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ASBESTOS-MEDIATED TRANSFECTION OF MAMMALIAN CELL CULTURES

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SUMMARY

The capacity of asbestos to mediate transfection was tested in a rapid and relatively simple system: picornavirus RNAs and mammalian cells in vitro. Thirteen asbestos samples, including amosite, anthophyllite, chrysotile, and crocidolite, 4 picornaviruses (poliovirus 1 and 2, echovirus 7, and encephalomyocarditis virus), and 4 cell lines (CLI, chimpanzee liver; KB, human carcinoma; eta, monkey kidney; NIH 3T3, mouse embryo) were tested. The results showed that all asbestos samples mediated transfection and that all cell lines were transfectible by viral RNA with asbestos. Transfection was much greater with asbestos added to the viral RNA inoculum than to the cells before or after the RNA. Transfection was directly proportional to asbestos concentration. Initiation of transfection events was rapid, with half becoming irreversible by washing 2 min postinoculation. DNA in the inoculum strongly interfered with asbestos-mediated transfection by the RNA but was ineffective when added, with or without asbestos, to the cells before or after the inoculum. Asbestos compared with six classical "insoluble" facilitators (bentonite, calcium phosphate, chromic oxide, ferric oxide, kaolin, talc) was of intermediate rank in transfection mediation. It is hypothesized that the prominence of asbestos in carcinogenesis is due to a combination of properties, including transfection mediation as well as chromosome mutagenicity, fiber dimensions, biological durability, hydrocarbon transport, and prevalence.

Key words: asbestos; transfection; carcinogenesis theory; picornavirus RNAs; CLI cells; NIH 3T3 cells; eta cells; KB cells.

INTRODUCTION

Asbestos is carcinogenic (4,35), with a latency in man of commonly more than 20 yr. Investigations have suggested that asbestos, under different circumstances, can play the role of initiator, cocarcinogen, or promoter in the carcinogenic process.

The finding that asbestos causes mesothelioma in man (35) seems to cast asbestos as a "complete carcinogen," and thus as an initiator, a role supported by results in rats, where inhaled asbestos causes lung cancer (34) and intrapleurally administered asbestos causes mesothelioma (33). Because initiation events seem to constitute a subset of mutations (23), and because asbestos is a *chromosome* mutagen (19,28,31,32), the initiator role probably derives from chromosome mutagenicity (2).

Studies (14) undertaken to explain synergism between asbestos and cigarette smoking in causing human bronchogenic carcinoma (30) support a role for asbestos as cocarcinogen, because asbestos was found to increase cellular uptake of the initiator benzo[*a*]pyrene, a smoke component, and the number of adducts of benzo[*a*]-pyrene derivatives with the DNA.

The promoter role derives from observations of asbestos effects similar to effects of classical promoters (24). Although promotion is often thought of as the provision of an environment selectively favorable for the multiplication of transformed, neoplastic, or malignant cells, mechanisms of promotion may include mutation and even gene transfer, because two or more different oncogenes seem to be required to transform a normal cell into a cancer cell (21). The initiating mutation would putatively result in the presence of just one oncogene in the cell, and the acquisition of the second oncogene could be viewed as part of promotion. The second oncogene could be acquired by mutation occurring in a descendant cell or possibly by transfer of a chromosome or chromosome fragment from a mutated cell of another lineage (8). As a chromosome mutagen, asbestos could induce the second mutation. Whether asbestos could mediate transfer of an oncogene is unknown, but asbestos is cytocidal (18,20) and an especially potent *genome* mutagen (19,27,28,31,32).

The present work was undertaken to determine whether asbestos can mediate gene transfer in a simple model system. The model system of transfection of mammalian cells in vitro by picornavirus RNAs was chosen because of the high transfectivity titers, rapid plaque development, and variety of host cells, coupled with the fact that other transfection methods, including

calcium phosphate (10,16), kaolin (15,17), and diethylaminoethyl-dextran (25,26), are effective with both RNA and DNA.

Part of the results have been presented: 12 April 1985, Annual Meeting, Nebraska Academy of Sciences, Lincoln (8); 25 April 1986, Missouri/Missouri Valley Regional Meeting, American Society for Microbiology (ASM), Kansas City, Missouri; 7 June 1986, Annual Meeting, Tissue Culture Association, Chicago, Illinois (11); 24 April 1987, Annual Meeting, Missouri Valley Branch, ASM, Manhattan, Kansas.

