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Transfection of Human Mesothelial Cells Mediated by Different Asbestos Fiber Types

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Several different asbestos fiber types mediate transfection of human mesothelial cells by exogenous DNA. We have employed the human MeT-5A mesothelial cell line, which allows the use of DNA replication as an assay for entry of DNA when plasmids bearing the SV40 origin of replication are used for transfection. We find that Canadian chrysotile, Calidria chrysotile, amosite, and crocidolite are each capable of introducing plasmid pSVod DNA into MeT-5A cells followed by subsequent replication of a fraction of the plasmid DNA. A significant fraction of the input plasmid DNA associated with the cells in the presence of asbestos is fragmented, and this fragmentation is particularly evident with crocidolite. Each of the fiber types is highly cytotoxic for the MeT-5A cells, and these cells actively accumulate the added fibers from the surrounding environment as visualized by phase-contrast microscopy. MeT-5A cells were transfected at higher efficiency with calcium phosphate than were several other primate cell lines. Calcium phosphate, however, did not induce fragmentation of the input plasmid DNA. Compared with several different mineral agents, including glass fibers, kaolin, and talc, Calidria chrysotile fibers were most effective at mediating transfection of the MeT-5A cells. Results provide a mechanism by which transfection can contribute to mutagenicity of asbestos fibers and indicate that this mechanism can operate in human mesothelial cells.

INTRODUCTION

Several different asbestos fiber types have been linked to the induction of malignant mesothelioma or bronchogenic carcinoma in humans (Wagner *et al.*, 1960; Selikoff *et al.*, 1964; Nicholson, 1986; Morinaga *et al.*, 1989; Begin *et al.*, 1992). At this time, however, no specific genetic locus is known to be invariably altered in asbestos-induced cancer. While cytogenetic evidence indicates several chromosomal alterations are associated with mesothelioma, and several such alterations are induced in cells by asbestos, at present these alterations cannot be ordered in any unique sequential pathway. In human mesothelial cell cultures exposed to asbestos a high percentage of chromosome breaks have been seen in chromosome 1, although several other chromosomes have also displayed abnormalities, and no distinctly recurrent abnormality has been reported in any particular band (Olofsson and Mark, 1989). A chromosome breakpoint at 1p11-22 has been observed as a recurrent, although not invariant, abnormality in mesotheliomas and is associated with a high asbestos burden (Tainen *et al.*, 1989). In that study, large cytogenetically detectable deletions in chromosomes 1 and 4 were also associated with a high asbestos burden. Asbestos has also been observed to cause changes in chromosomal DNA of cells indicative of DNA recombination (cf. Knuutila, 1991). For example, increased frequencies of sister chromatid exchange are induced in rat pleural mesothelial cells by crocidolite fibers (Achard *et al.*, 1987). It is most likely that induction of mesothelioma involves a combination

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of these mutational and/or recombinational events effective at more than one specific gene locus.

Little is known about the molecular mechanisms through which asbestos fibers can interact with chromosomal DNA to induce specific gene mutations. It is conceivable that mechanical interference with mitosis can account for aneuploidy induced by asbestos. Syrian hamster cell lines transformed by asbestos have been reported to display trisomy of chromosome 11 (Oshimura *et al.*, 1984). Polysomy of chromosome 7 is frequently seen in human mesothelioma (Knuutila, 1991). Asbestos has been observed to interact directly with chromosomes in rat pleural mesothelial cells (Wang *et al.*, 1987). In cells exposed to chrysotile, an increase in number of anaphase abnormalities, including lagging chromosomes, has been reported (Hesterberg and Barrett, 1985). While this could suggest an interaction of asbestos fibers with the mitotic spindle apparatus (Hesterberg *et al.*, 1986; Cole *et al.*, 1991), no direct evidence for this has been reported. In any case, such mechanical intervention may not account for the full spectrum of rearrangements and deletions induced by asbestos. Early studies have not detected significant asbestos-induced mutagenicity as measured at target gene loci either in bacterial (Chamberlain and Tarmy, 1977) or mammalian cell systems (Reiss *et al.*, 1982; Oshimura *et al.*, 1984). More recently, test systems have been developed which demonstrate the mutagenicity of asbestos fibers in cultured mammalian cells (Fasy, 1991; Hei *et al.*, 1991; Stankowski *et al.*, 1992). Many of these mutations affect large chromosomal loci and would not have been detected in earlier systems in which inactivation of important sequences flanking the target gene would be lethal. Nothing is presently known about the mechanisms by which such mutations are generated.

The ability of asbestos fibers to mediate transfection of cells by exogenous nucleic acids could help explain many aspects of fiber mutagenicity. We have previously reported that chrysotile fibers can mediate the transformation of monkey COS-7 cells by exogenous plasmid DNA (Appel *et al.*, 1988). Dubes and Mack (1988) have reported that chrysotile, amosite, and crocidolite are each capable of mediating transfection involving exogenous RNA. It is now well documented that DNA introduced into cells via transfection is highly recombinogenic, most of the recombination events being of the nonhomologous type and therefore mutagenic (Calos *et al.*, 1983; Razzaque *et al.*, 1984). Most types of mutations resulting from transfected DNA are independent of chromosomal insertion of that DNA (Bardwell, 1989). It has been demonstrated that transfection of Chinese hamster cells with highly repetitive human DNA sequences can induce chromosome aberrations, aneuploidy, and sister-chromatid exchange (Heartlein *et al.*, 1988).

