Thicker Fibres (>10 μm)	37177	Add Thinner Fibres (>10 μm)	30121	Add Thicker Fibres (>5 μm)	10978
Add Thinner Fibres (>5 μm)	37259	Add Thicker Fibres (> 0.4 μm)	30263	Add Thicker Fibres (>8 μm)	10986
Add Mass Dose	37552	Add Thicker Fibres (>20 μm)	30805	Add Thicker Fibres (>10 μm)	10991
Add Thinner Fibres (> 0.4 μm)	37871	Add Thicker Fibres (>15 μm)	31218	Add Biopersistence (>10 μm)	10992
Add Thicker Fibres (>15 μm)	38272	Add Mass Dose	31430	Drop Biopersistence (>15 μm)	26948
Add Thicker Fibres (>20 μm)	38283	Drop Thinner Fibres (>20 μm)	33499	Drop Thinner Fibres (>20 μm)	31037
Chosen action:		**Chosen action:**		**Chosen action:**	
Add Thinner Fibres (>20 μm)		Add Biopersistence (>15 μm)		???	

The smallest residual mean square was obtained with the variable representing the log of the number of fibres in the longest (> 20 μm) and thinner (< 0.95 μm) size category, explaining almost 20% of the variance of the median survival times. At this step, the closest alternative choices were variables quantifying either the numbers or the biopersistence of fibres in the longer cumulative length classes. Neither the injected dose expressed as a mass nor the dissolution rate coefficients were useful predictors at this stage.

At step 2, with the number of the longest thinner fibres in the regression model, the best additional predictor was the biopersistence of fibres of length >15 μm, bringing the explained variance to 73% of the total. The next closest candidates were all biopersistence ratios, and only they and the dissolution rates gave potential improvements to the model.

At step 3, the candidates for further model improvement were all variables representing numbers of fibres, and the best was the total number of thinner fibres regardless of length, bringing the variance explained to 86% of the total.

The models fitted after Steps 2 and 3 are shown in Table 5.5. With each of the estimated regression coefficients is given, in italics, the ratio of the estimate to its estimated standard error. Since each of the terms is associated with a single degree of freedom, this ratio can be interpreted as a two-tailed t-statistic testing the significance of including that term in the presence of (i.e. adjusted for) all the other terms in the model. Note that with the small numbers of residual degrees of freedom (d.f.), critical values of t-statistics (shown below the table) are larger than when d.f. are plentiful, and our Step 3 model has only 5 d.f. for the residual.

Comparison of the models from Steps 2 and 3 shows that the Step 2 model predicts that survival decreases with increasing numbers of longer fibres and with increasing biopersistence, and these trends are plausible. The Step 3 model is more problematic, in that the sign of the third variable is in the opposite direction, implying increasing survival with increasing fibre numbers over 10 μm in length. This seems an implausible conclusion and although, the addition of this variable was apparently statistically significant at 5%, its addition reduced the residual degrees of freedom from 6 to 5, and with a data set this size over-fitting is a real danger. In addition, there is the problem of multiple testing: where a number of variables are offered as predictors, the overall probability that one or more will appear significant at the 5% level is much greater than 5%. It is arguable that, in order to protect against declaring too many variables significant, we need to apply a more stringent significance level, e.g. 1% or smaller, to the individual tests.

Table 5.5
Summary of results of regression analysis of median lifetime from IP study. Table contains estimated regression coefficients, and t-statistic in italics. Glass microfibre 104E not included in analysis.

Terms in regression model	Step 2		Step 3	
	Coefficient	t-statistic ratio	Coefficient	t-statistic ratio
Constant	1421.0	*15.15*	1040.0	*5.08*
Thinner fibres (> 20 μm)	-141.1	*4.21*	-155.3	*6.29*
Biopersistence (> 15 μm)	-91.6	*3.82*	-100.9	*5.74*
Thinner fibres (> 0.4 μm)			67.6	*2.58*
Residual sum of squares	54968		23592	
Degrees of freedom	6		5	
Residual mean square	9161		4718	
Critical values of two-tailed t-statistic at 5% significance level, for low degrees of freedom (d.f.):	d.f.	6		5
	t (crit)	2.45		2.57

Table 5.6 compares the observed median survival with the values predicted by the Step 2 and Step 3 models. While Step 3 appears to fit some of the data points (e.g. 100/475) better than the Step 2 model, there are other types (e.g. RCF 2) for which the fit is made worse. Noting that the largest gain in variance explained came at Step 2, and the implausibility of increased fibre dosage promoting longer survival, we prefer the two-variable model from Step 2 to the Step 3 model. However, the limited numbers of data points carry a warning against over-interpretation.

Table 5.6 also shows the performance of both the Step 2 and Step 3 models in predicting the survival of the new fibre 104E. The Step 2 model predicts a median survival time of 352 days, very close to the observed 359 days. The Step 3 model, by contrast, over-predicts survival by 139 days. This and the other discrepancies of prediction must be interpreted with an eye on the standard errors quoted. (Note that for all fibres except 104E, the standard error is for the mean value of the regression curve at that point, whereas the rather larger standard error for 104E is for an individual point prediction).

Table 5.6
Results of regression analysis of median lifetime from IP study. Actual median survival time and prediction from fitted models, with standard errors. 104 E not included in analysis.

Fibre label	Median survival (days)	After Step 2		After Step 3	
		Predicted median survival	Standard error	Predicted median survival	Standard error
100/475	642	500.0	32.7	654.2	64.3
104E	359	351.7	105.7	498.2	94.8
SiC (ACMC)	250	378.7	39.8	345.1	31.4
Amosite	509	454.8	37.2	481.5	28.7
MMVF 10	643	675.1	65.2	618.9	51.6
MMVF 21	281	279.0	58.0	269.0	41.8
MMVF 22	658	650.3	64.3	650.4	46.1
RCF 1	337	436.5	35.0	399.3	29.0
RCF 2	376	311.4	52.5	281.4	39.5
RCF 4	725	735.2	87.9	721.2	63.3

5.1.3 Results including 104E

Table 5.7 shows the model fitting sequence for a second analysis, which included 104E as a data point. The results at both Step 2 and Step 3 were broadly similar to those from the analysis excluding 104E, although in the new analysis the model improvements at Steps 1 and 2 were slightly better, while that at Step 3 failed to reach statistical significance. Table 5.8 shows the survival predicted by the Step 2 model for each of the fibres. While the prediction for 104E is close to observed, that for 100/475 is too small by 141 days, while those for RCF1 and SiC are too large by 100 and 130 days respectively.

Table 5.7
Results of three steps of regression modelling. Response variable is median lifetime from IP injection study. All predictors are on logarithmic scale. Glass microfibre 104E included in analysis.

Step 1		Step 2		Step 3	
Candidate model change	**Residual mean square**	**Candidate model change**	**Residual mean square**	**Candidate model change**	**Residual mean square**
Add Thinner Fibres (>20 μm)	23862	Add Biopersistence (>15 μm)	7859	Add Thinner Fibres (> 0.4 μm)	5630
Add Thinner Fibres (>15 μm)	24871	Add Biopersistence (>10 μm)	8439	Add Thinner Fibres (>5 μm)	6951
Add Thinner Fibres (>10 μm)	27501	Add Biopersistence (>8 μm)	9284	Add Biopersistence (> 0.4 μm)	7114
Add Biopersistence (>15 μm)	28883	Add Biopersistence (>5 μm)	9486	Add Dissolution K_{dis} (adjusted)	7283
Add Biopersistence (>20 μm)	29177	Add Biopersistence (>20 μm)	10130	Add Thinner Fibres (>15 μm)	7785
Add Biopersistence (>5 μm)	29291	Add Biopersistence (> 0.4 μm)	10498	*No change*	7859
Add Thinner Fibres (>8 μm)	29634	Add Dissolution K_{dis} (adjusted)	13808	Add Biopersistence (>20 μm)	7906
Add Biopersistence (> 0.4 μm)	29697	Add Dissolution K_{dis}	13831	Add Thinner Fibres (>8 μm)	8069
Add Biopersistence (>10 μm)	29856	*No change*	23862	Add Dissolution K_{dis}	8105
Add Biopersistence (>8 μm)	29958	Add Thicker Fibres (>10 μm)	25499	Add Thinner Fibres (>10 μm)	8474
No change: Null model	31526	Add Thicker Fibres (>8 μm)	25521	Add Thicker Fibres (>15 μm)	8776
Add Thicker Fibres (>5 μm)	32488	Add Thicker Fibres (>5 μm)	25660	Add Biopersistence (>5 μm)	8934
Add Dissolution K_{dis}	32554	Add Thicker Fibres (> 0.4 μm)	25952	Add Thicker Fibres (>20 μm)	8971
Add Thicker Fibres (>8 μm)	32635	Add Thicker Fibres (>20 μm)	26423	Add Mass Dose	9113
Add Thinner Fibres (>5 μm)	32690	Add Thicker Fibres (>15 μm)	26837	Add Thicker Fibres (> 0.4 μm)	9115
Add Thicker Fibres (> 0.4 μm)	32699	Add Thinner Fibres (> 0.4 μm)	26933	Add Biopersistence (>8 μm)	9125
Add Dissolution K_{dis} (adjusted)	32799	Add Thinner Fibres (>15 μm)	26991	Add Thicker Fibres (>5 μm)	9148
Add Thicker Fibres (>10 μm)	32868	Add Thinner Fibres (>5 μm)	27150	Add Thicker Fibres (>8 μm)	9156
Add Thicker Fibres (>15 μm)	34863	Add Mass Dose	27168	Add Thicker Fibres (>10 μm)	9161
Add Thicker Fibres (> 20 μm)	35008	Add Thinner Fibres (>8 μm)	27230	Add Biopersistence (>10 μm)	9161
Add Thinner Fibres (> 0.4 μm)	35278	Add Thinner Fibres (>10 μm)	27254	Drop Biopersistence (>15 μm)	23862
Add Mass Dose	35410	Drop Thinner Fibres (>20 μm)	31526	Drop Thinner Fibres (>20 μm)	28883
Chosen action:		**Chosen action:**		**Chosen action:**	
Add Thinner Fibres (>20 μm)		Add Biopersistence (>15 μm)		???	

