LUNG CLEARANCE OF EXPERIMENTAL MAN-MADE MINERAL FIBRES, PRELIMINARY DATA ON THE EFFECT OF FIBRE LENGTH

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INTRODUCTION
The hazard from inhaled fibres is believed to be related to their dimensions, with long, thin fibres being potentially more carcinogenic than short, thick ones (Stanton et al., 1981; Pott, 1978) and to their biopersistence, as fibres which dissolve rapidly in the lung are unlikely to induce long-term pathological changes (Morgan and Holmes, 1986). Following deposition in the lung, short fibres (< 10–20 um in length) are ingested by alveolar macrophages, exposing them to the acidic environment of the phagolysome (pH 4.5). Longer fibres remain free in the lung tissue and are exposed to the neutral environment of the lung fluid. These longer fibres may be partially engulfed by macrophages, with macrophages gathering along the fibre like pearls on a string, exposing different parts of the fibre to different environments.

These pH differences can explain the length dependent biopersistence observed for experimental glass fibres (Eastes et al., 1995). For these fibre types, short fibres (< 20 μm) showed only limited decline in diameter with time, indicating low dissolution. For longer fibres, diameter reduction was faster, indicating higher dissolution, similar to that seen in in vitro studies. This is consistent with the fact that glasses are more soluble at neutral pH than acidic pHs. For stonewool fibres, which are more soluble at low pHs, the situation should be reversed, with short stonewool fibres showing lower lung biopersistence than longer fibres. If pH is the determining factor, adjustment of the chemical composition of fibres may result in higher dissolution of longer fibres within the lung, reducing the potential hazard from these fibres.

Studies on lung clearance of fibres (and hence biopersistence) present problems as thick fibres (respirable by humans) cannot be inhaled by rats. With intratracheal instillation, longer and thicker fibres may be administered to the lungs of animals and measurements of biopersistence made. In this study, initial lung biopersistence results for two experimental MMVFVs are compared and the effect of fibre length on clearance demonstrated. Corresponding data for biopersistence of both fibres in the peritoneal cavity and for HTN lung retention measured by SEM is currently being collated.
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METHODS

Two stonewool fibre types were studied, MMVF21 and HTN. The MMVF21 was obtained from the NAIMA (North American Insulation Manufacturers Association) repository and was the same material as that used in inhalation studies conducted at RCC (Research and Consulting Centre, Geneva, Switzerland) (McConnell et al., 1994). HTN was an experimental stonewool fibre developed and supplied by Rockwool International A/S, Denmark. Prior to administration, approximately 2 g of each fibre type was irradiated in the Imperial College reactor at Silwood Park, for 8 h in a neutron flux of $2.5 \times 10^{12}$, to activate some of the Na present to $^{24}\text{Na}$, detectable by external $\gamma$-counting. A preliminary test of the effects of hypochlorite digestion on the morphometry of neutron activated fibres showed that the methods of sample preparation did not significantly affect the fibres to be used.

Suspensions of each fibre type in sterile saline (0.2 ml; 6 mg fibres ml$^{-1}$) were administered by intratracheal instillation, to groups of 40 female Fischer F344 rats (Harlan Olac, U.K.) which were 12 weeks old, under halothane anaesthesia. Each rat received approximately 1.2 mg of fibres. Samples of the instillation suspension were taken at regular intervals during the administrations to act as $\gamma$-counting standards for the $\textit{in vivo}$ whole body counts. A group of 10 control animals received instillations of 0.2 ml of sterile saline only. Following recovery animals were returned to their cages and housed in standard conditions with food and water available \textit{ad libitum}.

The whole body $\gamma$-activity of $^{24}\text{Na}$ in each animal was measured using NaI detectors at 2 days. From this the original fibre burden in each animal was estimated by comparison with aliquot activity, normalised by the number of fibres which had been measured in the aliquot samples. Fibre exposed animals were killed at 2 days ($n = 5$) and at 7, 14, 30, 90, 141 and 180 days ($n = 3$) and control animals ($n = 2$) at 2, 30 and 360 days after administration. The lung lobes were removed and digested in 60 ml of hypochlorite at 4°C for ~ 4 h. Measured volumes of each digest were filtered onto cellulose filters. Four, 0.45 $\mu$m pore size mixed cellulose ester filters were prepared for phase contrast optical microscopy (PCOM). These were dried, mounted, and cleared on glass slides using the acetic acid/formamide method of Le Guen and Galvin (1981). Two, 0.22 $\mu$m pore size mixed cellulose ester filters were mounted on copper stubs and sputter coated with gold for scanning electron microscopy (SEM) examination.