The ability of transfecting DNA to replicate is a useful criterion for confirming the entry of that DNA into cells (Appel *et al.*, 1988). In that regard, monkey COS cells have provided a standard test system since they contain a partial, integrated SV40 genome, produce T-antigen constitutively, and will initiate replication of plasmids bearing an SV40 origin of replication (Mellon *et al.*, 1981). Recently, a human mesothelial cell line, MeT-5A, has been constructed which also produces T-antigen constitutively (Gerwin *et al.*, 1987; Ke *et al.*, 1989). The present report documents the ability of several different asbestos fiber types to mediate transfection of MeT-5A cells. This report also describes the potent cytotoxic effect of

asbestos fibers on the MeT-5A mesothelial cells and the striking ability of these cells to accumulate asbestos fibers.

MATERIALS AND METHODS

Asbestos fiber types and other mineral agents. Canadian chrysotile, amosite, and crocidolite asbestos fiber types used were samples standardized for research and distributed by the Union Internationale Contre le Cancer (UICC). Fiber sizes and other characteristics have been described by Rendall (1980). Canadian chrysotile was UICC Canadian chrysotile sample B. Calidria chrysotile was unmodified HPO fiber kindly provided by KCAC, King City, California. Fibers were sterilized dry by heating at 250°C for 6 hr prior to suspension in sterile 50 mM Hepes, pH 7.0, 0.1 M NaCl (HBS) for transfection. Glass fibers were prepared by milling Pyrex wool filtering fiber (Corning Catalog No. 3950) with a mortar and pestle as previously described (Appel *et al.*, 1988). Diameters of the prepared fibers ranged from 15 to 30 μm, and lengths varied from 30 to 600 μm. Calcium phosphate precipitates were formed and used in transfections for Fig. 4 as previously described (Graham and Van der Eb, 1973; Appel *et al.*, 1988). Mineral agents employed for experiments in Table I and Fig. 6 were: calcium phosphate (Mallinckrodt, No. 4265), kaolin (J. T. Baker, No. 2242), talc (Mallinckrodt, No. 8476); these minerals were kindly provided by Dr. George Dubes (Dubes and Mack, 1988). Each was sterilized dry as described and stored in sterile 50 mM Hepes, 0.1 M NaCl, pH 7.0, prior to use.

Cells and transfection conditions. MeT-5A cells were the generous gift of Dr. John Lechner. Cells were grown in medium consisting of three parts MCDB 105 medium to two parts medium 199 (both from Sigma), supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were transfected as described earlier for COS-7 cells (Appel *et al.*, 1988) using varying amounts of asbestos fibers and plasmid pSVod DNA, as indicated in figure legends, per 5×10^5 cells, the contents of one 10-cm-diameter culture dish. Cells were harvested at times indicated in the figure legends. Plasmids employed for transfection in this study were pSVod and pMAM-neoCAT, propagated in *dam*$^+$ *Escherichia coli* strain HB101. Plasmid pSVod contains an SV40 origin of replication in a truncated version of pBR322, and it replicates in monkey COS cells (Mellon *et al.*, 1981). Plasmid pMAM-neoCAT (Clontech) also contains an SV40 origin of replication. In addition, it contains a chloramphenicol acetyltransferase (CAT) gene linked to the dexamethasone-inducible LTR promoter of the murine mammary tumor virus.

Assay for DNA replication. Hirt supernatant DNA was prepared from harvested cells (Hirt, 1967) following transfection and treated with restriction endonucleases to assay for newly replicated DNA. Following treatment with restriction endonuclease Bam HI (New England Biolabs, 20 U/μg DNA), to linearize any circular plasmid DNA in the supernatant, samples were further treated with restriction endonuclease DpnI (New England Biolabs, 20 U/μg DNA), which digests unreplicated pSVod DNA (Mellon *et al.*, 1981), for 2 hr at 37°C. Agarose gel electrophoresis, blotting, and hybridization were performed as described previously (Appel *et al.*, 1988). Blots were probed with pBR322 DNA labeled with [α-^{32}P]dCTP to approximately 3×10^8 cpm/μg DNA. pBR322 is homologous to much of plasmid pSVod but not to other components of Hirt supernatant DNA.

Assay for CAT activity in cells transfected with pMAM-neoCAT. For this study all cell lines were transferred to Dulbecco's modified Eagle medium containing 10% fetal bovine serum. COS-7 monkey kidney cells, MeT-5A human mesothelial cells, GM2522 human fibroblasts, and human Hela cells were plated at 10^6 cells per 100-mm dish 24 hr before transfection. A standard calcium phosphate precipitation with 10 μg pMAMneo-CAT was employed. The calcium phosphate precipitate was removed 4 hr after transfection by washing with 1× PBS, followed by glycerol shock as described elsewhere (Lopata *et al.*, 1984). Dexamethasone (Sigma) was added to each dish at 1.0 μM after 24 hr. Cells were harvested 24 and 48 hr after adding dexamethasone, collected by centrifugation at 15,000*g* and resuspended in 100 μl of 0.25 M Tris–HCl buffer, pH 7.5. Cells were disrupted by freezing and thawing three times. After centrifugation at 15,000*g* for 5 min to remove cell debris, 20 μl of supernatant was used for assay of CAT activity. To each sample was added 76.5 μl H_2O, 20 μl of 4 mM acetyl-CoA (Boehringer-Mannheim), 32.5 μl of 1.0 M Tris–HCl, pH 7.5, and 1 μl of D-threo-[dichloroacetyl-1,2-^{14}C]chloramphenicol (8.8×10^{-4} M; 56.8 mCi/mmole; NEN). Samples were incubated for 1 hr at 37°C. Samples were subjected to thin-layer chromatography on silica gel 1B plates (J. T. Baker, No. 4-4462) essentially as previously described (Gorman *et al.*, 1982). Plates were dried and autoradiographed with an intensifying screen at −70°C on Kodak X-OMAT-AR film.

