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The Activity of Environmental Samples in a Cell Culture Test for Asbestos Toxicity[1]

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The inhibition of colony-forming efficiency of cultured human embryonic intestine-derived epithelial (I-407) cells was utilized in order to assay the toxic potential of six coded samples of particulate matter provided by the United States Environmental Protection Agency (EPA). The results of the assay indicated that the most toxic of the EPA samples tested "blindly" was amosite which was equal in toxicity to the amosite used, by chance, as a positive control. The toxicities of the particulates from drinking water were approximately 100-fold less than the amosite; of these, the order of toxicity of the samples was San Francisco > Seattle > Duluth. The samples of attapulgite clay and taconite tailings displayed approximately half the toxicity of the drinking water particulates. These results indicate that this assay provides a sensitive and accurate method for screening asbestos and asbestiform contamination for potential toxicity.

INTRODUCTION

Occupational exposure to airborne asbestos fibers has been implicated in the production of pulmonary asbestosis, lung cancer, and pleural and peritoneal mesotheliomas (Selikoff, *et al.*, 1964, 1972; McDonald *et al.*, 1971; Enterline *et al.*, 1972). Data are also suggestive of a relationship between exposure to asbestos and the development of various forms of gastrointestinal cancer as a result of swallowing inhaled fibers or ingesting food and beverages containing asbestos fibers (Hammond *et al.*, 1965; Selikoff *et al.*, 1974). Since asbestos fibers penetrate the mucosal lining of the gastrointestinal tract, they may also lead to the production of tumors in remote organs (Westlake *et al.*, 1965; Storeygard and Brown, 1977; Cook and Olson, 1979).

These possibilities have caused concern about the presence of asbestos fibers in municipal water supplies in this country (Masson *et al.*, 1974; Levy *et al.*, 1976) and in Canada (Cunningham and Pontefract, 1971). The discovery in 1973 of asbestos fibers in Lake Superior, the source of municipal water for Duluth, Minnesota, prompted investigation of Duluth's drinking water (Cook *et al.*, 1976; Kramer, 1976). Studies revealed the presence of 600 million amphibole asbestiform fibers per liter in samples of Duluth tap water (Cook *et al.*, 1976). Contamination of the water supply with asbestos apparently began in 1955 as a result of the dumping of taconite mine tailings containing amphibole impurities into the lake

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(United States of America vs Reserve Mining Company, 5-22 Civil 19). Although, to date, surveys have demonstrated no consistent increase in cancer incidence (Masson *et al.*, 1974; Levy *et al.*, 1976) or mortality (Wigle, 1977) that can be specifically attributed to exposure to asbestos in drinking water, with the exception of one study which did indicate an increased incidence (McCabe and Millette, 1979), ongoing cancer surveillance in regions with high levels of asbestos fiber contamination in the drinking water has been initiated. Furthermore, it should be borne in mind that among persons occupationally exposed to asbestos dust, the increases in death rates from cancer of the stomach, colon, and rectum do not reach measurable proportions until many years after the onset of exposure. Thus, the lapse of time since Duluth water was first heavily polluted with mineral fibers is not yet sufficient to have produced a significant increase in death rates from these cancers, particularly if the risk for residents of Duluth is not as great as for workers occupationally exposed but still of important proportions. If increased risk exists, it will become apparent within another 5 to 15 years (National Research Council, 1977).

In order to study the cellular effects of asbestos, we have developed a cytotoxicity assay that measures the inhibition of colony-forming efficiency of cultured human embryonic intestine-derived (I-407), adult rat liver-derived epithelial (ARL-6), and mouse colon-derived epithelial-like (MCE-1) cells (Reiss *et al.*, 1980). Using this assay we were able to quantitate the cytotoxic effects of the chrysotile and amphibole (amosite and crocidolite) forms of asbestos. The I-407 line was found to be the most sensitive of these cell lines to the toxic effects of all forms of Union Internationale Contre Le Cancer (UICC) asbestos (Timbrell and Rendall, 1971–1972) that were tested. This cytotoxicity assay was utilized in the present investigation to determine "in a blind fashion" the toxicity of six coded samples of particulate matter provided by the EPA. Among these samples were particulates extracted by filtration from the drinking water of three large American cities, San Francisco, Seattle, and Duluth (the Duluth sample was obtained prior to the installation of a filtration plant), as well as taconite tailings, attapulgite clay, and the amosite form of asbestos.

The results of the assay indicated that the most toxic of the EPA samples tested "blindly" was the amosite which was equal in toxicity to the amosite used, by chance, as a positive control. The toxicities of the particulates from drinking water were approximately 100-fold less than the amosite control; of these, the order of toxicity of the samples was San Francisco > Seattle > Duluth. The toxicities of the attapulgite clay and the taconite tailings were approximately half that of the drinking water particulates. Thus, the test displayed a wide range in sensitivity to particulates of different composition, and, of these, the asbestos sample was the most toxic.

MATERIALS AND METHODS

Samples of Particulates

Six samples of particulates were provided by the EPA. At the time of testing, the identity of the samples was unknown; however, following the completion of the assays, the EPA supplied us with the following descriptions:

A. *Sample 1.* prior to the amphibole c line fraction

B. *Sample 2.* P No filtration chrysotile a

C. *Sample 3.* I water. The analysis is s

D. *Sample 4.* S which had b phibole fibe

E. *Sample 5.* S eral which c chrysotile as from the mi checked by some water generally les bundles in di

F. *Sample 8.* A ingested asb tute of Envi Administrati

The UICC referenc a 50% decrease in th 0.025 g/liter also pro cytotoxicity assay.