Table 5.8
Results of regression analysis of median lifetime from IP study.
Actual median survival time and prediction from fitted models, with standard errors.
Glass microfibre 104E included in analysis

Fibre label	Median survival (days)	After Step 2	
		Predicted median survival	Standard error
100/475	642	500.6	28.9
104E	359	353.0	37.5
SiC (ACMC)	250	379.8	33.9
Amosite	509	455.4	33.3
MMVF 10	643	675.4	60.2
MMVF 21	281	280.7	48.9
MMVF 22	658	650.7	59.3
RCF 1	337	437.4	30.6
RCF 2	376	312.7	45.8
RCF 4	725	734.4	80.7

5.2 LONG-TERM INHALATION STUDY

5.2.1 Strategy

The objectives and strategy of the analysis of the lung cancer incidence were similar to those for the survival data from the injection study, both excluding and including fibre 104E. For the inhalation study, the response was the incidence of lung cancers, which was treated as a binomial response variable, requiring analysis by logistic regression methods (Collett, 1991). Airborne fibre concentrations were summarised using the same size categories as for the fibres injected intraperitoneally.

Miller *et al.* (1999b) reported on previous analyses of data from these experiments. The data available for the present analysis differed in that the biopersistence and dissolution data were new estimates, and the results for microfibre 104E were now available. In addition, a transcription error occurred in Miller *et al.* (1999b): for the amosite, the observed incidence of lung cancers had been misreported as 9 instead of 7, and the incorrect value 9 had been used in all the analyses. (A set of analyses, not reported here, using the corrected response and the older estimates for biopersistence and dissolution showed the published conclusions to be still valid.) All analyses reported here used the corrected value.

The main sources of uncertainty with respect to the conclusions of the statistical analysis arise from the relatively small number of fibre types used in the model and some aspects of experimental design. The IOM inhalation experiment had been designed so that animals were exposed to the same concentration of each of four fibre types and the dust clouds were well matched in terms of fibre size distribution. Although the RCC experiments incorporated multiple doses of some fibres, the dust clouds were again matched in terms of fibre size distribution. The model, therefore, has limited power to determine the specific effects of fibre size distribution on disease outcome. Differences in the exposure regimes used at IOM and RCC add further uncertainty to the analysis in terms of selection of appropriate input variables. Although the period of exposure was shorter in the IOM experiments compared with that used in the RCC experiments, the cumulative exposures were actually greater in the

IOM experiments than in the RCC experiments. Overall, however, uncertainties in the input data to the model are not thought to be substantial enough to significantly affect the model outcome. A recent investigation of the comparability of fibre measurements made at IOM and RCC suggests that differences between the two organisations should not have been a substantial source of error (Jones *et al.*, 1999).

5.2.2 Input variables used in regression modelling

Table 5.9 shows the complete set of results for tumour production in the IOM and RCC inhalation experiments. In the analyses, these were related to estimates of airborne fibre concentrations in selected size classes as summarised in Table 5.10 and the estimates of dissolution and biopersistence shown in Table 5.3.

Table 5.9:
Pathology results from IOM and TIMA studies

Fibre label	Mass conc (mg m^{-3})	Animals	Lung cancers	% Lung cancers	All lung tumours	% lung tumours	Mesothel-iomas	% Mesos
100/475	5.8	38	0	0	4	11	0	0
104E	4.6	43	7	16	10	23	2	5
SiC 1	11.4	42	5	12	10	24	10	24
Amosite	5.5	42	7	17	16	38	2	5
MMVF 10	*3*	*119*	*0*	*0*	*0*	*0*	*0*	*0*
MMVF 10	*16*	*121*	*0*	*0*	*1*	*1*	*0*	*0*
MMVF 10	*30*	*121*	*1*	*1*	*7*	*6*	*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*	*1*	*1*	*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*	*2*
RCF 4	*30*	*118*	*2*	*2*	*4*	*3*	*1*	*1*

Italics indicate RCC data from TIMA study

Table 5.10
Duration-weighted estimated exposure concentrations for IOM and TIMA/RCC inhalation experiments (fibre hr^{-1} $litre^{-1}$).

Fibre label	Mass conc. (mg m^{-3})	Diam. Class (µm)	Length class (µm)					
			> 0.4	> 5	> 8	> 10	> 15	> 20
100/475	5.8	< 0.95	6580	1729	811	533	138	51
		> 0.95	46	46	36	34	21	12
104E	4.6	< 0.95	3922	1622	983	666	244	120
		> 0.95	0	0	0	0	0	0
SiC 1	11.4	< 0.95	3268	1581	786	529	237	89
		> 0.95	26	26	16	16	3	0
Amosite	5.5	< 0.95	6311	1551	815	530	204	127
		> 0.95	102	83	78	77	36	21
MMVF 10	*3*	*< 0.95*	*27*	*22*	*16*	*13*	*8*	*5*
		> 0.95	*73*	*69*	*59*	*51*	*34*	*24*
MMVF 10	*16*	*< 0.95*	*136*	*110*	*81*	*63*	*38*	*24*
		> 0.95	*366*	*347*	*297*	*253*	*168*	*120*
MMVF 10	*30*	*< 0.95*	*218*	*176*	*129*	*101*	*61*	*38*
		> 0.95	*586*	*556*	*475*	*404*	*269*	*192*
MMVF 21	*3*	*< 0.95*	*54*	*44*	*36*	*32*	*23*	*18*
		> 0.95	*65*	*63*	*53*	*49*	*39*	*33*
MMVF 21	*16*	*< 0.95*	*238*	*195*	*160*	*141*	*102*	*77*
		> 0.95	*286*	*276*	*236*	*216*	*172*	*145*
MMVF 21	*30*	*< 0.95*	*385*	*316*	*259*	*228*	*164*	*125*
		> 0.95	*463*	*447*	*382*	*350*	*279*	*236*
MMVF 22	*3*	*< 0.95*	*58*	*47*	*38*	*32*	*23*	*17*
		> 0.95	*49*	*47*	*42*	*39*	*31*	*25*
MMVF 22	*16*	*< 0.95*	*254*	*205*	*164*	*141*	*102*	*73*
		> 0.95	*214*	*204*	*184*	*172*	*136*	*110*
MMVF 22	*30*	*< 0.95*	*414*	*334*	*266*	*229*	*165*	*119*
		> 0.95	*348*	*332*	*299*	*280*	*222*	*178*
RCF 1	*3*	*< 0.95*	*53*	*37*	*25*	*23*	*15*	*11*
		> 0.95	*47*	*46*	*40*	*39*	*32*	*27*
RCF 1	*9*	*< 0.95*	*153*	*106*	*73*	*65*	*43*	*31*
		> 0.95	*135*	*131*	*115*	*111*	*91*	*77*
RCF 1	*16*	*< 0.95*	*244*	*169*	*117*	*105*	*69*	*50*
		> 0.95	*216*	*210*	*184*	*178*	*146*	*122*
RCF 1	*30*	*< 0.95*	*398*	*296*	*222*	*186*	*135*	*106*
		> 0.95	*300*	*291*	*265*	*238*	*189*	*157*
RCF 2	*30*	*< 0.95*	*431*	*276*	*176*	*144*	*96*	*59*
		> 0.95	*434*	*425*	*369*	*337*	*290*	*235*
RCF 4	*30*	*< 0.95*	*198*	*58*	*22*	*9*	*2*	*0*
		> 0.95	*496*	*444*	*300*	*220*	*116*	*59*

Italics indicate JMTC data from TIMA study

5.2.3 Results excluding 104E

Table 5.11 shows, in the section labelled Step 1, the residual mean deviances achieved by models containing each of the potential predictors alone, for the fibres excluding 104E. Here and at later steps, a slightly better fit was obtained with all of the predictor variables on the logarithmic scale. The smallest residual mean deviances were obtained with variables representing dissolution rates, and the adjusted K_{dis} 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, and than those for biopersistence in the lung. None of the exposure variables for the numbers of thicker fibres were useful predictors. At the end of this step, adjusted K_{dis} was added to the model and the search begun for additional useful predictors.