Cytotoxicity measurements. MeT-5A cells were plated at 7.5×10^3 cells per dish in culture dishes with 1-cm grid markings. Dishes were treated with different amounts of asbestos fibers as indicated in figure legends. At different times after addition of asbestos, number of cells per square centimeter were determined by averaging over five grid units per dish.

RESULTS

Cytotoxic response of MeT-5A human mesothelial cells to chrysotile, amosite, and crocidolite asbestos fibers. In order to assess the propensity of asbestos fibers to kill mesothelial cells upon direct exposure, MeT-5A cells were exposed to varying concentrations of each of four different asbestos fiber types: Canadian chrysotile, Calidria chrysotile, amosite, and crocidolite. Figure 1a shows that, on a per-weight basis, all of the different fiber types had approximately equally lethal effects on the cultured mesothelial cells. In each case, greater than 40% mortality was observed using 2 μg of asbestos per culture dish containing 15 ml of medium. In a direct comparison of amosite and Canadian chrysotile (Figs. 1b and 1c), it can be seen that both fiber types reduce cell number by 45 to 70% at 6 days at doses ranging from 2 to 20 μg of fiber per dish. However, at the 2-μg dose with amosite, cells resume approximately normal growth rate by 7 days (Fig. 1c), whereas with the same dose of chrysotile, growth rate is further depressed with increasing time (Fig. 1b).

The cytotoxicity reported here for MeT-5A cells is much higher than that previously observed using COS-7 cells (Appel *et al.*, 1988). In that study no significant cell death was induced in 24 hr by a dose of 50 μg of chrysotile fibers per plate, and only moderate killing was observed after 10 days. These data indicate that the MeT-5A cells are highly susceptible to the cytotoxic effects of asbestos. On a per-weight basis, all fiber types are approximately equal in their cytotoxic effect. On average the chrysotile fibers are of smaller diameter than the amphibole fibers (Rendall, 1980), and thus more chrysotile fibers are represented by a given weight. Nonetheless, a broad range of fiber diameters can be observed for each

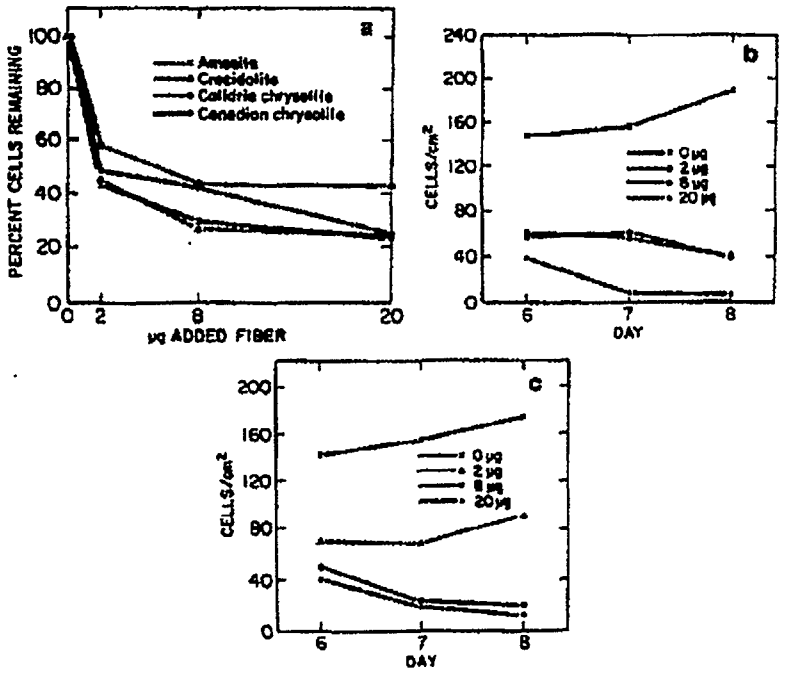

FIG. 1. Cytotoxic effects of different asbestos fibers on MeT-5A mesothelial cells in culture. (a) Cell-killing effects of four different asbestos fiber types at different doses. MeT-5A cells (7.5×10^3 cells per 10-cm dish) were treated with varying doses of Canadian chrysotile, Calidria chrysotile, amosite, or crocidolite as described under Materials and Methods. At 3 days after addition of fibers, cell counts were performed as described. Typical experiments are presented in each panel. (b) Time course of MeT-5A cell killing by Canadian chrysotile fibers at different doses. Cells were exposed to Canadian chrysotile fibers, and counts of surviving cells were performed at indicated times as described under Materials and Methods. (c) Time course of MeT-5A cell killing by amosite fibers at different doses. Cells were exposed to amosite fibers, and counts of surviving cells were performed at indicated times as described under Materials and Methods.

fiber type by electron microscopy, and a significant percentage of fibers <0.2 μm is seen for each type. Therefore, to facilitate comparison of our data to the work of others, we report effects of asbestos fibers in terms of fiber weight.

