DO VITREOUS FIBERS BREAK IN THE LUNG?

*Walter Eastes**
Granville, OH, U.S.A.

Paul A. Baron
U.S. Department of Health and Human Services, Public Health Service,
Centers for Disease Control and Prevention,
National Institute for Occupational Safety and Health,
Cincinnatti, OH, U.S.A.

Robert E. Baier
State University of New York at Buffalo,
Buffalo, NY, U.S.A

Marianne Guldberg
Rockwool International A/S, Hedehusene, Denmark

Russell Potter
Owens Corning, Granville, OH, U.S.A.

*to whom correspondence should be addressed at 288 Bryn Du Drive, Granville, OH 43023, U.S.A. Email:
eastes@infinet.com

ABSTRACT

In order to determine if breakage of long vitreous fibers in the lung could be responsible for removing significant numbers of these fibers, an intratracheal instillation study was done with a preparation consisting of mostly long fibers of two different types. Following instillation of both fibers, laboratory rats were sacrificed at 6 times up to 14 days. The NK (conventional borosilicate glass) fiber preparation had about 20% short fibers (length ≤15 μm) initially, and fibers recovered from the lungs remained at that proportion for the entire 14 days. But the HT (a new rock or stone wool) fiber preparation, which had about 5% short fibers initially, jumped to about 50% short fibers at 2 days and remained at that proportion for the rest of the study. The appearance of many short HT fibers where there were few initially is conclusive evidence that these long fibers break, and it explains their rapid removal from the lung. Since the HT fibers dissolve rapidly at acid pH, but slowly at the near neutral pH of the extracellular lung fluid, it is likely that acid attack by phagocytic cells is causing the long fibers to dissolve and break. The long NK fibers dissolve rapidly at neutral pH but slowly at acid pH and thus appear to clear by more or less uniform dissolution without apparent breakage. The long fibers of these two kinds are removed rapidly at about the same rate, but by a different mechanism.

Introduction

It is now well established that the dissolution and breakdown of fibers in the lung are important factors in the clearance of inhaled long fibers from the lung and consequent absence of adverse health effects (IARC, 2002). Fiber dissolution, leaching, and breakage are often mentioned as the mechanisms for clearing long fibers, but

there is very little evidence that fibers actually break in the lung. To provide such evidence, this paper reports the intratracheal instillation in laboratory rats of a fiber preparation consisting of nearly all long fibers of two different types, followed by serial sacrifice and measurements of the fiber sizes remaining in the animals' lungs. If significant numbers of short fibers were to appear in the lungs, when there were few initially, then it would provide direct evidence for fiber breakage of that fiber type.

It has been found that the disappearance of long borosilicate, refractory ceramic, slag wool, and several other types of synthetic vitreous fibers from animal lungs following inhalation or intratracheal instillation can be explained quantitatively by their respective dissolution rate measured in vitro at near neutral pH in cell free systems (Eastes & Hadley, 1995). For these fiber types, a nearly uniform dissolution of the fibers is sufficient to explain the observed lung clearance, and fiber breakage need not be invoked.

A new type of rock wool or stone wool fiber, here called an HT fiber, has been developed (Guldberg et al., 2000; Guldberg et al., 2002), the long fibers of which disappear rapidly from animals' lungs, and yet their dissolution rate at near neutral pH in vitro is not high enough to explain it. Since these types of fibers dissolve quite rapidly at acid pH (Knudsen et al., 1996; Guldberg et al., 2002), it has been proposed that these fibers are attacked by acid solutions excreted by phagocytic cells as they come into contact with the long fibers. This acid attack would then break the fibers into many pieces, allowing them to be cleared efficiently by macrophage mediated mechanical clearance (Kamstrup et al., 2001). Without such breakage, it is difficult to explain how these types of fibers clear so rapidly, as the long fibers are too long to be completely engulfed by macrophages and subjected to breakdown by the phagolysosomes (Eastes & Hadley, 1995).

Previous inhalation and intratracheal instillation studies have used fiber preparations consisting predominately of short fibers, typically with less than 10% of the fibers longer than 20 μm (Bernstein et al., 1996). Therefore these studies were unable to observe a buildup of short fibers resulting from long fibers breaking in the presence of an overwhelming number of short fibers overall (Eastes & Hadley, 1995). The study reported here aims to reverse that situation by starting with predominately long fibers so that the generation of any short fibers from breakage of long ones can be observed unambiguously.