Materials and Methods

Asbestos. Thirteen asbestos samples from five sources were tested. From R. E. G. Rendall, National Centre for Occupational Health (NCOH), Johannesburg, South Africa, we obtained five standards of the Union Internationale Contre le Cancer (UICC): amosite, anthophyllite, crocidolite, Rhodesian "A" chrysotile, and Canadian "B" chrysotile. UICC standard crocidolite and chrysotile were also obtained from Prof. Edward Bresnick, Eppley Cancer Institute (ECI), Omaha, NE. Four standards of the National Institute of Environmental Health Sciences (NIEHS) were received from Dr. E. E. McConnell and Ms. Martha Harris, NIEHS: amosite, log no. 3125; crocidolite, log no. 517V; chrysotile of short range in fiber length, log no. 031N; and chrysotile of intermediate range, log no. 128K. Asbestos, Powminco Brand, purified, acid-washed, medium fiber, catalogue no. 0918, lot 37 905, was purchased in 1967 from J. T. Baker Chemical Co. Phillipsburg, NJ; in 1985, William C. Broad of Baker Chemical Co. indicated that this asbestos was of the amphibole type and had come from the Powhatan Mining Co., Baltimore, MD. Prof. R. B. Nelson, Department of Geology, University of Nebraska at Lincoln, confirmed the identification as amphibole, using X-ray diffraction, and used electron probe analysis to identify the variety of amphibole as anthophyllite. This anthophyllite consists predominantly of long, thin, straight fibers (Fig. 1). Last, ASTM std 20-481 asbestos was obtained from Prof. Nelson as the amphibole highest-matched by X-ray diffraction to the anthophyllite from Powhatan (Pow).

Asbestos suspensions were prepared in deionized distilled water, and autoclaved. Just before use, asbestos was resuspended by vigorous pipetting and shaking.

Nonasbestos minerals and chemicals. Bentonite, U. S. P., powder, catalogue no. B-235, lot 770386, Fisher Scientific Co., Fair Lawn, NJ; $CaHPO_4 \cdot 2H_2O$, N. F., powder, catalogue no. 4265, control NBD, Mallinckrodt Chemical Works, St. Louis, MO; Cr_2O_3, purified powder, catalogue no. CB301, lot CX1600, Matheson, Coleman, and Bell, Norwood, OH; Fe_2O_3, Reagent, red powder, catalogue no. CB385, lot FX230, Matheson, Coleman, and Bell; kaolin, N. F., powder, catalogue no. 2242, lot 37 126, J. T. Baker Chemical Co.; and talc, U. S. P., talcum powder, catalogue no. 8476, control NAP, Mallinckrodt, were used. Suspensions were prepared, autoclaved, and resuspended, as for asbestos.

Cell cultures. CLI, a line of epithelioid cells from normal chimpanzee liver (5), was obtained from Dr. William V. Hartwell, USPHS Laboratories, Phoenix, AZ, via Dr. Vincent Marshall of Omaha in May 1968. Eta, a line of epithelioid cells from normal rhesus monkey kidney (3), has been in continuous culture since the primary cultures of October 1961. KB, a line of human carcinoma cells, and NIH 3T3, a fibroblast line derived from normal Swiss mouse embryo, were obtained from the American Type Culture Collection (ATCC), Rockville, MD, in 1966 and 1986, respectively. All 4 cell lines were cultured in Medium SA, which is Medium S (3) plus antibiotics (penicillin G, 50 U/ml; streptomycin sulfate, 50 μg/ml). Cell cultures for the experiments were grown to confluence in 60 $\times$ 15-mm polystyrene petri dishes for tissue culture (Lux Contur).

Tests of the cell lines for fungi and for bacteria including mycoplasma were negative. Direct tests for mycoplasma were under 95% N_2 and 5% CO_2 using a) Bacto PPLO agar medium with Bacto Mycoplasma Supplement (Difco, Detroit, MI), b) Hayflick agar medium, and c) Mycotrim-TC (Hana Media, Inc., Berkeley, CA) The indirect test was MycoTect (GIBCO Laboratories Life Technologies, Inc., Grand Island, NY) with 6-methylpurine deoxyriboside, 3T6 cells (ATCC no. CCL 96), and adenosine phosphorylase as positive control. In most tests, both direct and indirect, a positive control of *Mycoplasma orale* strain CH19299 (ATCC no. 23714) was included. The positive controls gave the expected results. The CLI, eta, and NIH 3T3 lines were tested in all four systems, KB only in a).

Picornaviruses. The Brunhilde strain of type 1 poliovirus and the MEF1 strain of type 2 poliovirus were used for the CLI and KB cells, the *r* mutant (1) of the Wallace strain of type 7 echovirus for the eta cells, and the tissue culture-adapted EMC strain (ATCC no. VR-129B) of encephalomyocarditis (EMC) virus for the NIH 3T3 cells.

Plaquing. Culture medium was removed from confluent cultures of CLI, eta, or KB cells in the 60-mm dishes, and the cultures were inoculated with virus, 0.30 ml/dish, incubated at 37° C for 60 min, overlayered with medium SA with washed agar at 10 mg/ml, 3.0 ml/dish, and incubated at 37° C in 5% CO_2 in air. Two or three days later, medium SA with washed agar at 10 mg/ml and neutral red at 100 μg/ml was added, 3.0 ml/dish; and the plaques were counted a few hours later and periodically thereafter.

To avoid spotty degeneration, the above protocol was modified for the NIH 3T3 cells, which require more generous feeding with Medium SA. There were three modifications: first agar-overlay, 5.0 ml/dish; second agar-overlay added not later than 2 d postinoculation; neutral red at 50 μg/ml.