Asbestos-mediated transfection of MeT-5A cells with plasmid pSVod. Since MeT-5A mesothelial cells synthesize SV40 T-antigen constitutively, they permit replication of plasmids containing an SV40 viral origin of DNA replication. Thus one can use an assay for replication to ascertain whether or not the added plasmid DNA has entered the cells and the extent to which the introduced DNA is functional. As described previously (Mellon *et al.*, 1981; Johnson and Jelinek, 1986; Appel *et al.*, 1988), we have employed a *Dpn*I digestion assay to detect newly replicated DNA. *Dpn*I cleaves at the recognition sequence GATC only when the adenine base is methylated, as it is when propagated in *dam*$^+$ strains of *E. coli*. Mammalian cells do not methylate adenosine, however, and after one round of replication in a mammalian system, plasmids originally methylated become *Dpn*I resistant. Since pBR322 has 22 *Dpn*I sites, plasmids based on pBR322 are cleaved into small fragments if they are not replicated. Figure 2 shows a blot hybridization analysis of DNA from Hirt supernatants extracted from MeT-5A cells transfected with plasmid pSVod mediated by Calidria chrysotile. Figure 2A shows that plas-

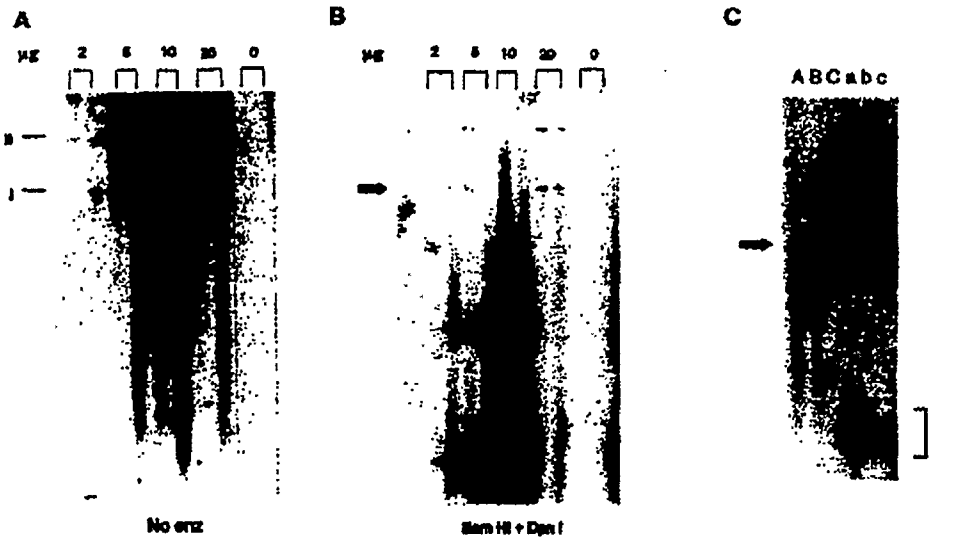

FIG. 2. Hybridization analysis of plasmid pSVod DNA transfected into MeT-5A mesothelial cells mediated by Calidria chrysotile fibers. (A) Analysis of transfected plasmid DNA uncleaved by restriction endonucleases. MeT-5A cells were transfected with plasmid pSVod, using 2, 5, 10, and 20 μg of asbestos and 10 μg of plasmid pSVod per 5×10^5 cells as described under Materials and Methods. Cells were harvested at 8 and 24 hr, Hirt supernatant DNA was prepared, and agarose gel electrophoresis was performed as described previously (Appel *et al.*, 1988) and under Materials and Methods. Amounts of asbestos are indicated above the gel lanes. For each pair of lanes, the leftmost is the 8-hr point and the rightmost is the 24-hr point. Following gel electrophoresis, DNA was blotted to membrane filters and hybridized with ^{32}P-labeled pBR322 DNA as described under Materials and Methods. Autoradiographs are presented. (B) DNA from the same samples as in A, treated with *Bam*HI and *Dpn*I. The arrow indicates the position of the 3.3-kb linear pSVod band. (C) Control pSVod DNA treated with either *Bam*HI (A, B, C) or *Bam*HI and *Dpn*I (a, b, c). Samples of 10, 5, and 1 μg of DNA were treated with 20 U of each enzyme for 2 hr at 37°C. Amounts of plasmid DNA loaded per gel lane were 100 ng (A, a), 50 ng (B, b), or 10 ng (C, c). The arrow indicates the position of the linear, full-length pSVod band. The bracket on the lower right indicates the position of the larger *Dpn*I digestion products of the linearized pSVod band.

mid DNA is detected in the Hirt supernatants when introduced with the asbestos fibers. The amount of plasmid detected is dependent upon the dose of fibers added to the dish up to 10 μg of fibers, above which the amount of detected plasmid declines. No plasmid is detected in Hirt supernatants when asbestos is not included in the transfection mix (lanes 0). Samples for Fig. 2A were not treated with any restriction enzyme, and DNA can be visualized in bands I and II, which represent supercoiled circular and relaxed circular forms of plasmid, respectively. However, a striking feature of Fig. 2A is that a significant fraction of the plasmid DNA detected in the Hirt supernatants is degraded and seen as a smear extending to low molecular weight. For Fig. 2B the plasmid in the Hirt supernatant samples has been linearized with *Bam*HI and treated with *Dpn*I. Gels in Figs. 2B and 2C have been run longer than that in Fig. 2A. The gel in Fig. 2A thus more clearly shows DNA breakdown to small fragments. The arrow denotes the position of the 3.3-kb pSVod molecule. *Dpn*I-resistant DNA, indicative of replication, can be seen at every asbestos concentration tested. In each case this amounts to a minor fraction of the total DNA detected by hybridization. (It is true of most cell transfections, including those of monkey COS cells, that only a small percentage of

entering DNA replicates (Mellon *et al.*, 1981; Johnson and Jelinek, 1986).) Figure 2B shows that more *Dpn*I-resistant DNA is detected at 8 hr with most concentrations of asbestos than at 24 hr. This most likely reflects the higher level of mortality of cells exposed for longer time to the asbestos fibers. Again, in Fig. 2B a vast amount of degraded plasmid DNA is seen, especially at 10 μg of mediating fiber.