Materials and Methods

The starting point for the production of the long fiber preparation was a quantity of each fiber type that had been crushed and separated by a waterborne process so that most of the fibers were respirable by laboratory rats. The NK fiber was the same as NK8340 studied previously (Eastes et al., 2000) and the HT fiber is the same as RIF42020-2 reported previously (Guldberg et al., 2000). Their compositions are given in Table 1.

Table 1. Composition of the fiber types used in this study.

Oxide	NK	HT
SiO_2	54.06	37.0
Al_2O_3	2.05	20.7
Na_2O	17.17	4.1
K_2O	0.83	0.6
B_2O_3	15.66	-
CaO	7.50	18.3
MgO	1.80	10.7
Fe_2O_3[a]	0.136	-

FeO	0.056	5.4
TiO_2	0.082	1.4
MnO	-	0.5
P_2O_5	-	0.2

[a]Total iron reported as Fe_2O_3.

The fiber preparations containing mostly rat respirable diameters were separated by length using the technique of differential dielectrophoretic classification of fibers in the airborne state (Baron et al., 1994; Baron et al., 2002; Deye et al., 1999). The bulk fibers were sieved and then aerosolized using an orbital motion fluidized bed. The fibers were humidified to make them sufficiently conductive, neutralized using several [210]Po sources, and introduced into the classifier. The separation voltage was selected so that the mean fiber length was approximately 24 μm and the classifier resolution was reduced somewhat to increase the throughput of classified fibers. This length was chosen to ensure that virtually all the size-selected fibers would be too large to be completely engulfed by rat macrophages. The dielectrophoresis technique separates particles according to their length while airborne, so shorter fibers attached to longer fibers or agglomerates consisting of shorter fibers are present as part of the classified material. The diameter distributions of the two fiber preparations thus produced are similar but not the same, with NK averaging 1.6 μm compared to 1.2 μm for HT and standard deviations of 0.7 and 0.5 μm respectively. These differences in diameter could influence how soon the fibers dissolve or break following administration but are not expected to impact the clearance mechanism, whether they dissolve or break.

The animals used in this study were Fischer 344 rats, which were 43 to 55 days old and weighed an average of 120 g before instillation. For the results reported in this paper, equal aliquots of the NK fiber suspension of 0.2 ml, containing 0.04 mg of fibers (about 250,000 NK fibers and about 125,000 HT fibers), were instilled into 6 animals. They were sacrificed at 2 hours, 1 day, and 2, 5, 11, and 14 days after instillation. Aliquots of the HT fiber suspension were instilled into 16 animals, one of which was sacrificed at 2 hours afterwards, and 3 each at the other 5 times. The lungs of each animal were excised and frozen. The 2 hour sacrifice was done to check that an adequate number of fibers had been administered. The other sacrifice times were chosen to cover the times when significant changes in fiber numbers in the lung were expected based on previous biopersistence studies. Since direct evidence of fiber breakage had not been observed before and was the point of the study, 3 animals with HT fibers were sacrificed at each chosen time compared to one animal at each time for NK.

An aliquot of the NK and HT fiber suspension was filtered and prepared for scanning electron microscope (SEM) measurement. The frozen lungs from each animal were thawed, low temperature ashed for 48 hr each, and the remains suspended in nanopure water and filtered onto 25 mm diameter 0.2 μm pore size mixed cellulose ester membrane filters. These were dried and cut into wedges, and each wedge cleared with acetone vapor and mounted for light microscope (LM) measurements.

The fiber measurements of both SEM and LM mounted samples were carried out using the conventional end selection rules for fiber counting (NIOSH, 1989; WHO, 1985) except that the fibers thus selected were not only counted, but their length and diameter were measured. An attempt was made to measure at least 100 fibers, but that number was not achieved for some samples that had very few fibers. In addition, the SEM initial aliquot and some of the 1 day LM samples were separately measured initially by a selection method that was approximately but not exactly end selected. These initial measurements were done to get an estimate of the numbers of fibers recovered to judge if this experiment was feasible.