Buffers. The buffers were: buffer P, the phosphate buffered saline of Dulbecco and Vogt (13); buffer B, buffer P minus $CaCl_2$; buffer A, buffer B minus $MgCl_2$; buffer Ah, buffer A with L-histidine· HCl at 150 μM; buffer M, buffer A with $MgCl_2$ at 2.5 mM.

Virus stocks in serum-free medium. Poliovirus stocks were prepared by growing virus in CLI cells, echovirus stocks in eta cells, and EMC virus stocks in eta and NIH 3T3 cells. Confluent cultures of the cells were inoculated with 5 to 100 plaque-forming units (pfu)/cell; incubated

at 37° C in 5% CO_2 in air for 60 min; washed three times with buffer P, 4 ml/wash; fed medium HA, which is medium SA without serum, 5 to 8 ml/dish; and incubated at 37° C in 5% CO_2 in air until viral cytopathology was complete or nearly complete. The infected cultures were then harvested, sterility-tested, and clarified by centrifugation at 23° C at 2800 *g* for 15 min.

Purification of virions. Brunhilde virions were purified essentially as already described (7).

Viral RNA preparations. Viral RNA prepared from the serum-free virus stocks and from the purified Brunhilde virions by extraction with phenol was diluted and kept in buffer with L-histidine, commonly buffer Ah. Inocula usually contained the viral RNA preparation at a dilution in the range 1:1000 to 1:300 but occasionally at 1:16 or 1:8, relative to the serum-free virus stock. Inocula prepared from the purified virions contained the RNA preparation at dilution 1:525; based on previous determinations (12), viral RNA in such 1:525 dilutions is at approximately 10 ng/ml.

Transfection tests. The medium over confluent cell cultures in 60-mm dishes was replaced with fresh Medium SA, 8.0 ml/dish. Three to eight hours later, the culture medium was discarded, and the cell sheets were washed twice with buffer M, 4 ml/wash, unless otherwise indicated. The washed sheets were inoculated with viral RNA, 0.30 ml/dish, incubated at 37° C for 10 min unless stated otherwise, and then overlayered with agar medium for plaquing as described above. The concentration of asbestos in the inoculum ranged from 0.10 to 10 mg/ml; and, because the dish culture surface was 21.2 cm^2, the