In one series of control experiments, varying amounts of plasmid pSVod DNA were digested with *Bam*HI and *Dpn*I in the absence of asbestos fibers or cell extracts (Fig. 2C). At each concentration of DNA, under digestion conditions similar to those of Figs. 2A and 2B, the plasmid DNA was completely digested by *Dpn*I. Additional control experiments were performed using either calcium phosphate or glass fibers as mediating agents for transfection. The results are not shown but can be summarized as follows. Calcium phosphate, used as described previously for COS cells (Johnson and Jelinek, 1986), mediates transfection of pSVod DNA into MeT-5A cells, and a readily detectable although minor percentage of introduced DNA replicates as indicated by the *Dpn*I digestion assay. However, in contrast to results using asbestos fibers, the calcium phosphate did not induce degradation of the DNA detected in Hirt supernatants. In this regard, results were generally similar to those previously reported for transfection of COS-7 cells using asbestos or calcium phosphate (Appel *et al.*, 1988). In the presence of glass fibers, no plasmid DNA was detected in Hirt supernatants from transfected MeT-5A cells, again confirming the reported inability of these larger, thick fibers to mediate transfection.

The abilities of crocidolite and amosite fibers to mediate transfection of MeT-5A cells by plasmid pSVod DNA are demonstrated in Fig. 3. In each case *Dpn*I-resistant DNA is detected in Hirt supernatant samples after using 5 and 10 μg of asbestos fiber per dish. Also in each case, no hybridizing DNA is detected in Hirt supernatants when either asbestos fibers or plasmid DNA is left out of the transfection. The arrows at left denote the positions of *Dpn*I-resistant pSVod DNA linearized with *Bam*HI. In both transfections *Dpn*I-resistant DNA bands can be seen migrating more slowly than the linearized plasmid band, as is characteristic of certain replication intermediates. In the case of crocidolite (Fig. 3A) degradation of the entering DNA is very extensive, as evident by the lack of distinction among the *Dpn*I cleavage bands normally seen at the bottom of the gel. In the case of amosite, degradation is less extensive, and the cleavage bands are clearly distinct at the bottom of the gel.

MeT-5A cells are especially amenable to transfection and accumulate asbestos fibers. Experiments were performed using calcium phosphate as a control transfection mediator in order to assess the amenability of various cell types to transfection. For this experiment vector pMAM-neoCAT was employed, a plasmid which contains the SV40 origin of replication as well as a CAT gene linked to the dexamethasone-inducible murine mammary tumor virus promoter. The cells transfected were Hela cells, derived from a human cervical adenocarcinoma, GM2522 fibroblasts, a human primary fibroblast line, HepG2 cells, derived from a human hepatoma, COS-7 cells, a monkey kidney cell line, and MeT-5A human mesothelial cells. The comparison between COS-7 cells and MeT-5A cells is especially meaningful since both possess a partial, integrated SV40 genome and would be expected to promote replication of plasmids bearing an SV40 origin (Mellon *et al.*, 1981; Gerwin *et al.*, 1987). Figure 4 shows that, of all cell lines

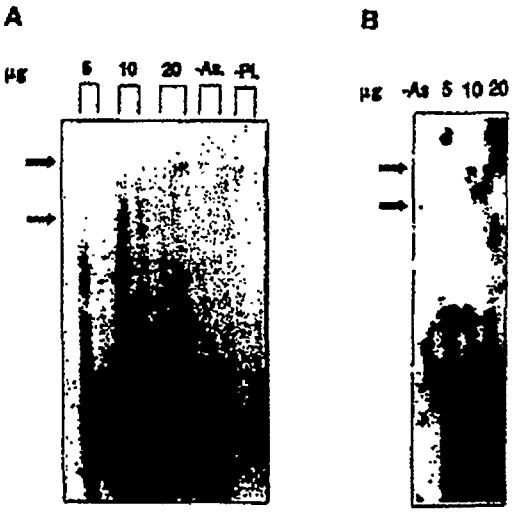

FIG. 3. Hybridization analysis of plasmid pSVod DNA transfected into MeT-5A mesothelial cells mediated by crocidolite or amosite asbestos fibers. Cells were transfected, and cellular Hirt supernatant DNA was extracted and analyzed as described in the legend to Fig. 2 and under Materials and Methods. Autoradiographs of blot hybridizations are shown. Amounts of asbestos are indicated above the gel lanes. Lanes marked −As are controls from cells transfected in the absence of asbestos fibers. Lanes marked −Pl are controls from cells transfected in the absence of plasmid DNA. The lower arrow indicates the position of the linear pSVod band and the upper arrow the position of a plasmid replicative intermediate. (A) Transfection mediated by crocidolite fibers. The 8- and 24-hr points are shown as described in the legend to Fig. 2. (B) Transfection mediated by amosite fibers. In this case only 24-hr points are shown.

tested, the MeT-5A cells produced the highest level of CAT activity both in the presence and absence of dexamethasone. Activity seen with both COS-7 cells and MeT-5A cells in the absence of dexamethasone indicates the imperfect dependence of the MMTV promoter on the steroid analogue in this plasmid construct. It is most likely that the high level of CAT activity in MeT-5A cells versus COS-7 cells is based on transfection efficiency rather than on plasmid replication or efficiency of the MMTV promoter. The MeT-5A cells possess one copy of the integrated gene for SV40 T-antigen (Ke *et al.*, 1989), whereas COS-7 cells possess several copies of the T-antigen gene (Gluzman, 1981). Plasmids bearing the SV40 origin of replication would thus be expected to replicate at least as well in COS-7 cells as in MeT-5A cells. Our controls eliminate the MMTV promoter from this comparison. Figure 4 (bottom) clearly indicates that MeT-5A cells show more CAT activity at 48 hr in the absence of dexamethasone than COS-7 cells do at the same time in the presence of dexamethasone. Using the CAT assay test of Fig. 4, the MeT-5A cells show the highest levels of transfection of any cell line tested by us thus far.