The main results are reported as the percent of short fibers, that is fibers less than or equal to 15 μm in length, out of all the fibers measured at that sacrifice time for that fiber type instilled. In addition, the standard error of that

percentage is reported, which is estimated as the square root of the number of short fibers observed divided by the total number of fibers measured, as is appropriate for such Poisson distributed quantities.

Although this study was not designed for it, an effort was made to estimate the number of long fibers remaining at each sacrifice time for each fiber type. These results are reported as the number of long fibers, just for this purpose defined as 20 μm or longer to be consistent with previous inhalation studies, per mg of dry lung, averaged over the number of samples measured at that sacrifice time. Additionally, the standard error of this average, or the standard deviation divided by the square root of the number of samples, is also reported. When only one sample was measured, then no standard error is available.

Results

A useful check on the consistency of the fiber measurements is to compare the percent of short fibers (15 μm long or shorter) as measured by the standard, end selected method with the measurements of the initial aliquot and 1 day samples done as an initial scan of these samples, but not exactly by consistent end selection rules. These are compared in Table 2, which shows the percent, the standard error of this percent, and the total number of fibers measured in parentheses. The standard error limits overlap in all cases.

Table 2. Percent of short fibers ± standard error (total number of fibers) measured in two cases independently.

Time	Method	NK	HT
Initial aliquot	Standard	18 ± 3 (256)	5 ± 1 (251)
	Non-standard	28 ± 8 (209)	7 ± 2 (123)
One day	Standard	20 ± 4 (122)	18 ± 4 (133)
	Non-standard	16 ± 5 (62)	18 ± 4 (101)

The main results of this experiment are shown in Table 3. The NK fiber preparation began with about 20% short fibers and remained at that level for the entire two weeks. Some of the later sacrifices had large standard errors, because so few fibers were found. The HT fiber began at 5% short fibers but jumped to 50% or more short fibers by 2 days after instillation, remaining there for the entire two weeks studied. As with NK, some later sacrifices of HT had large standard errors, because so few fibers could be found.

Table 3. Percent of short fibers ± standard error (total number of fibers) measured for each fiber type at each sacrifice time following installation.

Time	NK	HT
Initial	18 ± 3 (256)	5 ± 1 (251)
2 hours	8 ± 3 (100)	33 ± 6 (100)
1 day	20 ± 4 (122)	18 ± 4 (133)
2 days	19 ± 7 (42)	55 ± 17 (20)
5 days	26 ± 11 (23)	56 ± 6 (167)
11 days	25 ± 11 (20)	60 ± 6 (156)
14 days	9 ± 3 (100)	55 ± 9 (76)