Table 5.11

Results of three steps of logistic regression modelling. Response variable is whole-study lung cancer incidence in long-term inhalation study. All predictors are on logarithmic scale. Glass microfibre 104E not included in analysis.

Step 1		Step 2		Step 3	
Candidate model change	**Residual mean deviance**	**Candidate model change**	**Residual mean deviance**	**Candidate model change**	**Residual mean deviance**
Add Dissolution K_{dis} (adjusted)	1.703	Add Thinner Fibres (>20 μm)	1.093	Add Biopersistence (>10 μm)	0.998
Add Dissolution K_{dis}	1.739	Add Thinner Fibres (>15 μm)	1.168	Add Biopersistence (>8 μm)	0.999
Add Thinner Fibres (> 0.4 μm)	1.915	Add Thinner Fibres (>10 μm)	1.208	Add Thicker Fibres (>8 μm)	1.016
Add Thinner Fibres (>5 μm)	1.989	Add Thinner Fibres (>8 μm)	1.210	Add Thicker Fibres (>5 μm)	1.016
Add Thinner Fibres (>8 μm)	2.204	Add Thinner Fibres (>5 μm)	1.248	Add Biopersistence (>15 μm)	1.017
Add Thinner Fibres (>10 μm)	2.465	Add Thinner Fibres (> 0.4 μm)	1.349	Add Biopersistence (>5 μm)	1.018
Add Biopersistence (>20 μm)	2.839	*No change*	1.703	Add Thicker Fibres (>10 μm)	1.025
Add Biopersistence (>15 μm)	2.863	Add Thicker Fibres (>20 μm)	1.704	Add Thicker Fibres (> 0.4 μm)	1.026
Add Thinner Fibres (>15 μm)	2.923	Add Thicker Fibres (>10 μm)	1.751	Add Thicker Fibres (>20 μm)	1.030
Add Biopersistence (>10 μm)	3.052	Add Thicker Fibres (>15 μm)	1.753	Add Thicker Fibres (>15 μm)	1.035
Add Biopersistence (>8 μm)	3.107	Add Thicker Fibres (>8 μm)	1.779	Add Biopersistence (>20 μm)	1.046
Add Thinner Fibres (>20 μm)	3.116	Add Biopersistence (> 0.4 μm)	1.786	Add Biopersistence (> 0.4 μm)	1.072
Add Biopersistence (>5 μm)	3.236	Add Biopersistence (>20 μm)	1.795	*No change*	1.093
Add Biopersistence (> 0.4 μm)	3.700	Add Biopersistence (>15 μm)	1.797	Add Thinner Fibres (>10 μm)	1.114
Add Thicker Fibres (>20 μm)	3.733	Add Thicker Fibres (>5 μm)	1.802	Add Thinner Fibres (>15 μm)	1.126
Add Thicker Fibres (>15 μm)	3.798	Add Thicker Fibres (> 0.4 μm)	1.803	Add Thinner Fibres (>8 μm)	1.158
No change	3.830	Add Dissolution K_{dis}	1.806	Add Dissolution K_{dis}	1.159
Add Thicker Fibres (>8 μm)	4.009	Add Biopersistence (>5 μm)	1.814	Add Thinner Fibres (>5 μm)	1.169
Add Thicker Fibres (>5 μm)	4.021	Add Biopersistence (>10 μm)	1.814	Add Thinner Fibres (> 0.4 μm)	1.170
Add Thicker Fibres (>10 μm)	4.022	Add Biopersistence (>8 μm)	1.816	Drop Thinner Fibres (>20 μm)	1.703
Add Thicker Fibres (> 0.4 μm)	4.036	Drop Dissolution K_{dis} (adjusted)	3.830	Drop Dissolution K_{dis} (adjusted)	3.116
Chosen action:		**Chosen action:**		**Chosen action:**	
Add Dissolution K_{dis} (adjusted)		Add Thinner Fibres (>20 μm)		*No change*	

At Step 2, the best additional predictors, which were also highly statistically significant, were variables representing the numbers of the longer, thinner fibres (weighted for duration of exposure). 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_{dis} 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 close to 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. These conclusions were similar to those reported by Miller *et al.* (1999b).

The coefficients for the logistic regression model achieved at the end of Step 2 are shown in Table 5.12, 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 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 statistically significant contributions to the model, even if significance levels are adjusted to allow for multiple testing.

Table 5.12
Summary of results of logistic regression analysis of whole-study cancer incidence from long-term inhalation study, after two model-building steps. Glass microfibre 104E not included in analysis. Table contains estimated regression coefficients, and z-statistic in italics.

Terms in regression model	Coefficient	Standard error	z-statistic ratio
Constant	-7.07	0.79	
Dissolution K_{dis} (adjusted)	-0.4775	0. 0782	*-6.11*
Thinner fibres (> 20 μm)	0.5190	0.1910	*2.72*

This model predicts the risk r that an individual animal will develop lung cancer as a function of x_1, the natural log of the adjusted dissolution rate K_{dis}, and x_2, the natural log of the number of thinner fibres > 20 μm in length (exposure duration weighted), using the logistic function

$$r = [\, 1 + \exp(7.07 + 0.4775\, x_1 - 0.5190\, x_2)]^{-1}$$

Table 5.13 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. For 104E, the standard error is for the prediction for a new data point.

We note that the model under-predicts considerably for 104E. As before, the evidence for a relationship with airborne concentration within individual fibres is weak except from the highest level of RCF 1, and that the model under-predicts for this level and for RCF 2.

Table 5.13

Results of logistic regression analysis of whole-study cancer incidence from long-term inhalation study, after two model-building steps. Glass microfibre 104E not included in analysis. Observed cancers and prediction from fitted model, with standard error.

Fibre label	Mass concn. ($mg.m^{-3}$)	Animals	Observed with lung cancers	Predicted with lung cancers	Standard error of prediction
100/475	5.8	38	0	0.2	0.07
104E	4.6	43	7	0.7	0.72
SiC (ACMC)	11.4	42	5	6.1	1.49
Amosite	5.5	42	7	7.1	1.76
MMVF 10	*3*	*119*	*0*	*0.2*	*0.10*
MMVF 10	*16*	*121*	*0*	*0.4*	*0.26*
MMVF 10	*30*	*121*	*1*	*0.6*	*0.19*
MMVF 21	*3*	*114*	*1*	*0.9*	*0.28*
MMVF 21	*16*	*115*	*1*	*2.0*	*0.45*
MMVF 21	*30*	*114*	*1*	*2.6*	*0.68*
MMVF 22	*3*	*116*	*1*	*0.5*	*0.16*
MMVF 22	*16*	*115*	*0*	*1.0*	*0.30*
MMVF 22	*30*	*115*	*1*	*1.3*	*0.43*
RCF 1	*3*	*125*	*0*	*0.9*	*0.34*
RCF 1	*9*	*128*	*1*	*1.6*	*0.37*
RCF 1	*16*	*126*	*1*	*2.0*	*0.41*
RCF 1	*30*	*121*	*7*	*2.9*	*0.67*
RCF 2	*30*	*121*	*5*	*2.4*	*0.45*
RCF 4	*30*	*118*	*2*	*1.4*	*0.92*

5.2.4 Results including 104E

Table 5.14 shows the model-fitting results when the data set is augmented with 104E, and these are quite different from those without 104E. The best predictor was the number of thinner fibres, regardless of length, and at Step 2 the best addition was biopersistence of fibres >8 μm in length. However, the residual mean deviance after Step 2 was 40% larger than in Table 5.11, indicating a worse overall fit, and no further additions gave any worthwhile improvement.

Table 5.14
Results of three steps of logistic regression modelling. Response variable is whole-study lung cancer incidence in long-term inhalation study. All predictors are on logarithmic scale. Glass microfibre 104E included in analysis.