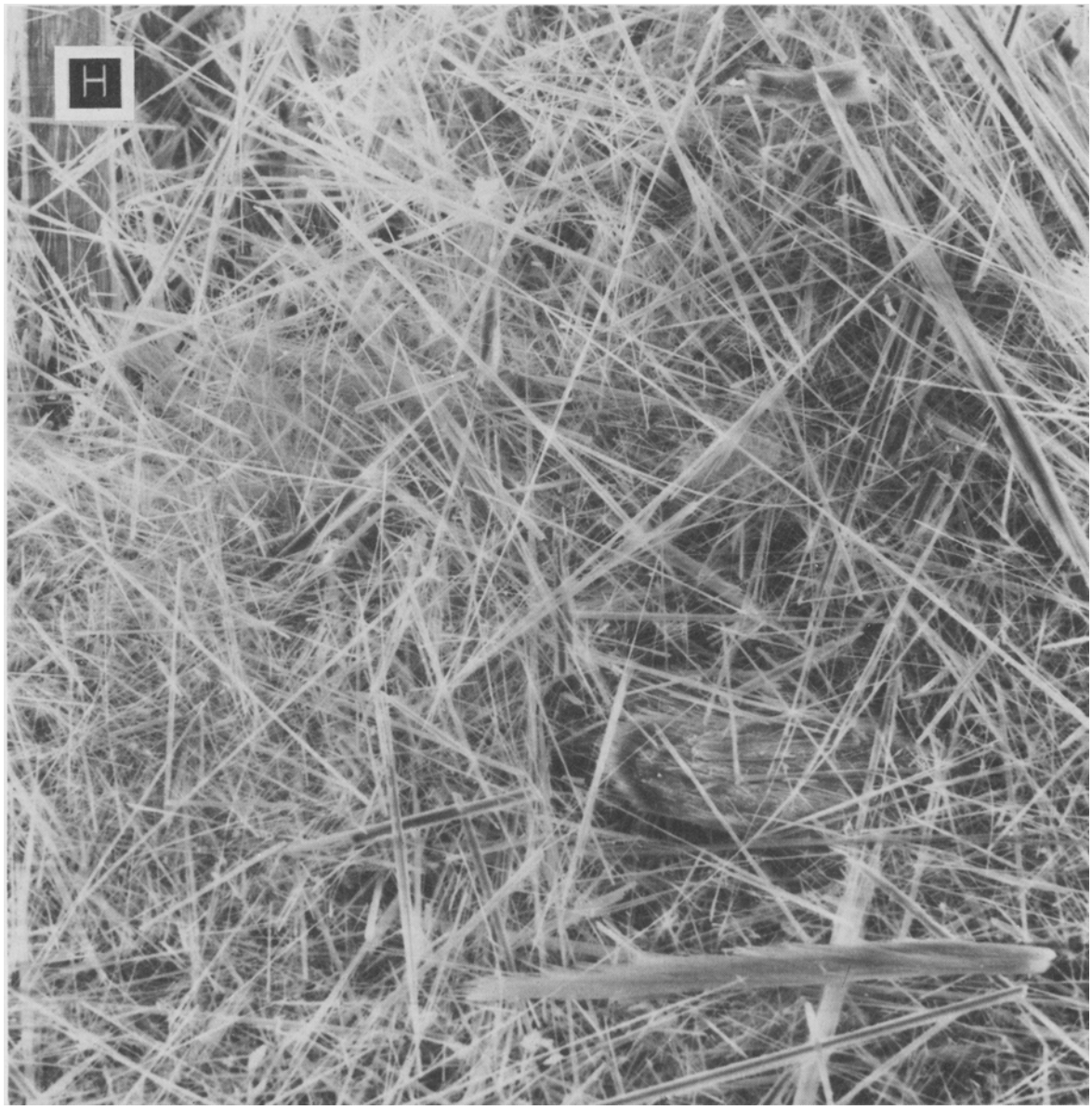

FIG. 1. Anthophyllite (Pow) examined uncoated in an Etec Autoscan scanning electron microscope at 20 kV. *Bar* is 10 μm.

TABLE 1

ASBESTOS-MEDIATED TRANSFECTION

Cell	Viral RNA	Source[b]	Plaques/Plate, $\bar{X} + s$[a] Without Asbestos	With Asbestos[c]
CLI	Poliovirus 1	stock	0	164 ± 94
		purified	0	15 ± 6
	Poliovirus 2	stock	0	208 ± 28
KB	Poliovirus 1	stock	0	55 ± 28
	Poliovirus 2	stock	0	59 ± 24
Eta	Echovirus 7	stock	0	14 ± 2
NIH 3T3	EMC Virus	stock	0.3 ± 0.8	17 ± 7

[a]Data in each row based on two experiments and expressed as mean ± SD calculated from pooled error variance. Inter-row comparisons not justified except for rows 3 to 5.

[b]Stock: serum-free virus stock. Purified: purified virions.

[c]Anthophyllite (Pow) at 10 mg/ml in inoculum, except at 2.5 mg/ml on the NIH 3T3 cells.

area density of asbestos was 1.42 to 142 $\mu g/cm^2$. Control inocula included RNA without asbestos and asbestos without RNA.

Statistics. Comparisons based on single experiments are presented using arithmetic means and standard errors of the means.

Comparisons based on multiple experiments had to take into account the fact of interexperimental variation. In most cases, each experiment was done with just one *batch* of cells, a *batch* being a group of replicate cultures originally seeded with replicate samples from the same cell suspension, incubated and fed together, et cetera. Despite close attention to repeating standard protocol, cell batches often differed, commonly up to two or three fold, occasionally more, in transfectibility. Thus, when data from two or more experiments were used for calculating means and standard deviations for making specific comparisons, the proportion of the cultures from each experiment was the same for the different treatments or materials being compared. The mean was calculated as the total plaque counts divided by the total number of cultures, and the standard deviation was calculated as the square root of the pooled error variance for that treatment or material.

In one case, the kinetics of transfection initiation, an internal standard was used in each experiment. Plaque counts of other groups in an experiment were expressed as fractions of the internal standard, these fractions were log transformed to provide a more normal distribution, means and standard errors of the mean of the logs were calculated, and antilogs were plotted.

For tests of statistical significance of differences based on multiple experiments, ANOVA was used, after log transformation.

RESULTS

Tests of control inocula with asbestos but without viral RNA. Each of the 13 asbestos samples was tested using CLI cells. Asbestos concentration in the inoculum was 5.0 mg/ml, except that anthophyllite (Pow) was at 2.5 and 10 mg/ml. Anthophyllite (Pow) at 2.5 mg/ml was also tested on NIH 3T3 cells. Transfection and plaquing protocols were used, but without virus or viral RNA. Neutral red was added on Day 2 postinoculation. In all cases, red staining of the cell sheets indicated survival of a high percentage of the cells.

Asbestos mediation of transfection by viral RNA. Asbestos mediated transfection by RNA prepared from all four picornaviruses, including RNA prepared from purified picornavirions as well as RNA prepared from serum-free virus stocks (Table 1). Velocity-type ultracentrifugation showed that the single-stranded RNA from the virions accounted for the great majority of the transfectivity in RNA preparations made from serum-free virus stocks (Fig. 2).

In all the experiments done, no plaques were obtained from control inocula with picornavirus RNA but without asbestos, except for one culture with two plaques from EMC viral RNA.