The average number of long fibers, just for this purpose defined as fibers 20 μm long or longer, remaining per milligram of dry lung are shown in Figure 1 for each fiber type at each sacrifice time with error bars denoting the standard error. Not shown in Figure 1 are the counts at 2 hr after instillation, which are off the scale at 313 long fibers/mg for NK and 162/mg for HT. No standard error estimates are available for the 2 hr samples since only one sample was measured. For the same reason, no standard error bars are given for the NK outlier at 14 days. The reason for this outlier is unclear, since no fewer fibers were counted for this sample than for many others (Table 3). However, the number of fields scanned to find these fibers amounted to less than 1 mg of dry lung for this sample, whereas 5 to 10 mg was searched for most other samples.
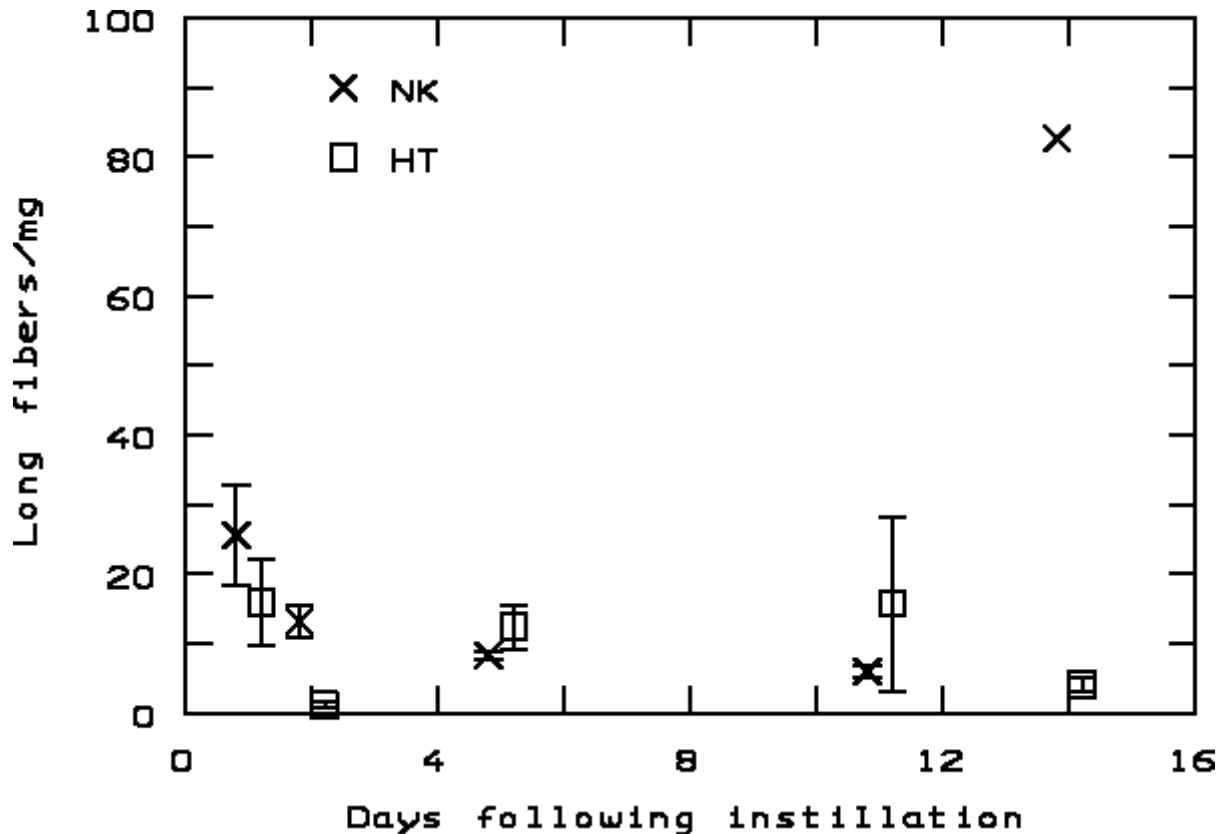

Figure 1. Average number of long fibers per mg dry lung recovered for each fiber type and sacrifice time with standard error bars. The NK and HT values are offset by a small amount horizontally in opposite directions from the exact time in order to show each clearly. Two values at 2 hr (or 0.083 days) are off the scale and are not shown: NK = 313 and HT = 162. The data in this graph are available here in tab separated text format.

It is clear from Figure 1 that long fibers of the two types clear very rapidly and at about the same rate, reaching 50% of the 1 day level by 5 days.

Discussion

The original intention for this experiment was to have a vanishingly small number of short fibers so that, when any such short fibers were observed, they could have come only from the breaking of long fibers. What length defines a short fiber depends partly on the purpose and partly on what is achievable. Some conventions consider the length limit to be 5 μm (WHO, 1985), because anything shorter is easily phagocytized. Other studies (Bernstein et al., 1996) take the limit to be 20 μm, since any fiber longer than this is unlikely to be subject to macrophage mediated physical clearance, at least in rats, but human macrophages are somewhat larger (Zeidler-Erdely et al., 2006). For the present purpose, then, the length limit reasonably could be anywhere between 5 and 20 μm. Since the NK preparation turned out to have a significant number of fibers between 15 and 20 μm long by the time the instillation suspensions were prepared, 15 μm was chosen as the length less than or equal to which is defined as a short fiber.

With this definition, the NK preparation had 18% short fibers in the initial aliquot, more than desired, but still feasible. The HT fiber preparation, on the other hand, had only 5% short fibers, a much better proportion for this purpose.