Step 1		Step 2		Step 3	
Candidate model change	**Residual mean deviance**	**Candidate model change**	**Residual mean deviance**	**Candidate model change**	**Residual mean deviance**
Add Thinner Fibres (> 0.4 μm)	1.919	Add Biopersistence (>8 μm)	1.443	Add Thinner Fibres (>20 μm)	1.316
Add Thinner Fibres (>5 μm)	1.953	Add Biopersistence (>5 μm)	1.443	Add Thinner Fibres (>8 μm)	1.379
Add Thinner Fibres (>8 μm)	2.142	Add Biopersistence (>10 μm)	1.445	Add Thinner Fibres (>15 μm)	1.382
Add Thinner Fibres (>10 μm)	2.430	Add Biopersistence (>15 μm)	1.453	Add Thinner Fibres (>5 μm)	1.407
Add Dissolution K_{dis}	2.777	Add Biopersistence (> 0.4 μm)	1.486	Add Thinner Fibres (>10 μm)	1.422
Add Dissolution K_{dis} (adjusted)	3.078	Add Biopersistence (>20 μm)	1.510	*No change*	1.443
Add Thinner Fibres (>15 μm)	3.101	Add Dissolution K_{dis}	1.695	Add Dissolution K_{dis} (adjusted)	1.462
Add Thinner Fibres (>20 μm)	3.504	Add Dissolution K_{dis} (adjusted)	1.709	Add Dissolution K_{dis}	1.486
Add Biopersistence (>20 μm)	3.662	*No change*	1.919	Add Biopersistence (> 0.4 μm)	1.508
Add Biopersistence (>5 μm)	3.679	Add Thinner Fibres (>20 μm)	1.975	Add Thicker Fibres (>5 μm)	1.515
Add Biopersistence (>8 μm)	3.696	Add Thicker Fibres (>5 μm)	1.995	Add Thicker Fibres (> 0.4 μm)	1.518
Add Thicker Fibres (>20 μm)	3.719	Add Thicker Fibres (> 0.4 μm)	2.000	Add Thicker Fibres (>8 μm)	1.524
Add Biopersistence (>15 μm)	3.736	Add Thicker Fibres (>15 μm)	2.000	Add Thicker Fibres (>15 μm)	1.524
Add Thicker Fibres (>15 μm)	3.746	Add Thicker Fibres (>10 μm)	2.002	Add Thicker Fibres (>10 μm)	1.525
Add Biopersistence (>10 μm)	3.750	Add Thicker Fibres (>8 μm)	2.003	Add Biopersistence (>15 μm)	1.528
Add Thicker Fibres (>8 μm)	3.978	Add Thicker Fibres (>20 μm)	2.016	Add Biopersistence (>5 μm)	1.529
Add Thicker Fibres (>5 μm)	3.983	Add Thinner Fibres (>15 μm)	2.018	Add Thicker Fibres (>20 μm)	1.533
Add Thicker Fibres (>10 μm)	4.008	Add Thinner Fibres (>5 μm)	2.020	Add Biopersistence (>10 μm)	1.538
Add Thicker Fibres (> 0.4 μm)	4.023	Add Thinner Fibres (>8 μm)	2.029	Add Biopersistence (>20 μm)	1.539
Add Biopersistence (> 0.4 μm)	4.263	Add Thinner Fibres (>10 μm)	2.037	Drop Biopersistence (>8 μm)	1.919
No change: Null model	4.640	Drop Thinner Fibres (> 0.4 μm)	4.640	Drop Thinner Fibres (> 0.4 μm)	3.696
Chosen action:		**Chosen action:**		**Chosen action:**	
Add Thinner Fibres (> 0.4 μm)		Add Biopersistence (>15 μm)		*No change*	

Table 5.15 shows the predicted values for each of the fibres from the model after Step 2. Although the prediction for RCF 2 appears improved, the model under-predicts for 104E and now over-predicts for 100/475.

Table 5.15
Results of logistic regression analysis of whole-study cancer incidence from long-term inhalation study, after two model-building steps. Glass microfibre 104E included in analysis. Observed cancers and prediction from fitted model, with standard error.

Fibre label	Mass concn. ($mg.m^{-3}$)	Animals	Observed with lung cancers	Predicted with lung cancers	Standard error of prediction
100/475	5.8	38	0	4.3	1.05
104E	4.6	43	7	5.2	4.69
SiC (ACMC)	11.4	42	5	4.2	0.80
Amosite	5.5	42	7	5.5	1.23
MMVF 10	*3*	*119*	*0*	*0.1*	*0.07*
MMVF 10	*16*	*121*	*0*	*0.3*	*0.19*
MMVF 10	*30*	*121*	*1*	*0.4*	*0.25*
MMVF 21	*3*	*114*	*1*	*0.5*	*0.19*
MMVF 21	*16*	*115*	*1*	*1.5*	*0.35*
MMVF 21	*30*	*114*	*1*	*2.1*	*0.42*
MMVF 22	*3*	*116*	*1*	*0.2*	*0.11*
MMVF 22	*16*	*115*	*0*	*0.5*	*0.27*
MMVF 22	*30*	*115*	*1*	*0.7*	*0.37*
RCF 1	*3*	*125*	*0*	*0.8*	*0.27*
RCF 1	*9*	*128*	*1*	*1.6*	*0.41*
RCF 1	*16*	*126*	*1*	*2.2*	*0.47*
RCF 1	*30*	*121*	*7*	*3.0*	*0.54*
RCF 2	*30*	*121*	*5*	*4.9*	*1.20*
RCF 4	*30*	*118*	*2*	*3.1*	*0.96*

6. DISCUSSION

6.1 BIOPERSISTENCE

6.1.1 Biopersistence in injection experiments

Precision associated with biopersistence estimates

There has been much interest in the reliable assessment of fibre biopersistence as part of the regulatory process of hazard classification. The confidence limits associated with our estimates of clearance halftimes reflect a relatively low level of precision, but are, however, comparable with those cited by other authors such as Muhle and Bellmann (1995). This imprecision largely reflects the effects of interanimal differences in retained lung burden which is a feature of both injection and inhalation experiments (Searl *et al*, 1999).

The reduced size of the confidence limits associated with the persistence estimates for most of the fibre types following additional SEM analysis reflects the increased precision of our estimates of individual lung fibre burdens. The relatively small overall improvement in precision, however, reflects the overriding importance of interanimal differences as a source of variability associated with the determination of mean lung fibre burdens at each time point. The limited effect on our persistence estimates that arose from adding the new data to our old data suggests that there may be little value in counting as many fibres as advocated by the EU protocol. The ideal number may lie between our original totals of 100 fibres per lung and the EU stopping rules of 400 WHO fibres or 1000 fibres of all sizes. The biopersistence of long fibres is thought to be of most relevance to health, but as only a small proportion of the fibres counted had lengths greater than 20μm, there was only a small improvement in the information for these fibres.

The precision in our estimates of biopersistence would have been better had we had greater numbers of animals in each experimental group. The number of animals available for lung burden analysis was, however, determined by the original experimental design dating from the early 1990s. At that time four animals per treatment group was deemed to be reasonable. The number of animals recommended in the ECB protocols for the assessment of biopersistence is five, only slightly greater than the groups of four that we used.

Clearance processes

The observed differences in biopersistence between long and short fibres, and the relationship of the persistence of long fibres to their dissolution characteristics, are consistent with current understanding of fibre clearance processes. The clearance of short fibres is believed to be primarily by macrophages, and that of long fibres by dissolution and disintegration (Morgan *et al.*, 1982; Davis, 1994; Morris *et al.*, 1995; Eastes and Hadley, 1994; Bernstein *et al.*, 1996).

The efficiency of macrophage clearance is related both to lung burden and particle toxicity. As lung burden increases beyond a certain threshold, the rate of macrophage clearance is progressively reduced (Bolton *et al*, 1983). For most of the fibre types the clearance half times of fibres within the macrophage size range (<15 m) are of the same order of magnitude as for the unimpaired clearance of nonfibrous particles of low toxicity (Morrow *et al*, 1989; Tran et al, 1997). The slower clearance of short fibres of MMVF10 combined with the rapid clearance of longer fibres of MMVF10 is consistent with the creation of additional short fibres through the disintegration of longer fibres (Searl, 1994). Tran *et al* (1996) showed that

breakage probably played an important role in the removal of long fibres of MMVF10 from rat lungs following chronic exposure by inhalation in the experiments described by Hesterberg *et al* (1994). More recently Yu *et al* (1998) have shown that disintegration played an important role in the removal of long fibres of MMVFs 10, 21 and 22, from rat lungs following short term inhalation (experiment described by Musselman *et al*, 1994). Although clear evidence for fibre breakage was confined to MMVF10 in our data, it is possible that the relatively low precision of our biopersistence estimates obscured lower rates of breakage that may have occurred with other fibre types. It is also possible that we would have been able to track the process better had we had more time points during the first six months of the experiment. The data for MMVF21, 22, the glass microfibres and RCF1 all show slow rates of disappearance of fibres in the 5-10 m length category, that may be consistent with the creation of additional fibres through the breakage of longer fibres. Biopersistence data for 100/475 following short term inhalation exposure also suggests that disintegration of long fibres of code 100/475 is an important mechanism in their clearance (Searl, 1996).

The relatively slow clearance of fibres within the macrophage size range of RCF4 and SiC is not coupled with the disappearance of long fibres. It may, instead, reflect some toxic effect of these particles on macrophage clearance processes as is observed with toxic dusts such as silica (see reviews by Soutar *et al*, 1997; Donaldson and Borm, 1998).