At each time point, bivariate size measurements were made on at least 600 (or as many as are found in a maximum of 300 fields) fibres sampled from all the animals killed, by SEM (Leica, S440 SEM, Leica, U.K.). At each time point, the median fibre length and diameter, the fraction of WHO fibres, and the fraction of fibres < 5 $\mu$m, 5–10 $\mu$m, 10–20 $\mu$m and > 20 $\mu$m in length were calculated. The number of fibres in each sample was estimated by PCOM. At least 200 fibres, up to a maximum of 200 fields were scored per filter, using modified WHO (1985) and NIOSH (1989) counting rules. From the known area of each field and the total filter area, the number of fibres on the filter was estimated. Two filters per lung digest sample were randomly selected and scanned. The number of fibres present in each lung was calculated from the average counts per animal corrected by appropriate scaling factors for sample volume and dilution.
Table 1. Median length and diameter of fibres recovered from lungs at various times after intratracheal instillation (μm)

<table>
<thead>
<tr>
<th>Days</th>
<th>MMVF21 Length</th>
<th>MMVF21 Diameter</th>
<th>HTN Length</th>
<th>HTN Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.00</td>
<td>1.22</td>
<td>20.21</td>
<td>1.09</td>
</tr>
<tr>
<td>2</td>
<td>15.71</td>
<td>1.19</td>
<td>24.22</td>
<td>1.14</td>
</tr>
<tr>
<td>7</td>
<td>18.17</td>
<td>1.25</td>
<td>23.82</td>
<td>1.26</td>
</tr>
<tr>
<td>15</td>
<td>15.44</td>
<td>1.25</td>
<td>17.43</td>
<td>1.29</td>
</tr>
<tr>
<td>30</td>
<td>17.55</td>
<td>1.17</td>
<td>14.83</td>
<td>1.13</td>
</tr>
<tr>
<td>90</td>
<td>15.78</td>
<td>1.15</td>
<td>12.66</td>
<td>1.13</td>
</tr>
<tr>
<td>141</td>
<td>17.94</td>
<td>1.28</td>
<td>15.50</td>
<td>1.84</td>
</tr>
<tr>
<td>181</td>
<td>17.27</td>
<td>1.10</td>
<td>16.39</td>
<td>1.88</td>
</tr>
</tbody>
</table>

RESULTS

The median length and diameter of the fibres recovered from the lungs at the different time points are given in Table 1. For MMVF21, there was little change in diameter over the course of the study, with a trend towards a slight increase in fibre length, indicating some preferential removal of shorter fibres with time. For HTN, there was a decrease in length with time, with the minimum length being found 90 days after administration. The diameter of the fibres remained relatively constant up to 90 days and then showed a marked increase.

The fraction of the fibres falling into each size category at each time point is given in Table 2. Changes in the distribution of fibres between size categories with time (2–181 days) were not seen for MMVF21, but were noticeable for HTN fibres. For HTN, there was a decrease in the proportion of WHO fibres with time, increases in the proportion of fibres in fractions 5–20 μm and decreases in the proportions < 5 and > 20 μm.

Initial fibre burdens, measured at 2 days after administration, were similar for
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Fig. 1. Retention of MMVF21 fibres in the lung of rats following intratracheal instillation. (% of burden at 2 days post-administration, mean n = 3–5.)

Both groups (6.2 ± 0.3 x 10⁶ and 4.8 ± 0.4 x 10⁶ fibres for MMVF21 and HTN respectively, mean ± SE, n = 5) and very few fibres were found in the control animals (350 ± 200, n = 3). From the total number of fibres recovered from each animal and the fraction of fibres in each size category (Table 2), the number of fibres in each size category remaining in the lungs of each animal was calculated. For each animal, the retention of each size fraction of fibres was calculated by comparing the number of fibres in that category at death with the number of that category present originally (2 days). Retention curves for each size fraction and fibre type are given in Figs 1 and 2. For MMVF21, there was some clearance of all

Fig. 2. Retention of HTN fibres in the lung of rats following intratracheal instillation. (% of burden at 2 days post-administration, mean n = 3–5.)
Table 3. Clearance half times and 95% confidence intervals for fibres and size fractions of fibres following intratracheal instillation (days)

<table>
<thead>
<tr>
<th></th>
<th>All fibres</th>
<th>WHO fibres</th>
<th>Fibres &lt; 5 μm long</th>
<th>Fibres 5-10 μm long</th>
<th>Fibres 10-20 μm long</th>
<th>Fibres &gt; 20 μm long</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMVF21</td>
<td>231 (167-375)</td>
<td>246 (174-415)</td>
<td>174 (130-261)</td>
<td>188 (139-290)</td>
<td>250 (175-433)</td>
<td>261 (186-437)</td>
</tr>
<tr>
<td>HTN</td>
<td>64 (58-71)</td>
<td>61 (55-68)</td>
<td>49 (43-56)</td>
<td>96 (85-110)</td>
<td>73 (65-81)</td>
<td>53 (47-61)</td>
</tr>
</tbody>
</table>

size fractions, with slightly faster clearance of shorter fibres than longer ones (retention at 181 days of < 5 μm fibres = 26.6 ± 6.4 and for > 20 μm fibres = 39.9 ± 9.6, mean ± SE). For the HTN fibres, clearance of all size fractions was faster than for MMVF21 and the shortest and longest fractions (< 5 μm and > 20 μm) cleared faster than fibres in the intermediate size ranges (5–20 μm).