All 13 asbestos samples mediated transfection (Table 2). Because of logistical difficulties, all 13 were not tested in the same experiment. However, the 7 experiments of Table 2 show several comparisons of interest; and it seems clear that there are quantitative differences in transfection mediation among the 13 samples. Anthophyllite (Pow), because of its inclusion in six of the seven experiments,

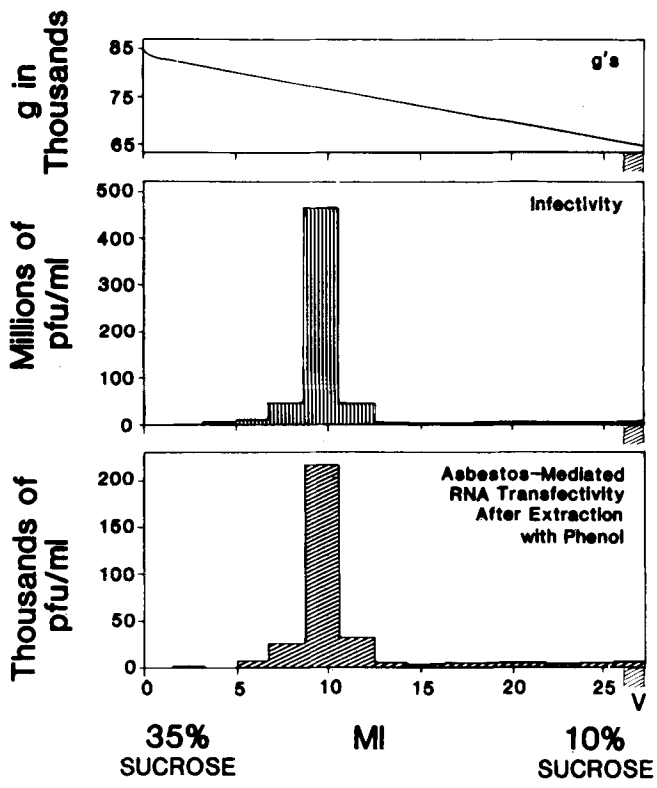

FIG. 2. Coincidence of infectivity (virions) and source of asbestos-mediated transfectivity after ultracentrifugation. A 1.0-ml sample of serum-free stock of poliovirus type 1 was centrifuged in a 10 to 35% sucrose gradient in buffer A with L-histidine·HCl at 300 μM at 4° C for 3.0 h. Yield of infectivity, titrated on CLI cells, was 112%. Yield of transfectivity, titrated on CLI cells using anthophyllite (Pow) at 10 mg/ml, was 87%. V = virus at start.

TABLE 2

COMPARISONS OF ASBESTOS SAMPLES IN TRANSFECTION MEDIATION

Asbestos[b]	Plaques/Plate[a] in Experiment						
	T74	T75	T76	T77	T78	T79	T80
Amosite (NCOH)	29 ± 9			5 ± 1			
Amosite (NIEHS)					379 ± 25	232 ± 10	
Anthophyllite (NCOH)	237 ± 56			93 ± 17			182 ± 11
Anthophyllite (Pow)	144 ± 20	94 ± 13		67 ± 5	184 ± 25	71 ± 3	177 ± 8
ASTM std amphibole 20-481		35 ± 16				70 ± 24	
Chrysotile (ECI)	3 ± 1		14 ± 2				3 ± 1
Chrysotile, Canadian (NCOH)	8 ± 0.9		12 ± 0.4				43 ± 8
Chrysotile, Rhodesian (NCOH)	12 ± 2			5 ± 1			
Chrysotile, medium (NIEHS)					17 ± 5	24 ± 4	
Chrysotile, short (NIEHS)					100 ± 9	67 ± 1	
Crocidolite (ECI)	43 ± 7		26 ± 9				36 ± 7
Crocidolite (NCOH)	32 ± 3		23 ± 8			32 ± 15	32 ± 5
Crocidolite (NIEHS)					274 ± 24	150 ± 20	

[a] Arithmetic mean ± its SE; $n = 2$ to 4. Interexperiment comparisons of different varieties unjustified. No entry: not done.
[b] Inocula of asbestos at 5.0 mg/ml and poliovirus type 1 RNA at 1:525 from serum-free stock plated on CLI cells.

can be used as an internal standard to facilitate comparison. The amosite from NIEHS seems to be the most effective asbestos. Interestingly, the UICC (from NCOH and ECI) and NIEHS crocidolite standards differed substantially in number of transfection events initiated.

Holding inocula. With all 13 samples of asbestos, the effect of holding the inoculum of poliovirus type 1 RNA at dilution 1:525 and asbestos at 5.0 mg/ml in buffer Ah at 0° C for 45 min while preparing the cell cultures for inoculation was tested by directly comparing the amount of transfection obtained with such holding vs. that obtained when inoculation was made promptly. i.e. < 1 min, after mixing the viral RNA and asbestos together. The results showed that holding did not affect subsequent transfection.

Asbestos dose response. The number of transfection events was found to be directly proportional to asbestos concentrations (Fig. 3).

Dependence on dilution of viral RNA. With constant asbestos concentration and varying dilution of viral RNA in the range of 1:300 and higher dilutions, the number of plaques per plate was approximately directly proportional to the concentration of viral RNA.