Role of dissolution in fibre disappearance

The ranking of the test fibre types with respect to the biopersistence of the long fibres in lung tissue is largely consistent with our own and published *in vitro* dissolution data (Eastes and Hadley, 1995; Searl, 1994; Christensen *et al.*, 1994; Bauer *et al.*, 1994; Scholze and Conradt, 1987; Potter and Mattson, 1991). Despite the uncertainties associated with both the *in vivo* and *in vitro* measurement of persistence, this suggests that dissolution may be an important mechanism in fibre clearance. The diameter data for long fibres of MMVF10 recovered in the injection experiment is consistent with diameter reduction through dissolution, but that for the other fibre types does not show a similar effect. This may reflect the importance of disintegration as a process for the removal of long fibres (below). Fibres weakened by hydration and partial dissolution may disintegrate into smaller fragments that can be removed by cellular processes and thus disappear more rapidly than if dissolution was the main mechanism of removal (Searl, 1994; Moore *et al.*, 1998). Yu *et al* (1998) found that the breakage rate of five fibre types in animal lungs following short term inhalation was correlated with *in vitro* measures of dissolution.

Fluid composition and the presence of macrophages may be additional sources of difference between dissolution behaviour in the lung and in *in vitro* experiments. The simulated lung fluids used in *in vitro* experiments do not contain the trace organic chemicals that are present in lung fluids. These trace components may enhance the dissolution of some cations, promoting dissolution of the entire glass structure (Searl, 1994). Luoto *et al* (1995) have shown that the incubation of fibres within cell cultures favours the dissolution of glass compositions rich in iron and aluminium, but inhibits the dissolution of fibres with little of these components in their composition. This may partly explain the more rapid disappearance of MMVF21 and RCF1 relative to more soluble fibre compositions from animal lungs than would be expected from *in vitro* dissolution measurements. Overall *in vitro* measures of dissolution appear to be a useful predictor of fibre.

Validity of intratracheal methods for measuring biopersistence

There has been considerable debate about the validity of the intratracheal biopersistence assay. Other authors have expressed concerns that because the material is injected within a small volume of saline, fibres may be insufficiently dispersed within the lung for normal

processes of cellular clearance and dissolution to occur (Bernstein *et al*, 1997). Our results demonstrate that the intratracheal assay can successfully discriminate between fibre types and that the normal clearance mechanisms (macrophage mediated and dissolution/ disintegration) are active following injection of fibres into the lung.

6.1.2 Biopersistence following inhalation

Deposition and Clearance

The greater accumulation of 100/475 and 104E within lung tissue relative to the amosite and SiC parallels the higher concentration of fibres within the aerosol. The relatively small number of long code 100/475 fibres compared with the other fibre types also parallels the smaller concentration of long fibres within the aerosol.

The relatively poor clearance of all four fibre types may be related to overload. Most of the inhaled fibres were in the macrophage size range and the total inhaled volume was within the range where overload would be expected (Bolton *et a.l*, 1983; Morrow, 1988; Morrow *et al.*, 1989; Tran *et al.*, 1997). The extremely poor clearance of silicon carbide may be related to its greater toxicity and slightly thicker mean diameters relative to the other three fibre types used in the inhalation experiments. The toxicity may have led to inflammation and retardation of cellular clearance processes at lower levels of lung loading than for the other fibres. Similar effects have been observed following the inhalation of other toxic dusts such as quartz (Soutar *et al.*, 1997). The thicker diameters of the silicon carbide may have increased the probability of volumetric overload for a given number of inhaled fibres.

The poor quantification of persistence in the inhalation experiments is largely due to interanimal variability in the uptake and clearance of fibres. All animal experiments are subject to interanimal variability, but it possible that after a whole year of exposure, differences in uptake and clearance may have led to more substantial interanimal variability than for shorter treatments.

The change in composition of fibres of 100/475 following retention in rat lungs for at least 12 months is consistent with an incongruent dissolution process. Elements that are weakly held within the glass structure such as alkali metals (sodium and potassium) and alkali earths (calcium and barium) are preferentially removed in solution relative to silicon. This leads to the development of a hydrated silica gel on fibre surfaces. The 104E contains a similar total proportion of metals (calcium and barium) that might undergo preferential leaching but preferential leaching was not detected using EDX. It is possible that the higher proportion of alumina in the 104E glass network, in comparison to 100/475, affects the bonding of the alkali earth metals within the glass so that they become less susceptible to leaching. Another possibility is that any hydrated silica rich phase that forms on the surface of fibres of 104E may contain more alumina and be much less stable than that which develops on fibres of 100/475. This would restrict its thickness such that it would not be detectable by EDX.

Comparison of biopersistence following inhalation and injection

The significance of the apparent differences in clearance rate between the inhalation and injection experiments is unclear because of the wide confidence intervals associated with the estimated mean lung burdens in each experiment. The apparently smaller overall degree of clearance of amosite, code 100/475 and silicon carbide from lungs over 12 months following inhalation relative to that following injection might be a function of the relative fibre dose (Table 3.8). This is probably of particular relevance to silicon carbide where the inhaled dose appears to have been more than thirty times greater than the injected dose. It is less clear, however, why inhalation exposure to 104E did not similarly lead to retarded clearance relative

to that observed in the injection experiment. The apparently slowed clearance could also be related to the animals' health at the end of exposure as the inhalation animals had already been challenged with these fibres for 12 months before the measurement of clearance began. Again, it is difficult to understand why 104E should be an exception, given the relatively high incidence of tumours eventually developed by animals exposed to aerosol.

Differences in the size dependence of fibre clearance in the two experiments might be related to the longer mean residence within lung tissue of fibres recovered in the inhalation experiment relative to those in the injection experiment. The greater clearance of long fibres (>20μm) of both amosite and code 100/475 in the inhalation study might suggest that these fibres start to disintegrate after residence of more than 12 months in the lung. The 12 month recovery period in the injection experiment may have been too short for extensive degradation of amosite fibres or for more complete disintegration of code 100/475 to occur. An alternative explanation would be that the method of fibre delivery has affected the chemistry of the immediate environment surrounding long fibres in the lung and this in turn has affected their susceptibility to chemical degradation. For example, fibres may be more or less likely to be partially engulfed by macrophages and consequently exposed to low pH intracellular fluids.

6.1.3 Comparison of IOM estimates of biopersistence with published data from other studies

Only limited comparisons of our persistence estimates with other published results for the test fibres are possible, because only a few, relatively recent reports present separate persistence estimates for long fibres. Comparisons are also limited by the differences in the protocols used by different laboratories. There have been no published systematic investigations of the reproducibility of any of these biopersistence protocols. Kamstrup *et al* (1998), however, demonstrated the apparent persistence of long fibres of MMVF34, a new stone wool, increased significantly with increasing length of exposure by inhalation. They also found that the half time of clearance of long fibres of MMVF21 following intratracheal injection of a 2mg dose into rat lungs was more than double that following short term inhalation. Their findings of dose dependence are consistent with our finding that the greater lung burdens in our inhalation experiment than in our injection experiment gave rise to longer retention periods. Their estimated half time of clearance of long fibres following injection of 2mg of MMVF21 was, however, only 60% of our estimate based on a 1mg dose. It is possible that the clearance process is not well described by a simple exponential model such that the spacing of time points used in the half life calculation may have affected estimated half times (see Searl, 1996).

Our observations of the relative persistence of MMVFs 10, 21 and 22 following injection are consistent with the relative persistence of these fibres following short term inhalation (Musselman *et al*, 1994). Rates of fibre disappearance following inhalation were, however, somewhat faster than in our injection experiment. This is consistent with earlier comparisons between inhalation and injection made by Muhle and Bellmann (1995). Contributory factors may include the lower effective doses in short inhalation experiments, better dispersion within the lung and the slightly thinner diameter distribution of the rat respirable (as opposed to human respirable fibres) in the inhalation experiment. The better dispersion and thinner diameters would have made fibres more susceptible to removal by dissolution and disintegration. Clearance rates following chronic inhalation of these fibres and RCF1, however, also appear to be more rapid than in our injection experiments, despite initially higher lung burdens (Hesterberg *et al*, 1995). The relative rates of clearance of long fibres of these four fibre types also appear have been slightly different following chronic inhalation than in our injection experiment. Given the relatively poor precision of any biopersistence estimates, however, it is not clear whether these differences in relative ranking between experiments are significant.

Mast *et al* (1995) present extensive data from a chronic inhalation study with RCFs 1, 2 and 4, but do not provide specific information about the fate of long fibres. However, fibres of all sizes of RCF1 were cleared much more effectively than those of RCFs 2 and 4. This is consistent with our *in vivo* data and with the greater rate of *in vitro* dissolution displayed by RCF1. It could, however, also be caused by factors such as differences in the cytotoxicity of these fibre types.

The relative persistence of long fibres of amosite asbestos in our injection experiment was consistent with earlier injection studies showing very limited clearance of long fibres of amphibole asbestos (crocidolite; Bellmann *et al.*, 1994; Bellmann *et al.*, 1995).

6.1.4 *In vitro* measurement of dissolution

The development of an *in vitro* assay that could be used to predict reliably the persistence of fibres in lung tissue would be an extremely useful tool in the assessment of fibre toxicity. There is a widely held consensus that *in vitro* assessments of dissolution are best made using continuous flow tests. We have found however, that a simple static experiments in which fibres are simply put in a beaker of sodium oxalate was just as effective in predicting the relative persistence of fibres in lung tissue (Searl and Buchanan, 1999).