The repeated measurements of the initial aliquot by SEM and the 1 day lung samples by LM showed adequate agreement (Table 2), even though they were done independently. It indicates a certain robustness of the results. The initial aliquots were measured by SEM and not by LM because the entire aliquot was needed to make the SEM stub. It was decided to measure the lung samples by LM so that more slide area could be covered more quickly to find enough fibers. The higher available resolution of SEM was not needed to measure these fiber lengths adequately, and accurate diameter measurements were not needed.

Unambiguous evidence that long HT fibers are breaking in large numbers is shown by the fact that the proportion of short fibers jumps to about 50% within 2 days of instillation compared to 5% initially (Table 3). That this breakage is a continuing process is indicated by the fact that the proportion of short fibers remains at the 50% level for the rest of the two weeks observed. The continuing large proportion of short fibers while the number of long fibers in the lung is decreasing with time by a factor of 3 from 5 to 14 days (Figure 1) suggests that breakage is converting the long fibers to short fibers, which in turn are efficiently cleared by macrophage action.

In contrast, the proportion of short NK fibers is essentially unchanged at about 20% throughout the study. Thus there is no evidence of long fibers breaking, although a small amount of long fiber breakage would not be observed in the presence of the initial 18% short fibers. Clearly, the extent of breakage observed for HT does not happen with NK, or else it would be observable even with the comparatively large initial short fiber fraction.

The lack of observable fiber breakage in the NK fibers is consistent with the finding (Eastes & Hadley, 1995) that the dissolution rate of these fibers measured in vitro at near neutral pH is sufficient to explain the long fiber clearance rate following inhalation or intratracheal instillation. It is likely that some breakage of NK fibers happens near the end of their dissolution, given that the lung environment is not homogeneous, but breakage is not the mechanism that clears significant numbers of these fibers.

The in-vitro measured dissolution rate of HT fibers at neutral pH is too small to explain the observed rapid clearance of long HT fibers following inhalation (Eastes et al., 2000), but they do dissolve very rapidly at acid pH (Knudsen, et al., 1996). Since some phagocytic cells are known to generate quite acid pH within the phagolysosomes (Nyberg et al., 1991) or at their surfaces where they attach to objects too big to engulf (Etherington et al., 1981), it is likely that this acid attack dissolves HT fibers at certain points, causing them to break. The dissolution rate of HT fibers measured in vitro (Knudsen, et al., 1996) is sufficient to dissolve through the fibers in the 2 days after which extensive breakage was observed (Table 3). The apparent increase in the fraction of short fibers at 2 hours compared to the initial aliquot is more likely caused by breakage in sample handling than to phagocyte action. The breakage process appears to be continuous while long fibers are present, rapidly removing them. No doubt, the NK fibers are subjected to the same phagocytic action, but, since they do not dissolve rapidly at acid pH, they do not break. The question is still open about whether different surface compositions of vitreous mineral fibers can induce different phagocytic responses in the lung (Baier et al., 2000) in a manner similar to that demonstrated by Carter and co-workers (Carter, et al, 2000) for particulate matter in a related live-chick membranous model system.

Thus it would appear that at least two different dissolution mechanisms are available to clear long fibers. If the fibers dissolve readily in the near neutral lung fluid as with NK and several other vitreous fiber types, then they dissolve more or less uniformly. If they dissolve readily in acid solution, as with HT, then phagocyte attack breaks them into short fibers readily cleared by macrophage physical action. For these two particular fiber compositions, HT and NK, the two mechanisms happen to clear them equally rapidly. Future investigations can reveal the functional dependence of the actual phagocytic mechanisms that may be modulated by particular fiber compositions, as a useful step toward employment of fiber glass compositions in tissue engineering (DeDeigo, et al., 2000).

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REFERENCES

Baier, R., Meyer, A., Glaves-Rapp, D., Axelson, E., Forsberg, R., Kozak, M., and Nickerson, P., 2000. The body's response to Inadvertent Implants: Respirable particles in lung tissues. *J. Adhesion* 74:103-124.

Baron, P. A., Deye, G. J., Fernback, J. 1994. Length separation of fibers. *Aerosol Science and Technology* 21: 179-192.

Baron, P., Deye, G. J., Aizenberg, V., Castranova, V. 2002. Generation of size-selected fibers for a nose-only inhalation toxicity study. Inhaled Particles IX, Oxford, British Occupational Hygiene Society, vol. 54A, pp. 243-259.