Cell washing. In work with primary monkey kidney cell cultures (9,10), the specific omission of calcium from the buffer used to wash the cells markedly increased cell sensitivity to transfection by viral RNA. Similar tests using CLI cells and anthophyllite (Pow) showed no significant effect of calcium omission. Various cell washing experiments using CLI cells, poliovirus type 1 RNA, and anthophyllite (Pow) can be summarized as follows: After two to four washes, 4 ml/wash, the cells showed good sensitivity to transfection, whether the wash buffer was buffer P, B, A, or M. With unwashed cell sheets, there was very little transfection, possibly because of residual ribonuclease from the serum.

Inactivation of viral RNA by culture medium-treated asbestos. Anthophyllite (Pow) at 20 mg/ml in water was diluted two fold into medium SA, incubated at 37° C for 60 min, washed twice with water by centrifugation and resuspension, and then resuspended in water. Control anthophyllite (Pow) was treated and washed in the same

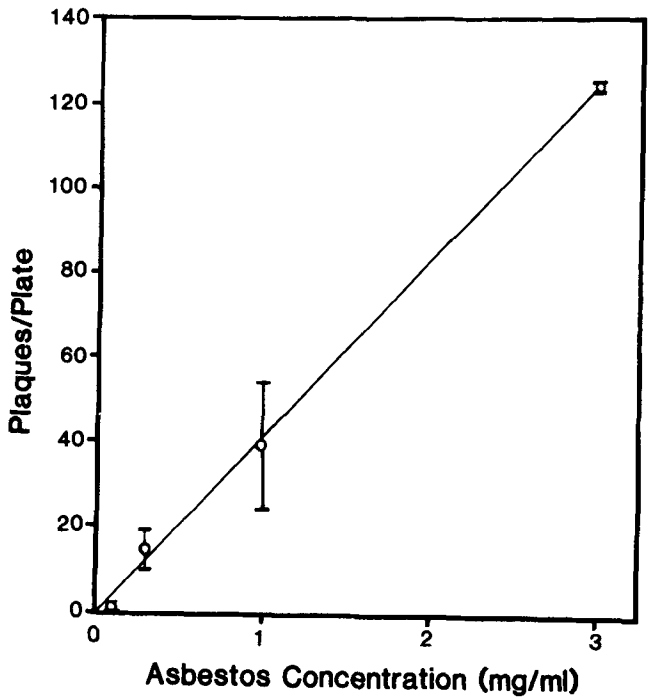

FIG. 3. Proportionality of transfection to concentration of asbestos in inoculum. Anthophyllite (Pow) was used. Poliovirus type 1 RNA concentration was held constant. Arithmetic means ± their SEs are presented.

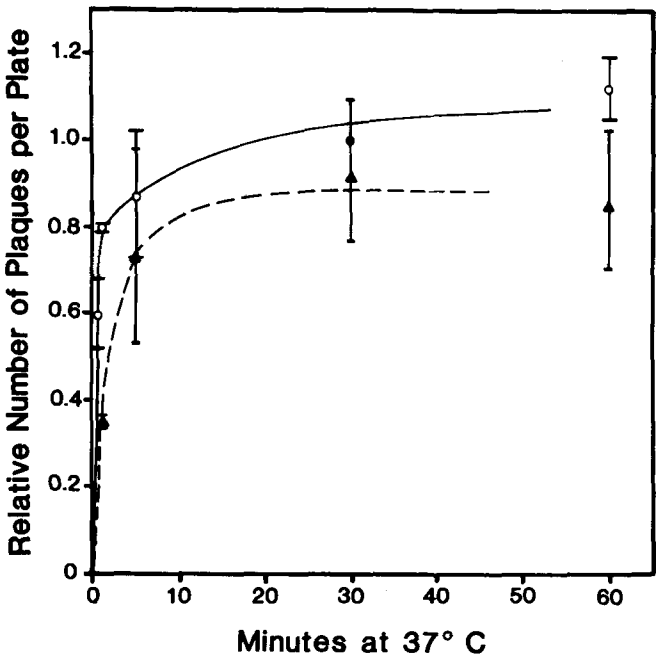

FIG. 4. Kinetics of initiation of transfection. Buffer Ah with poliovirus type 1 RNA at 1:300 and anthophyllite (Pow) at 10 mg/ml was inoculated onto washed CLI cell sheets. Inocula and sheets had been warmed briefly at 37° C just before inoculation Termination was by agar-overlayering: ● = 30-min internal standard; O——O = other times; or washing three times with buffer M and then agar-overlayering: ▲---▲. The data are from five experiments.

way but with the initial dilution into water. The medium SA-treated anthophyllite (Pow) rapidly inactivated RNA from poliovirus type 1, whereas the control anthophyllite (Pow) did not inactivate the RNA. This result suggests that anthophyllite (Pow) can adsorb some RNA-inactivating component.

Kinetics of transfection initiation. The initiation of asbestos-mediated transfection was rapid, with half of the transfection events already irreversible by washing 2 min postinoculation (Fig. 4).

Point of addition of asbestos. Asbestos premixed with the viral RNA was much more effective than asbestos added directly to the cells before or after the RNA (Table 3).