Published data from *in vitro* dissolution experiments show a wide range of variation in estimated K_{dis} values for individual fibre types. This is partly because the method is technically difficult as it involves measuring trace concentrations of solute in solutions with high background levels of salts. In addition, relatively small differences in protocol can lead to substantial differences in reported values of K_{dis} (Searl and Buchanan, 1999). The current interlaboratory comparison that is being run by EURIMA involving 12 laboratories should provide valuable information on the reproducibility of an *in vitro* assay performed to a common protocol. Our own experience suggests that it would be necessary to make at least 10 repeat determinations of K_{dis} to reduce the confidence levels around any estimate of K_{dis} to 10% (Searl and Buchanan,1999).

At the current level of development, *in vitro* dissolution assays can only reliably distinguish between fibres with very different dissolution characteristics (Searl and Buchanan,1999). This might well be sufficient in practical terms to identify fibres that would be expected to have a low biopersistence and therefore to have a low toxicity. Given the imprecision with which K_{dis} can be estimated, it would be difficult to set a simple cut off value to define low persistence in the *in vitro* assay. It would also be impossible to reliably rank fibres of intermediate persistence, if that were required.

6.2 PATHOGENICITY OF E GLASS

6.2.1 Short term biological tests

In vitro assays

The processes of inflammation and epithelial cell injury leading to cell proliferation may play important roles in the development of fibre-related disease (Rom *et al.*, 1991; Barrett 1994; Bissonette and Rola-Pleszczynski 1989; McGavran and Brody 1989; Davis *et al.*, 1996). During the course of the CFRP, we have examined the behaviour of the test fibre types in various short term *in vitro* and *in vivo* assays in relation to their pathogenicity in chronic experiments. The drawback of these short term studies, however, is that they do not generally allow for any changes to fibres during longer term retention within the lung. One approach is

to pre-treat fibres *in vitro* with a solution which causes partial dissolution (Donaldson *et al.*, 1994).

It has been suggested that macrophage cytokines are central to the inflammation produced by inhaled fibres. Increased release of cytokines and factors mitogenic for fibroblasts by BAL macrophages from asbestos-exposed rats, have been reported in many short-term studies (Donaldson and Brown, 1993). The greater activity of 104E in stimulating the production of the pro-inflammatory cytokine TNF in *in vitro* tests than the MMVFs was consistent with its greater long term biological activity as demonstrated in the intraperitoneal experiment. The similar activity of 104E to 100/475 in the TNF assay was, however, at odds with their very different carcinogenicity in long term inhalation and injection experiments. One factor that may contribute to this difference in long term activity may be that the surface properties of 100/475 are more extensively modified by dissolution than those of 104E. In addition, the TNF assay only examines one small component of the disease process and other components may be as or more important in determining carcinogenic potential. This may partly explain why the relative activity of 104E and amosite in the TNF assay differs despite apparently similar carcinogenic potential.

The relative activity of the two microfibres in the epithelial cell detachment assay did not reflect their long term pathogenicity. This assay was a poor predictor of long term carcinogencity of the test fibre types, despite its apparent promise in earlier experiments with asbestos fibres (Donaldson *et al.*, 1993; Goodglick and Kane 1990) using similar dose ranges.

Overall, the results of the *in vitro* toxicity assays with 104E supported our earlier conclusions and those of other authors that these assays have limited value in the prediction of fibre pathogenesis (Jones *et al*, 1998; Hart *et al*, 1994).

Short term inhalation

In the short term inhalation experiments, all four fibre types caused recruitment of inflammatory cells and increased concentrations of protein in lavage fluid. These responses were not, however, clearly related to long term pathogenicity. In contrast, our previous experiments with amosite, silicon carbide and 100/475 had suggested that increased proliferation following short term inhalation may be a useful predictor of carcinogenicity (Jones *et al.*, 1998; Cullen *et al.*, 1997). The proliferative response following exposure to 104E was greater than following exposure to 100/475, consistent with the apparently greater carcinogenic potential of 104E. The proliferative response following exposure to 104E was, however, less than that following exposure to amosite, despite the apparently similar potential of these materials to cause cancers in long term experiments. This suggests that there is not a simple relationship between the stimulation of cell proliferation and carcinogenesis. Proliferation is probably a useful index of carcinogenic potential but other factors must also influence the relative carcinogenicity of different fibre types.

It is not clear what physical or chemical properties of the test fibre types influenced the response in the proliferation assay. The aerosols for all four fibre types were well matched in terms of concentrations of WHO and long fibres, so that the differences in proliferation, reflect differences in some fibre property other than simply fibre dimension. Biopersistence is unlikely to have played an important role over such a short time period and this suggests that fibre surface properties may have been important in determining proliferative response.

6.2.2 Carcinogenicity

Inhalation experiment

The carcinogenicity of 104E microfibres (an E-glass) in rats exposed to an aerosol for 12 months was similar to that of amosite and much greater than that of 100/475. The production of tumours was consistent with predictions made by Eastes and Hadley (1996) which were based on the relative durability of E-glass in *in-vitro* dissolution experiments. The Eastes and Hadley prediction was for E glass fibres with the same size distribution as RCF1 and in experiments following the RCC protocol. The E glass that we used contained a lower proportion of long fibres than RCF1. In addition, the animals were exposed to much lower mass concentrations (5mgm^{-3} compared with 30mgm^{-3}) over a shorter time period (one year rather than two) than in the RCC experiments. This suggests that the 104E was actually more active than predicted by the Eastes and Hadley model. Our own *in vitro* dissolution experiments showed that our sample of 104E dissolves only slightly more slowly than our 100/475 sample and the K_{dis} values for the two materials are not significantly different (Searl and Buchanan, in preparation). The Eastes and Hadley model would therefore predict these two fibre types to have similar potentials to cause tumours, which is not consistent with their observed effects.

Mineral fibres are thought likely to be pathogenic if they are long, thin and sufficiently durable to remain in the lung for long periods. (World Health Organisation, 1986; Miller *et al.*, 1999b). 104E glass microfibres were expected to have a durability intermediate between the code 100/475 microfibres and amosite (Davis *et al.*, 1996). In fact, in terms of fibre numbers, the biopersistences of all three of these fibre types were similar, but the 100/475 was the only fibre type to undergo substantial changes in composition as a result of retention in lung tissue. The selective leaching of glass components from fibres of 100/475 leaves a relatively porous silica gel, which may have a greatly reduced structural strength compared with the original glass. This would increase the probability of long fibres disintegrating into shorter fragments that could be cleared by macrophages. In contrast, there is little evidence for extensive differential leaching of glass components from 104E fibres so that although fibres may become thinner during residence in lung tissue, they may retain their structural integrity longer. This would have allowed a relatively greater accumulation of 104E fibres over a 12 month period. At the end of 12 months inhalation, most fibres in the lung were a survivor population that had been in the lung for a period of months. This potential difference in the persistence of long fibres of the two microfibres may partly explain the greater accumulation of long fibres in the lungs of exposed to the 104E aerosol than those exposed to 100/475. The greater pathogenicity of the 104E glass microfibres might, therefore, be because approximately twice the dose of long (>15 μm) fibres was maintained in the lung over some critical period for disease production compared with 100/475 fibres. However, the differences in response to the two fibre types in the proliferation assay seems too substantial for the difference in carcinogenicity to be due entirely to a dose differential of this relatively small magnitude.

Differential dissolution is also likely to have greatly modified the surface properties of 100/475, but not of 104E fibres. It is possible that a glass surface that has been greatly modified by dissolution is less toxic to cells than a surface that has retained much of its original structure. If this is true, a more complete definition of fibre durability is required than merely the retention of long fibre shapes in lung tissue. Pathogenic fibres may be those that are long, thin and can present an unchanged surface to the lung tissue for long periods of time. This difference in the extent to which surface properties may be modified through time is again unlikely to be the sole cause of the difference in carcinogenicity between the two microfibres, given the differences in their short term proliferative potential.

The phenomenon of overload in which the normal macrophage-based clearance of particles from the lung becomes overwhelmed (Bolton *et al.*, 1983; Morrow, 1988) can also lead to the development of tumours. This can occur even with relatively non-toxic dusts given severe overload (Lee *et al.*, 1985, 1986; Driscoll, 1996). We have shown that overloading of the rat lung follows the same kinetics for both fibrous and non-fibrous particles (Tran *et al.*, 1997). It has been estimated that the effects of overload become apparent when the volume of dust in the rat lung is approximately 1500 nl and start to become severe at 10^4 nl (Morrow, 1988). There is experimental evidence from tracer studies that some impairment of clearance could be initiated at lung burden volumes as low as 100 nl (Bellmann *et al.*, 1991). The volume burdens after 12 months inhalation in our study were estimated to be about 3000 nl for 104E and 100/475 and 2000 nl for amosite indicating that overload was likely. The extent of overload would not have been sufficient to cause severe impairment of clearance as evidenced by the clearance of these fibres following cessation of exposure. Overload is unlikely to have been important to the development of tumours in the animals exposed to 104E given the absence of tumours in animals exposed to 100/475 that had experienced similar levels of overload.