Clearance half-times ($t_{1/2}$) for each size fraction were estimated by fitting a single exponential function to the data. A linear regression was performed on the natural logarithm of the retention data for individual animals. This method was chosen over fitting a single exponential to the basic retention as the standard deviation on loge retention is relatively constant. The linear regression analysis provided a best estimate of the gradient and the 95% confidence limits on this estimate, from which the $t_{1/2}$ and its 95% confidence limits were derived (Table 3). The two fibre types showed marked differences in their clearance characteristics, with $t_{1/2}$ all fibres of 231 and 64 days for MMVF21 and HTN, respectively. For MMVF21, shorter fibres cleared faster than longer fibres. For HTN, length related differences in clearance were more marked. The longest and shortest fibres (< 5 and > 20 μm) had $t_{1/2}$ values which were considerably lower than the fibres in the intermediate size ranges. SEM of the fibres indicated that whilst MMVF21 fibres were relatively unchanged in appearance with time, HTN fibres were eroded and fragmented.

DISCUSSION

Instillation has been criticised as a route of administration as mechanical clearance from the lung may be impaired or even halted by overloading of the lungs. In this study, the lungs of animals exposed to both fibre types were loaded to a similar extent both in terms of mass and fibre number, so any effects on mechanical clearance would have been similar for both groups. The differences in clearance between the groups therefore directly reflect differences in lung biopersistence of the fibres.

For MMVF21, clearance was relatively slow and there was a slightly faster clearance of shorter fibres than longer fibres. This is consistent with the fact that stonewool fibres are more soluble in the macrophages, than in the neutral conditions of the lung tissue. The longer the fibre, the more likely it is to be in a neutral environment, therefore the slower its dissolution in the lung as indicated by the results of this study. The HTN fibre has a relatively low dissolution rate in vitro at pH 7.5, similar to that of normal stonewool, but a high dissolution rate at pH 4.5,
Knudsen et al., (in press). Chemically compared to the traditional stonewool (MMVF21), the HTN fibre type is relatively low in silicon oxide and higher in aluminium oxide, Knudsen et al. (in press). For HTN, all fractions cleared considerably faster than MMVF21. The longest and shortest fibres appeared to be clearing faster than intermediate sized fibres. The > 20 μm fraction is too long for complete ingestion by alveolar macrophages (Morgan et al., 1982) and following lavage of lungs, these fibres have been observed with macrophages clustered along their length like pearls on a chain. As HTN fibres are more soluble in vivo than MMVF21, the longer fibres would be more likely to dissolve at the sites of macrophage contact and hence more likely to fragment into shorter fibres. Evidence for this was seen in the appearance of the fibres under the SEM, with the longer HTN fibres appearing eroded and fragmented. Fractionation of the longer fibres will “enrich” the pool of fibres in the lower size ranges, reducing the overall clearance of these fractions. This phenomena has also been observed following inhalation studies with MMVF fibres (Mussleman et al., 1994). In the present study, this enrichment appears to be occurring up to about 90 days after administration. Beyond 90 days far fewer longer fibres were available to enrich the shorter fractions and so clearance from the shorter fractions appeared to increase. Over the interval 141–181 days, clearance of the shortest fibre fraction is the fastest, with the other fractions clearing at similar rates.

This study has demonstrated that fibre length, fibre chemistry and fibre fragmentation all affect biopersistence. All length fractions of the HTN fibres were cleared much faster from the lungs than the traditional stonewool fibres (MMVF21). Shorter stonewool fibres were found to dissolve faster in the lung than longer fibres, confirming the hypothesis that the pH within the macrophages and lung environments determines fibre dissolution within the lung. Alterations in fibre chemistry can produce fibres with significantly shorter clearance half times which may prove to be less hazardous if inhaled. When fibre solubility at low pHs is high, digestion by macrophages at points along the length of the fibre, may result in significant fragmentation into shorter fibres which will enrich the shorter fibre populations. This study has shown the low biopersistence of the HTN fibre type which has a high in vitro solubility at pH 4.5, but low at pH 7.5.

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REFERENCES