Test for adsorption of transfective RNA onto asbestos. Poliovirus type 1 RNA at dilution 1:300 was incubated with anthophyllite (Pow) at 10 mg/ml in buffer Ah with $MgCl_2$ at 0, 2.5, or 6.0 m*M*, or in hypotonic (0.007 X) buffer Ah, at 0° C for 15 min and then centrifuged at 1900 to 2300 *g* at 23° C for 15 min. Tight pellets were obtained, and the supernatant fluids were clear or only slightly opalescent. Controls included sample without asbestos and uncentrifuged sample. Transfectivity of decantate, resuspended pellet, and controls was titrated. All inocula contained anthophyllite (Pow) at 5 mg/ml. Analysis of the results indicated no significant adsorption of the transfective RNA on the anthophyllite (Pow) except for a possibly significant adsorption averaging 15 to 20% when the milieu for adsorption was buffer Ah with $MgCl_2$ at 2.5 or 6.0 m*M*. Because of this result, the effect of $MgCl_2$ on transfection mediated by anthophyllite (Pow) was also tested. The results showed that, with anthophyllite (Pow) at 5.0 or 10 mg/ml in the inoculum, the number of transfection events was not significantly affected by $MgCl_2$ at 0.40, 1.0, or 2.5 m*M* in the inoculum and was decreased slightly (38%; $0.01 < P < 0.05$) by $MgCl_2$ at 6.0 m*M*.

Interference by DNA. The presence of double-stranded DNA (from salmon sperm) in the inoculum of viral RNA and asbestos decreased transfection. With poliovirus type 1 RNA at 1:1000, the decrease was 90% for DNA at 25 μg/ml in the inoculum and 95% for DNA at 50 μg/ml; with the RNA at 1:300, the corresponding values were 83 and 92%. All inocula contained anthophyllite (Pow) at 4.0 mg/ml. The decrease in transfection did not seem to depend on whether the DNA was preincubated alone with the anthophyllite (Pow) or was added to the premixed inoculum of viral RNA plus anthophyllite (Pow) only just before (< 1 min) inoculation. In contrast, DNA, with or without anthophyllite (Pow), added directly to the cells before or after inoculation did not affect transfection (Table 4).

To determine whether the DNA a) was interfering with transfection initiation or b) had some adverse effect not requiring the presence of cells, the DNA was incubated together with the viral RNA plus asbestos before dilution and inoculation onto cells. Diluents were selected to maintain the same asbestos concentration but to vary the DNA concentration. Corresponding inocula with DNA added just before inoculation were also prepared. The results (Table 5) showed that the DNA interferes with asbestos-mediated transfection by RNA.

Comparison of asbestos with classical "insoluble" facilitators. Two asbestos samples were compared with bentonite (6), calcium phosphate (9,10), chromic oxide (9,10), ferric oxide (9,10), kaolin (15), and talc (9,10) (Table 6). The calcium phosphate tests were done in three ways: a) using commercial $CaHPO_4 \cdot 2H_2O$; b) adding the RNA to calcium phosphate which had been freshly precipitated by mixing solutions of Na_2HPO_4 and $CaCl_2$; and c) adding in the sequence Na_2HPO_4, RNA, $CaCl_2$ so that precipitation of the calcium phosphate occurred in the presence of the viral RNA,

TABLE 3

COMPARISONS OF POINTS OF ADDITION OF ASBESTOS

Sequence of Inoculation[a]			
First	Second	Third	Plaques/Plate, $\bar{X} \pm s$[b]
Buffer	mixture	buffer	298 ± 32
Buffer	RNA	asbestos	3.8 ± 0.5
Asbestos	RNA	buffer	19 ± 9

[a] CLI cell cultures were washed twice with buffer M and inoculated, 0.30 ml/culture, three times in the sequences shown. Each inoculation was followed by incubation at 37° C for 30 min and then three washes with buffer M. The four inocula: RNA: Poliovirus type 1 RNA from serum-free stock at 1:300 in buffer Ah. Buffer: Control preparation made using medium HA in buffer Ah. Asbestos: Like buffer control but with anthophyllite (Pow) at 10 mg/ml. Mixture: Like RNA inoculum but also with anthophyllite (Pow) at 10 mg/ml.

[b] Data are from two experiments and are presented as means ± SD.

TABLE 4

IMPORTANCE OF POINT OF ADDITION OF DNA IN ITS INHIBITION OF ASBESTOS-MEDIATED TRANSFECTION BY VIRAL RNA

Expt. Set[b]	Inocula[a] First	Inocula[a] Second	Sequence Designation	Plaques/Plate, $\bar{X} \pm s$[c]
I	Buffer control	asbestos + RNA	A	111 ± 9
	Buffer control	asbestos + RNA + DNA	B	27 ± 12
	Asbestos	asbestos + RNA	C	132 ± 28
	DNA	asbestos + RNA	D	102 ± 10
	Asbestos + DNA	asbestos + RNA	E	107 ± 18
II	Asbestos + RNA	buffer control	F	58 ± 12
	Asbestos + RNA + DNA	buffer control	G	12 ± 5
	Asbestos + RNA	asbestos	H	45 ± 11
	Asbestos + RNA	DNA	I	52 ± 5
	Asbestos + RNA	asbestos + DNA	J	45 ± 4

[a]Inocula: Buffer Ah with additions as listed of anthophyllite (Pow) at 4.0 mg/ml, poliovirus type 1 RNA at 1:300, and salmon sperm DNA at 50 μg/ml. Inocula without viral RNA were control preparations from medium HA in buffer Ah. Cell sheets were washed twice with buffer A, inoculated, incubated at 37° C 15 min, washed three times with buffer A, inoculated with second inoculum, incubated at 37° C 15 min, and agar overlayered.