Overall, there is no single simple difference between 100/475 and 104E that explains their differing pathogenicity in the chronic inhalation experiment. Factors that may have contributed to the greater pathogenicity of 104E include the unknown factor that provoked a more intense initial proliferative response, the greater resistance of fibre surfaces to modification by dissolution and the greater accumulation of long fibres in lung tissue during exposure.

Intraperitoneal injection study

In the intraperitoneal injection study, peritoneal mesotheliomas appeared much more quickly with 104E than with amosite, and we conclude that 104E is the more pathogenic of the two. The 100/475 microfibre produced considerably fewer mesotheliomas, and at a slower rate, than the other fibre types. Other workers have also reported significant tumour production in this assay using fibres from Johns Manville similar to our 104E fibre. These fibres were labelled 104/1974 in Pott *et al.*, (1989) and 104E in Pott *et al.*, (1991).

We have previously reported that a wide range of other fibre types will produce tumours in the rat peritoneal cavity (Davis *et al.*, 1996; Miller *et al.*, 1999a). In these earlier reports, we found that, comparing median survival time, glasswool (MMVF10), slagwool (MMVF22) and 100/475 had less activity than amosite, silicon carbide whiskers, refractory ceramic fibres RCF1 and RCF2, and rockwool (MMVF21). Only RCF4 failed to induce mesotheliomas. Comparing 104E with these other fibres indicates that, in this assay, this fibre is probably more carcinogenic than amosite but possibly less active than silicon carbide whiskers, RCF1 and RCF2, and MMVF21. Somewhat surprisingly, a data summary given by Pott (1995) shows 104E to be slightly less potent than 104/475. There does not seem to be any obvious reason why 104/475 should be substantially more carcinogenic than 100/475 as both fibre types should have the same composition and only differ marginally in fibre size. This does raise questions about the consistency of glass fibre composition between different batches of test fibres. It also suggests that relatively small variations in composition could have a substantial effect on carcinogenicity.

6.3 PREDICTIVE MODELS

6.3.1 Intraperitoneal injection experiment

Miller *et al.* (1999a) reported on earlier analyses of the results from the intraperitoneal injection experiment, in relation to quantitative estimates available at that time of dissolution *in vitro* and of biopersistence in the lung. Those analyses suggested that the two best predictors of survival were the number of injected fibres in the longest (> 20 μm) thinner (< 0.95 μm) size category, and the biopersistence of fibres >5 μm in length. The present re-analyses used revised estimates of biopersistence based on more time points, and new dissolution estimates based on CFT techniques. The new analyses produced very similar results, although biopersistence of fibres >15 μm long was now a better predictor (by the smallest possible margin) than that for fibres >5 μm. This minor interchange in the strength of two highly correlated variables does not necessarily have any practical significance.

When the results of the current analyses (excluding data for 104E) were used to predict the median survival time for 104E, the prediction of 352 days agreed very well with the observed 359 days. However, the residual mean square from the two-variable regression model was 9161, giving a residual standard error of 95.7. Predictions from the model have standard errors in excess of 100 days. This reflects the poor fit of the model for several of the fibres, for example under-predicting survival for 100/475 and RCF1, and over-predicting for silicon carbide. Combined with a 5% t-value of almost 2.5, the variation produces 95% confidence intervals for predictions of about ± 250 days.

When the statistical analysis was extended to include the newly available data for 104E, the regression equation was not substantially changed, and the same two variables were chosen as best predictors. The residual mean square was somewhat reduced at 7859, giving a standard deviation of 88.7, summarising the poor fit achieved for some of the fibres. Predictions for new fibre types would still be associated with 95% confidence intervals of at least ± 200 days.

This degree of uncertainty, and the poor fit for some of the fibre types, suggest that there are other factors, with important influences on fibre pathogenicity, that are not included among the predictor variables that were tested. One influence on fibre pathogenicity may be related to some aspect of fibre surface properties, but it is not clear from the existing literature what particular surface property or properties are likely to be useful predictors of pathogenicity.

6.3.2 Inhalation experiment

Miller *et al.* (1999b) reported on analyses of the combined results of long-term inhalation studies at the IOM and at RCC, identifying the numbers of long thinner fibres and the adjusted dissolution coefficient as the best two predictors. Regrettably, there was a transcription error in the number of animals inhaling amosite that later developed lung cancers, and the published results reflect this. However, subsequent re-analyses of the corrected data showed almost identical conclusions (not given here).

The new analyses presented here include this correction, as well as revised estimates of biopersistence in the lung and dissolution *in vitro*. These analyses identified the same two variables as best predictors. This revised model predicted a tumour incidence of 9.1 tumours for the amosite following inhalation exposure to 2000 fml^{-1} over 12 months, which compares well with the 8 tumours observed (Davis *et al*, 1986). However, this model failed to predict the incidence of lung tumours observed in animals inhaling 104E.

Inclusion of 104E in the data set for analysis gave a much poorer fitting model. While this created a somewhat better fit for 104E, there was over-prediction for 100/475. In addition,

the model now selected as best predictors the exposure to all thin fibres, regardless of length, and the biopersistence of fibres >5 μm long. This contrasted strongly with both the analysis excluding 104E and the results from the injection experiment, both of which had highlighted the importance of length as a predictor of hazard. Factors that would have influenced the outcome of the statistical modelling would include the relatively small number of fibre types compared with the number of predictor variables. Additionally the outcome would be affected by the selection of the potential predictor variables. Fibre dose, for example, was assessed from fibre concentrations in the aerosol. Differences between the fibre types in terms of deposition and clearance means that the received dose in terms of lung burden might have been a more relevant variable, if data had been available for comparable time points in both sets of experiments. Additionally other factors may influence disease outcome more strongly than the variables included in the model. As with the injection experiment, it seems very likely that not all relevant predictor variables had been available to the analysis.

6.3.3 Comparison and overview of carcinogenicity following injection and inhalation

Both injection and inhalation experiments demonstrated that numbers of long thin fibres and the persistence of long fibres, whether measured in animal experiments or assessed from an *in vitro* assay, are important determinants of carcinogenic potential. The results of the regression modelling for both experiments suggest that there are factors that contribute to carcinogenic potential that have still to be fully identified. Both experiments suggest that the carcinogenic risks associated with 100/475 are rather lower than might be expected from its dimensions and persistence properties whereas the risks associated with silicon carbide are very much higher than would be expected. Both experiments also show RCF4 is remarkably benign in comparison to its untreated precursor, RCF1, although RCF4 does fit well with model predictions as it contains few long thin fibres.

The lower than expected risks associated with 100/475 may be related to the mechanism by which these fibres dissolve in the lung. The EDX data suggest that these fibres undergo selective leaching of alkali metals and alkali earths to leave a silica gel. The interaction of cells with this gel is presumably very different from their interaction with a less modified silicate glass surface. The difference in the short-term proliferative response following inhalation to 100/475 and 104E, however, suggests that the long-term modification of fibres of 100/475 by dissolution can only be one factor contributing to their low toxicity. It is possible that the special electrical properties of E glass may affect the interaction of 104E fibres with cells within the lung.

The higher than expected risks associated with silicon carbide may be related to its unusual shape (Miller *et al*, 1999a). It may also be related to its surface properties. Silicon carbide is similar to quartz in that both substances have a propensity to form "dangling bonds" that can interact with surrounding molecules to generate free radical species (Fubini, 1993). This is thought to contribute to the pathogenicity of quartz (Fubini, 1993). SiC whiskers have a greater propensity to generate free radical species than SiC in normal granular form (Fubini, 1993).

6.4 RELEVANCE OF STUDY FINDINGS TO HUMAN EXPOSURE

The data from the CRFP suggest that under similar levels of exposure (as measured by numbers of WHO fibres) some made-made fibres, most notably SiC whiskers and 104E microfibres, may have a similar carcinogenic potential to amosite asbestos.

The experiments were conducted at high concentrations so that excess incidence of tumours can be detected even within a small number of test animals. The statistical analysis suggests that not all the factors that influence fibre carcinogenicity have yet been identified and are, therefore, doubts about the dose-response relationships that might be expected with different fibre types.

If the hazards appear similar for similar exposure, then it is obviously important to consider whether equal levels of exposure are likely to occur. These man-made fibrous materials are much less dusty than traditional asbestos materials. Therefore, for similar handling or processing of equivalent quantities of bulk material, the potential for human exposure is therefore much lower and consequently the risks of adverse health effects in humans would be much lower for the less dusty materials.

The intraperitoneal injection assay, at a set dose of WHO fibres, produced results which suggest that MMVF21 may have more potential than MMVFs10 and 22 to give rise to mesothelioma. However, the intraperitoneal assay is recognised as being limited by the non-physiological route of administration and the implications for inhaled fibres of MMVF21 is probably also dependent on the extent to which such fibres could penetrate to the mesothelium.