High levels of transfection observed with the human mesothelial cell line MeT-5A may reflect the propensity of mesothelial cells to accumulate mineral particulate substances. Previous studies have documented the cytotoxic effects of asbestos fibers on mesothelial cells (Gabrielson *et al.*, 1991) and the ability of asbestos fibers to enter into the cytoplasm of living mesothelial cells (Jaurand, 1991). Figure 5 shows phase-contrast micrographs of human MeT-5A mesothelial cells in contact with four types of asbestos fibers: Canadian chrysotile, Calidria

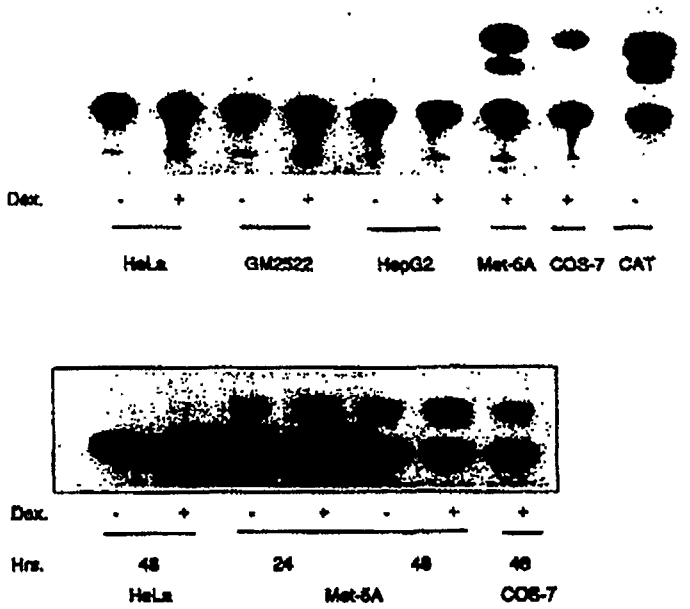

FIG. 4. Chloramphenicol acetyltransferase (CAT) activity measured in various cultured cells transfected with plasmid pMAM-neoCAT using calcium phosphate as a mediating agent. Cultured cell lines were transfected with 10 μg of pMAM-neoCAT by a standard calcium phosphate-mediated procedure as described under Materials and Methods. After 24 hr, dexamethasone (10^{-6} M) was added to dishes to be tested for induction of CAT activity. Cells were harvested 24 or 48 hr later, lysed, and aliquots of cell supernatants were assayed for CAT activity as described under Materials and Methods. Cell lines employed for transfection were human HeLa cells, GM2522 fibroblasts, HepG2 hepatoma cells, MeT-5A mesothelial cells and monkey COS-7 kidney cells. Autoradiographs of thin-layer chromatographic plates are presented. The lowest migrating spot represents unacetylated D-*threo*-[dichloroacetyl-1,2-^{14}C]chloramphenicol. The faster migrating bands represent acetylated forms of this substrate. Lanes for the experiment at top were allowed to migrate farther than those for the experiment at bottom. The lane marked CAT, at top, is a control reaction performed with 3 units of purified *Escherichia coli* CAT enzyme (Boehringer-Mannheim).

chrysotile, crocidolite, and amosite. In each case the cells have accumulated asbestos fibers from the surrounding area so that a higher concentration of fibers can be seen in association with the cells than that in the immediate area of the culture dish. For example, in the case of amosite (Fig. 5D) myriad fibers are associated with the MeT-5A cell while relatively few fibers are seen in the vicinity of the cell. The distribution of the accumulated fibers strongly suggests that they are inside the cell. The fibers are primarily located tangentially around and juxtaposed with the cell nucleus. If the fibers were merely in contact with the surface of the cell, they would be visualized in equal concentration over the nucleus as well as around it. Also, many fibers located outside the cell would be seen to extend beyond the cell's boundaries. This is not found. These observations corroborate data based on DNA replication (Fig. 2) indicating that asbestos fibers do enter into the cytoplasm of MeT-5A cells, a certain percentage of which remain alive and functional. In studies in which human mesothelial cells were not proliferating (data not shown), individual cells could be seen to migrate considerable distances over the culture dish. When asbestos fibers were added to the medium, cells were observed to accumulate fibers in their migratory path.