[b]I, Expts. T44, T45, T47; II, Expts. T49, T50: 2 CLI cell sheets per sequence per experiment.

[c]Mean ± SD calculated from pooled error variance. Interset comparisons unjustified. B vs. A, and G vs. F, $P < 0.01$; C vs. A, D vs. A, E vs. A, E vs. C, H vs. F, I vs. F, J vs. F, and J vs. H, $P > 0.05$.

a procedure recommended for DNA (16). The results showed the chrysotile and crocidolite to be of intermediate rank in transfection mediation.

Discussion

The most important conclusion from this work is that asbestos can mediate transfection of mammalian cells by viral RNA. Current and planned research has the objectives of determining whether asbestos can mediate a) transfection by viral DNA, b) cell transformation by cellular DNA, or c) cell transformation by cellular chromosomes or chromosome fragments such as those containing oncogenes or those produced through the chromosome mutagenicity of asbestos itself. A positive result in c) might have two consequences.

First, the transfer could, at least in part, account for the strong *genome* mutagenicity of asbestos. Second, the transfer could result in combinations of two or more oncogenes in the same cell; and some of these combinations may confer malignancy. Thus, a role for transfection mediation or chromosome transfer in carcinogenesis is hypothesized. Under this hypothesis, transfection mediation would join chromosome mutagenicity, fiber diminsions (33), biological durability (34),

TABLE 5

INHIBITION BY DNA OCCURS AT INITIATION OF ASBESTOS-MEDIATED TRANSFECTION BY VIRAL RNA

Concentration of Salmon Sperm DNA, μg/ml[a] During Preincubation	During Incubation with Cells	Inoculum Designation	Plaques/Plate, $\bar{X} \pm s$[b]
0	0	A	100 ± 20
50	8 1/3	B	56 ± 30
50	50	C	25 ± 5
0	8 1/3	D	48 ± 7
0	50	E	17 ± 5

[a]Preincubation: Poliovirus type 2 RNA at 1:100, anthophyllite (Pow) at 5.0 mg/ml, DNA at 0 or 50 μg/ml, in buffer Ah, 37° C, 30 min. Preincubated mixtures were diluted six-fold into diluents selected to give inocula of RNA at 1:600, anthophyllite (Pow) at 5.0 mg/ml, and DNA at 0, 8 1/3, or 50 μg/ml, in buffer Ah. CLI cell sheets were washed four times with buffer B, inoculated, incubated at 37° C 10 min, and agar overlayered.

[b]Mean ± SD calculated from pooled error variance. Data from two experiments, 2 CLI cell cultures per inoculum per experiment. D vs. B, and E vs. C, $P > 0.05$.

TABLE 6

ASBESTOS VS. CLASSICAL "INSOLUBLE" FACILITATORS

Facilitator[b]	Plaques/Plate, $\bar{X} \pm s_{\bar{X}}$[a] Experiment BV1	Experiment BV2
None (control)	0	0
Bentonite	>15 ± 0.9	>33 ± 4
Calcium phosphate (Mallinckrodt)	146 ± 13	82 ± 24
Calcium phosphate (fresh)	78 ± 8	34 ± 1
Calcium phosphate ("coprecipitated")	48 ± 4	29 ± 11
Chromic oxide	241 ± 97	357 ± 44
Chrysotile, short (NIEHS)	198 ± 22	222 ± 23
Crocidolite (NIEHS)	234 ± 3	240 ± 22
Ferric oxide	70 ± 39	48 ± 27
Kaolin	*ca.* 1000	*ca.* 1000
Talc	428 ± 84	554 ± 58

[a]Mean ± SE of the mean; $n = 3$ except $n = 2$ for control in BV2. CLI cells were used. Due to cytotoxicity of bentonite at 2.5 mg/ml (6), plaques were countable on less than half of the cell culture area.

[b]Inocula: Buffer Ah with poliovirus type 1 RNA at 1:525 from serum-free stock; facilitator concentration, 2.5 mg/ml. Solutions of Na_2HPO_4 and $CaCl_2$ were calculated to give calcium phosphate at approximately 2.5 mg/ml for the *fresh* and "*coprecipitated*" tests.

hydrocarbon transport (14), and prevalence as asbestos properties important to the prominence of asbestos in carcinogenesis.

The lack of prominence of other "insoluble" facilitators in carcinogenesis can be ascribed to deficiencies in one or more of the above properties, especially fiber dimensions and prevalence. However, evidence suggests that ferric oxide (29), kaolin (24), and talc (22) may have some significance in human or experimental carcinogenesis.

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