The results of the regression modelling suggest that the potency of fibres is directly related to the numbers of long fibres and the durability of fibres (measured either by *in vitro* dissolution assays or by *in vivo* persistence in the lung). The results also suggest, however, that this is not the complete explanation. In particular, the two microfibres showed markedly different responses (in terms of fibrosis, numbers of tumours, and survival time in the intraperitoneal injection assay) and the difference was greater than could be accounted for by differences in dose or durability of these two fibres. Caution is therefore needed in assuming that relative hazard of new fibre types is not substantially different from that which would be predicted solely on the basis of durability in *in vitro* dissolution assays.

6.5 SUGGESTED FUTURE WORK

6.5.1 Fibre properties affecting toxicity

The finding that the two tested microfibres produced markedly different responses indicates that these materials may provide an opportunity to clarify the fibre properties that affect toxicity.

The very different pathogenicity of the two microfibres despite their generally similar fibre size distributions and biopersistence suggests that there is some other property of fibres that can have a profound effect on their toxicity. The difference between the two fibre types has an immediate effect on the lung, as shown by the proliferation assay following short term inhalation exposure, and therefore cannot be directly related to their different durability. This suggests that the surface properties of fibres could be very important. This possibility could be investigated and confirmed by examining specific surface properties that are thought to be of relevance. The desired outcome would be more detailed definitions of "durability" and/or "toxicity" which would help define the relative hazard of new fibre types.

6.5.2 Short term assays of fibre toxicity

Given the expense and timescale involved in performing long term experiments to assess the carcinogenic potential of fibrous materials, there is a need for the development of reliable short term predictive tests.

The differences in epithelial proliferation detected after short term inhalation exposure appeared to be a useful guide to the subsequent development of fibrosis and tumours. These tests were limited to four fibre types. Since the test appeared to be indicative of the difference in pathogenicity between the two microfibres, it may be able to provide information where it is most needed, i.e. for fibres whose differences are not fully explained by dose and durability. There is now a need to validate the epithelial proliferation assay by testing a range of other fibres of known pathogenicity.

The results of the short term tests performed within the CFRP showed a disappointing absence of a correlation of measures of inflammation with pathogenicity. Work by Warheit and colleagues (Warheit 1993; Warheit *et al.*,1994a), however, suggests that better discrimination between fibres can be achieved by studying persistence of inflammation at intervals following cessation of exposure. This could be validated by undertaking a series of short term inhalation experiments with fibrous materials of known pathogenicity

The range of *in vitro* cellular (biological) assays tested in this programme did not help to predict the tumour production of the fibres. This is useful in that it indicates the need to take the search for *in vitro* tests capable of reducing dependence on animal tests to a greater sophistication. More sophisticated and complex *in vitro* tests could therefore usefully be evaluated in parallel with further development of short-term *in vivo* tests (for epithelial proliferation). Given that carcinogenesis is a multi-step process, it seems unlikely that a single *in vitro* assay could be developed that would be an adequate predictor of carcinogenic hazard. It may, however, be possible to devise a series of assays to examine different aspects of cell function, from which some indications of carcinogenic potential could be drawn. There is a need to review the component steps in current models of carcinogenesis and the *in vitro* assays available to investigate specific aspects of these steps. From such a review, a series of potentially relevant assays could be designed and then tested using the fibres tested within the CFRP with the aim of developing a streamlined *in vitro* testing programme for assessing the carcinogenic potential of fibres.

Fibres in the lung may be active over a period of months and at the time of disease initiation are likely to have been modified by residence in the lung. The predictive power of short term tests may therefore be improved by pre-treatment of fibres to reproduce the effects of long term residence in the lung. This could be done by exposing fibres to simulated lung fluids within continuous flow through experimental test rigs. The effects of pre-treatment with oxalic acid to simulate dissolution in the lung have already been examined for a limited number of fibres. It would be instructive to investigate a wider range of fibres using a range of assays, using an improved pre-treatment process. The pre-treatment process could be improved by using a more relevant fluid composition and by using a continuous flow through experimental set to reproduce the kinetics of dissolution in the lung.

6.5.3 The relationship between exposure and dose

The regression analysis has confirmed the importance of two parameters (exposure, and durability) which largely determine the cumulative dose over time. The relationship between exposure and cumulative dose to the target tissue is therefore an important step, which if modelled, might clarify the significance of fibre characteristics for humans. The IOM has developed models that account for the changing pattern of size distribution of fibres in the rat

lung during and after exposure to MMVF10, MMVF11, MMVF21 and RCF1. Further development of this model could form a basis for making theoretical predictions of the dose expected given exposure to any of the ten tested fibres. This could be calibrated to the data available for the lung burdens in rats and then converted to predictions in humans by modifying the values of coefficients in the model in accord with the physiological differences and well characterised differences in mechanical clearance rates. The difference in life span and clearance kinetics between humans and rats might well be expected to amplify the consequences of differences (between fibres) in durability.

6.5.4 The measurement of biopersistence

Since the data supported the importance of biopersistence/durability (in explaining much of the observed differences in response in the assays of carcinogenicity), there is clearly good reason to ensure that techniques for measuring these quantities are reliable and consistent.

6.5.5 Other fibre compositions

The finding that dose, dimension and durability explained so much of the observed response in the carcinogenicity assays also prompts the question as to how far the same (or similar) relationship would also apply to fibres of very different composition (such as durable organic fibres). This may become of greater practical importance as more products are manufactured with very fine durable organic fibres. The existing data set would provide an ideal basis for comparing and evaluating new fibres of different composition in similar experiments.

7. CONCLUSIONS

The biopersistence of ten fibre types following intratracheal injection was consistent with the hypothesis that short fibres are largely cleared by cellular processes whereas long fibres are cleared by the combined processes of dissolution and disintegration. The relative persistence of long fibres of different types in lung tissue was similar to their relative persistence in continuous flow dissolution experiments.

The main source of uncertainty associated with our biopersistence estimates was interanimal differences in lung burden. Additional counting and sizing of fibres recovered from animals used in the injection experiment for five of the test fibres reduced the width of the confidence limits associated with our estimates of biopersistence, but did not substantially change the estimated mean values.

The lung burdens of animals exposed by inhalation to four fibre types over 12 months inhalation were much greater than following injection of 1mg of fibre. There was no evidence of any significant subsequent clearance of silicon carbide following inhalation. This was probably due to the toxicity of the silicon carbide, combined with relatively large lung burdens that would have overwhelmed the normal cellular clearance mechanisms. Clearance rates for the other three fibre types were retarded relative to those in the injection experiment, consistent with greater overloading of cellular clearance mechanisms. The most important difference between the fibre types was that the composition of 100/475 microfibres retained in lung over 12 months was substantially modified by differential leaching whereas the composition of retained fibres of 104E microfibres and amosite were not.

The 104E had a similar activity in cellular tests to the other microfibre tested (100/475) and was less active than asbestos. Animals exposed to 104E in a short term inhalation experiment showed evidence of inflammation similar to that seen in animals exposed to 100/475, amosite or silicon carbide. A significant difference, however, was that the 104E caused increased cell proliferation but the 100/475 had no proliferative effect. In a chronic inhalation experiment, animals exposed to 104E, amosite or silicon carbide developed tumours whereas those exposed to 100/475 did not. The incidence of tumours was similar in animals exposed to 104E as in animals exposed to amosite. In a long term intraperitoneal injection experiment, the median survival time of animals treated with 104E was shorter than for those treated with amosite and substantially shorter than for animals treated with 100/475. Overall, it appeared that the 104E had a similar or greater carcinogenic potential to amosite asbestos.

Statistical analyses of data from both the inhalation and injection studies suggested that important explanatory variables of the carcinogenic potential of fibres included the numbers or concentrations of long thin fibres, and measures of their ability to persist in the lung. However, for neither study did the final model perform satisfactorily, and the variability of pathogenic outcomes could not be predicted sufficiently using the chosen variables. This suggests that there must be some other factor influencing fibre toxicity, most probably related to the surface properties of the fibres. Certainly, such a factor is required to explain the very different responses to the microfibres 100/475 and 104E, given their relatively similar dimensions, dissolution characteristics and abilities to persist in the lung. If such a factor were identified and included in statistical models, it cannot be predicted how important other variables would then appear.

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HEAD OFFICE:

Research Avenue North,
Riccarton,
Edinburgh, EH14 4AP,
United Kingdom
Telephone: +44 (0)870 850 5131
Facsimile: +44 (0)870 850 5132

Tapton Park Innovation Centre,
Brimington Road, Tapton,
Chesterfield, Derbyshire, S41 0TZ,
United Kingdom
Telephone: +44 (0)1246 557866
Facsimile: +44 (0)1246 551212

Research House Business Centre,
Fraser Road,
Perivale, Middlesex, UB6 7AQ,
United Kingdom
Telephone: +44 (0)208 537 3491/2
Facsimile: +44 (0)208 537 3493

Brookside Business Park,
Cold Meece,
Stone, Staffs, ST15 0RZ,
United Kingdom
Telephone: +44 (0)1785 764810
Facsimile: +44 (0)1785 764811

Email: iom@iom-world.org