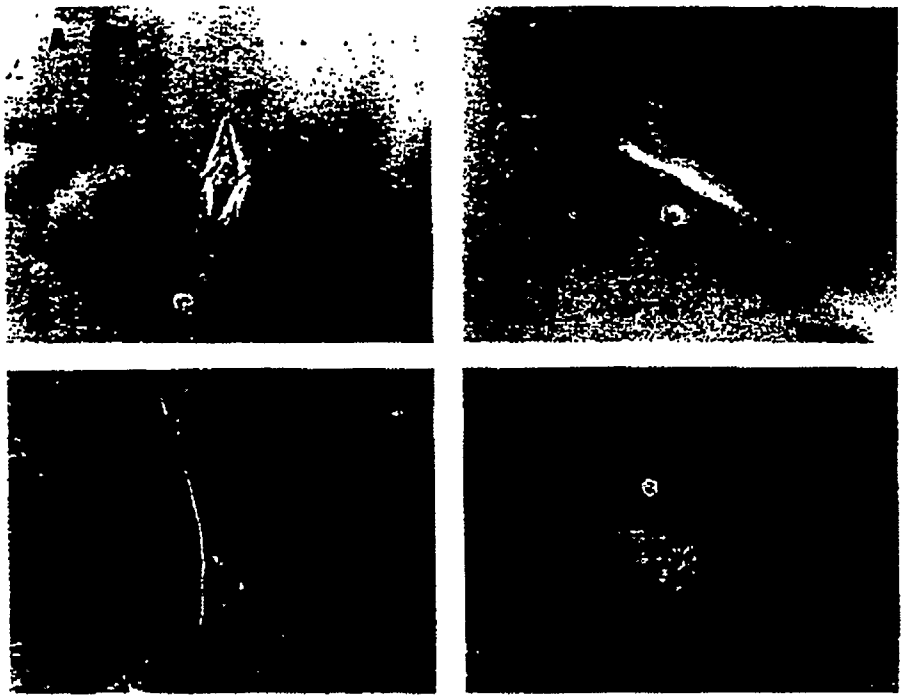

FIG. 5. Accumulation of asbestos fibers by cultured MeT-5A cells. MeT-5A cells were exposed to four different asbestos fiber types using 5 μg of each fiber as described in the legend for Fig. 1. Individual cells were photographed in the presence of (A) Canadian chrysotile, (B) Calidria chrysotile, (C) crocidolite, (D) amosite. Photos were taken at 3 days. Magnification as published is 400×.

Comparison of several mineral particulate agents with regard to transfection efficiency and cytotoxicity toward MeT-5A cells. Figure 6 shows the cell-killing effects of several different mineral agents at two concentrations, 2 and 20 μg, per dish of MeT-5A cells. At either concentration Calidria chrysotile is the most cytotoxic of the agents tested. Addition of only 2 μg of this agent on Day 3 causes a sharp drop in cell number, resulting in about 10% of the control cell number at Day 6. In addition to chrysotile, of the different agents tested, including talc, kaolin, calcium phosphate, and glass fibers, only calcium phosphate exhibited cytotoxicity toward the mesothelial cells at 2 μg, causing an approximate 50% drop in cell number, relative to control values, at Days 5 and 6. At 20 μg per dish, talc also caused an approximate 50% drop in cell number by Day 6. Kaolin and glass fibers showed only slight effects on cell number at 20 μg.

Transfection efficiency of the various mineral agents correlates approximately with cytotoxicity toward MeT-5A cells. Table 1 compares transfection of MeT-5A cells mediated by the agents based on replication of entering pSVod DNA at 24 hr. Highest values were obtained with Calidria chrysotile which was allowed to remain in contact with the cells in the presence of plasmid DNA for 2 hr before being washed away. Lower values were obtained if the chrysotile was allowed to remain in contact with the cells for the full 24 hr, presumably because cell-killing effects begin to predominate with longer contact. Calcium phosphate was slightly less effective at mediating transfection. With this agent, lengthening time of contact improved transfection efficiency over 24 hr (data not shown). It should be noted

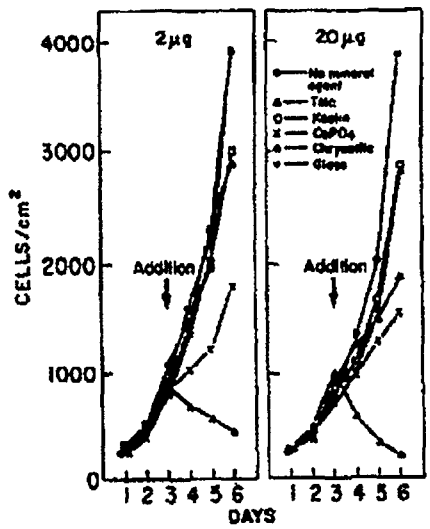

FIG. 6. Cytotoxic effects of various mineral agents toward cultured MeT-5A cells. Different mineral agents were added to culture dishes of MeT-5A cells, as described under Materials and Methods and in the legend to Fig. 1, at final concentrations of either 2.0 or 20 μg per dish in the absence of added DNA. Results of a typical experiment using one dish per dose are presented. Cell numbers were obtained as described under Materials and Methods before and after addition of the mineral agents on Day 3 as indicated.

that the amount of calcium phosphate used here, 20 μg per dish, and the method of use as a mediating agent are very different from those frequently employed for calcium phosphate as a transfecting tool (Graham and van der Eb, 1973). Neither glass fibers, talc, nor kaolin was effective in mediating transfection of MeT-5A cells.

DISCUSSION

This study reveals particular features of different asbestos fibers and particular features of a human mesothelial cell line, both of which cooperate to enhance potential deleterious effects of transfection of mesothelial cells. Asbestos fibers are able to introduce exogenous DNA into MeT-5A cells, and the fibers promote the degradation of the entering DNA (Fig. 2). The human MeT-5A cells are highly susceptible to the cytotoxic effects of asbestos fibers (Figs. 1 and 6), accumulate asbestos fibers to an unusual degree (Fig. 5) and are unusually susceptible to transfection (Fig. 4).