Bernstein, D. M., Morscheidt, C., Grimm, H.-G., Thvenaz, P., Teichert, U. 1996. Evaluation of soluble fibers using the inhalation biopersistence model: A nine-fiber comparison. *Inhal. Toxicol.* 8:345-385.

Carter, L. C., Carter, J. M., Nickerson, P. A., Wright, J. R., and Baier, R. E., 2000. Particle-induced phagocytic cell responses are material dependent: Foreign body giant cells vs osteoclasts from a chick chorioallantoic membrane particle-implantation model. *J. Adhesion* 74:53-77.

DeDeigo, M. A., Coleman, N. J., Hench, L. L., 2000. Tensile properties of bioactive fibers for tissue engineering applications. *J. Biomed. Mater. Res. (Appl. Biomater.)* 53:199-203.

Deye, G. J., Gao, P., Baron, P. A., Fernback, J. E. 1999. Performance evaluation of a fiber length classifier. *Aerosol Science and Technology* 30:420-437.

Eastes, W., & Hadley, J. 1995. Dissolution of fibers inhaled by rats. *Inhal. Toxicol.* 7:179-196. Also available here.

Eastes, W., & Hadley, J. 1996. A mathematical model of fiber carcinogenicity and fibrosis in halation and intraperitineal experiments in rats. *Inhal. Toxicol.* 8:323-343. Also available here.

Eastes, W., Potter, R. M., and Hadley, J. G. 2000. Estimation of dissolution rate from in vivo studies of synthetic vitreous fibers. *Inhal. Toxicol.* 12:1037-1054. Also available here.

Etherington, D. J., Pugh, D., and Silver, I. A. 1981. Collagen degradation in an experimental inflammatory lesion: studies on the role of the macrophage. *Acta. Biol. Med. Germ.* 40:1625-1636.

Guldberg, M., deMeringo, A., Kamstrup, O., Furtak, H., and Rossiter, C. 2000. The development of glass and stone wool compositions with increased biosolubility. *Regulatory Toxicology and Pharmacology* 32:184-189.

Guldberg M., Jensen, S. L., Knudsen, T., Steenberg, T., Kamstrup, O. 2002. High-Alumina Low-Silica HT Stone Wool Fibers: A Chemical Compositional Range with High Biosolubility. *Reg. Toxicology and Pharmacol.* 35:217-226.

International Agency for Research on Cancer, 2002. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 81, *Man-made Vitreous Fibres*, pp. 255-269. Lyon: IARCPress.

Kamstrup, O., Ellehauge, A., Chevalier, J., Davis, J. M. G., McConnell, E. E., Thvenaz, P. 2001. Chronic inhalation studies of two types of stone wool fibers in rats. *Inhal.Toxicol* 13:603-621.

Knudsen, T., Guldberg, M., Christiansen, V. R., and Lund Jensen, S. 1996. New type of stonewool (HT Fibres) with a high dissolution rate at pH = 4.5. *Glastech. Ber. Glass Sci. Technol.* 69:331-337.

National Institute for Occupational Safety and Health 1989. Fibers, Method 7400 (Revision #3). In: *NIOSH Manual of Analytical Methods*, 3rd ed. Vol 1. DHHS (NIOSH) Pub. No. 79-127, NTIS Pub. No. PB-297-731/A05, National Technical Information Service, Springfield VA.

Nyberg, K., Johansson, U., Johansson, A., Camner, P. 1991. Phagolysosomal pH and location of particles in alveolar macrophages. *Fundamental and Applied Toxicology* 16:393-400.

World Health Organization 1985. *Reference Methods for Measuring Airborne Man-Made Mineral Fibres (MMMF)*. WHO, Copenhagen.

Zeidler-Erdely, P. C., Calhoun, W. J., Ameredes, B. T., Clark, M. P., Deye, G. J., Baron, P., Jones, W., Blake, T., Castranova, V. 2006. Cytotoxicity of Manville code 100 glass fibers: Effect of fiber length on human alveolar macrophages. *Particle and Fibre Toxicology*, http://www.particleandfibretoxicology.com/content/3/1/5