The ability to degrade entering DNA is a property that differs among different asbestos fiber types. Degradation of entering DNA is most severe after crocidolite transfection and least severe after amosite transfection. Chrysotile transfection is intermediate in this regard. Calcium phosphate and glass fibers do not promote significant degradation of plasmid DNA in transfection experiments. We have found that plasmid DNA is not degraded in the presence of chrysotile fibers in physiological saline solutions or in cell culture medium in the absence of cells (J. Appel and E. M. Johnson, unpublished results). In this regard it may be relevant that oxygen radicals resulting from the interaction of crocidolite fibers with H_2O_2 cause DNA strand breaks *in vitro* (Jackson *et al.*, 1987). This same phenomenon

TABLE 1
TRANSFECTION EFFICIENCY: RELATIVE HYBRIDIZATION INTENSITY OF THE DpnI-RESISTANT 3.0-kb pSVod BAND

	24 hr
Glass fibers[a]	0
Glass fibers[b]	0
Talc	0
Kaolin	0
$Ca-PO_4$	60
Cal. chrysotile[a]	48
Cal. chrysotile[b]	100

Note. Following transfection of MeT-5A cells with plasmid pSVod, mediated by the indicated mineral fibers, DNA was isolated at 24 hr and treated with restriction endonucleases *Bam*HI (to linearize plasmid DNA) and *Dpn*I (to digest nonreplicated plasmid DNA) as described under Materials and Methods and in the legend to Fig. 2. DNA samples were subjected to agarose gel electrophoresis, blotted, and hybridized with ^{32}P-labeled pBR322 DNA as described under Materials and Methods. Transfections were performed with each mediating mineral agent at 20 μg per 15 cm^2 dish (10 ml of culture medium) and 2.0 μg of plasmid pSVod DNA. All agents were sterilized dry prior to suspension in 50 mM Hepes buffer, pH 7.0, 0.1 M NaCl as described under Materials and Methods. Suspensions of each mineral agent corresponding to 20 μg were incubated in the Hepes buffer–NaCl solution with the pSVod DNA for 1 hr at 37°C prior to addition to the cell culture medium. Cal. chrysotile represents Calidria chyrsotile.

[a] Culture medium containing the transfecting agent and DNA was kept in contact with the MeT-5A cells for 24 hr prior to harvesting cells.

[b] Culture medium containing the transfecting agent and DNA was kept in contact with the MeT-5A cells for 2 hr, after which cells were washed two times with fresh medium and incubated for the remaining 22 hr in fresh medium until harvesting.

might also be expected to occur with DNA adsorbed to asbestos fibers inside cells.

We report that human MeT-5A cells are highly susceptible to the cytotoxic effects of asbestos fibers (Figs. 1 and 6). Other investigators have recently reported that human mesothelial cells in primary cultures are selectively sensitive to cell killing by asbestos and related fibers (Gabrielson *et al.*, 1991). These workers suggest a correlation between this type of cytotoxicity and carcinogenicity leading to mesothelioma. We report here that MeT-5A cells are highly amenable to transfection, mediated not only by asbestos fibers but also by calcium phosphate (Figs. 2–4). Our experiments with calcium phosphate employed CAT activity as a marker for DNA transfection. Controls performed with COS-7 cells suggest that the high levels of CAT activity seen with MeT-5A cells actually reflect a high efficiency of DNA uptake by these cells, mediated by the mineral particulate agent. The unusual transfectability of MeT-5A cells may be a consequence of the propensity of these cells to accumulate mineral particulates, since these cells can be observed to accumulate several types of asbestos fibers from a broad expanse of surrounding medium (Fig. 5).

Recent experiments have indicated that several types of asbestos fibers and certain other mineral particulates can mediate transfection of cultured mouse cells by polyoma virus DNA (Dubes, 1991). While in most experiments anthophyllite asbestos was observed to be more effective at mediating transfection, other particulates, e.g., kaolin and calcium phosphate were also effective. It is notable that the fiber doses employed by Dubes (1 to 5 mg/ml) are approximately 1000- to 5000-fold higher than the highest dose employed in the present study. At the

lowest doses tested by Dubes, asbestos fiber types anthophyllite, crocidolite, and chrysostile were each more effective than either kaolin or calcium phosphate. Under the conditions we employed, kaolin was not able to mediate transfection of MeT-5A cells. The ability to mediate transfection per se may be a useful criterion to identify mineral particulate substances as suspect carcinogens. However, as recently detailed (Johnson *et al.*, 1991), this ability must be considered in the context of target cells to which a given agent has access and in the context of the mutagenicity of the incoming DNA.

Human mesothelial cells may represent a uniquely susceptible target for the mutagenic effects of transfection by asbestos fibers. We have demonstrated that MeT-5A cells are highly amenable to transfection and that they can accumulate asbestos fibers to a high degree. *In vivo*, asbestos fibers do gain access to pleural tissue (Sebastien *et al.*, 1979; Kohyama and Suzuki, 1991). Finally, we have demonstrated that asbestos fibers have the capacity to damage the DNA they introduce into cells. This has not been observed for calcium phosphate. Therefore, the particular properties of both asbestos fibers and mesothelial cells render their combined transfection system highly susceptible to potential genotoxic effects. It is difficult at this point to fully assess the consequences of delivering severely damaged DNA into the nucleus, but these likely include the triggering of DNA repair processes as well as recombination events which may lead to both deletions and insertions. Moreover, asbestos-catalyzed oxidant damage to the entering DNA may be associated with the intracellular formation of highly toxic substances such as base propenals (Grollman *et al.*, 1985; Jackson *et al.*, 1987). Asbestos fibers may exert their carcinogenic effects on cells through multiple pathways, not all of which involve transfection. At this point, however, transfection represents a demonstrated pathway to mutagenesis and a process to which mesothelial cells are highly susceptible.

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