Before weighing, t exposed to 130 erg/m contamination in a m chemical properties o inaccuracies in weig

ANALYSIS BY E

Sample
Duluth
San Francisco
Seattle
Attapulgite

Single crystal shapes an Circular/1977 IC-8751.

A. *Sample 1.* Particulates filtered from Duluth, Minnesota drinking water prior to the installation of the filtration plant at Duluth. Known to contain amphibole crystals, red clay, and biological fragments. From the crystalline fraction, the particle analysis is shown in Table 1.

B. *Sample 2.* Particulates filtered from Seattle, Washington drinking water. No filtration is used in water treatment. The sample is known to contain chrysotile asbestos. The particle analysis is shown in Table 1.

C. *Sample 3.* Particulates filtered from San Francisco, California drinking water. The sample is known to contain chrysotile asbestos. The particle analysis is shown in Table 1.

D. *Sample 4.* Sample of the less than 2-μm-size fraction of taconite tailings which had been prepared by a sedimentation separation procedure. Amphibole fibers were identified among the particulates in sample 4.

E. *Sample 5.* Sample of attapulgite (palygorskite) clay, a nonasbestos mineral which consisted of fibers of the same or smaller diameter range as chrysotile asbestos. The attapulgite was obtained in relatively pure form from the mine in Attapulgus, Georgia. The purity of the sample was checked by X-ray diffraction. Attapulgite fibers have been identified in some water supplies in Georgia and Florida. The attapulgite fibers are generally less than 2 μm in length and tend to clump and form small bundles in drinking water.

F. *Sample 8.* Amosite fibers currently being used to study the effects of ingested asbestos on rats and hamsters sponsored by The National Institute of Environmental Health Sciences (NIEHS), The Food and Drug Administration (FDA), and the EPA (Moore, 1978).

The UICC reference sample of amosite at a CD_{50} (cytotoxic dose that produced a 50% decrease in the number of colonies formed in the cytotoxicity assay) of 0.025 g/liter also provided by the EPA was used as a positive control for the cytotoxicity assay.

Before weighing, the coded samples and the known sample of amosite were exposed to 130 erg/mm²/sec ultraviolet light for 2 hr in order to suppress viable contamination in a manner that would be least likely to alter the physical and chemical properties of the particulates (Reiss *et al.*, 1980). In order to eliminate inaccuracies in weighing caused by the electrostatic character of asbestos,

TABLE 1
ANALYSIS BY ELECTRON MICROSCOPY OF THE CRYSTALLINE FRACTION OF THE SAMPLES OF PARTICULATES

Sample	Fibers[a] (%)	Equants (%)	Aggregates (%)	Acciculars (%)
Duluth	4.2	61.1	9.3	0.9
San Francisco	7.7	37.4	18.1	0.0
Seattle	2.0	80.9	1.5	0.0
Attapulgite	100	Trace		

[a] Single crystal shapes and aggregate classification as described in the Bureau of Mines Information Circular/1977 IC-8751.

aliquots of the coded samples or amosite were weighed in a known weight of water or culture medium immediately prior to use.

Cell Cultures

I-407 cells were used for the cytotoxicity assay. These cells were maintained at 37°C in Williams' Medium E (Williams and Gunn, 1974; Flow Laboratories, Rockville, Md.) supplemented with 10% fetal bovine serum (Flow Laboratories), and containing 50.0 units/ml mycostatin (Gibco, Grand Island, N.Y.) and 100 μg/ml gentamycin (Schering, Kenilworth, N.J.).

Cytotoxicity Assay

The cytotoxicity of the coded samples was quantified as described previously (Reiss *et al.*, 1980) by measuring the inhibition of I-407 epithelial cell colony formation following exposure. For this assay, 10 I-407 cells/cm² were inoculated into 25-cm² culture flasks. Twenty-four hours after the cells were seeded, the medium was replaced with medium containing an aliquot of the sample. Following 3 days of exposure, the cells were washed twice and reincubated in fresh medium. Between 1 and 2 weeks after the initiation of treatment, the cells were fixed in 10% formalin and stained with Giemsa for the determination of colony formation.

RESULTS

The dose–response for each unknown was determined over a range of concentrations from 0.001 to 5.0 g/liter. Testing was initiated by adding between 0.01 and 0.05 g/liter of each sample to the cultures. Depending upon the level of toxicity that the samples exhibited in these early experiments, a range of concentrations was chosen at quarter-log intervals in order to obtain several values of toxicity approximating a CD_{50} for each sample.

The results of the cytotoxicity assay (Table 2) demonstrated that by assaying the inhibition of I-407 cell colony formation, the cytotoxic levels of samples coded only by reference number could be easily and reliably evaluated. The order of toxicity of these samples, as determined by the assay, was: sample no. $8 > 3 > 2 > 1 \geq 5 \sim 4$. The most toxic sample with a CD_{50} of approximately 0.01 g/liter was sample 8, amosite. The similar toxicity of this amosite sample to the amosite control indicates the reliability of this assay.

The sample from San Francisco's drinking water (sample 3) was found to have a CD_{50} between 0.75 and 1.0 g/liter. The CD_{50} of the Seattle drinking water sample (sample 2) was between 1.0 and 2.5 g/liter and the CD_{50} of the Duluth drinking water sample (sample 1), taken before installation of a filtration plant, was between 2.5 and 5.0 g/liter. The CD_{50}'s of both the samples of attapulgite clay (sample 5) and taconite tailings (sample 4) were at concentrations greater than 5.0 g/liter. Because of the difficulty of obtaining a homogeneous suspension at high concentrations, the assay was not performed at concentrations above this value.

TABLE 2
TOXICITY TO THE I-407 CELL LINE OF SAMPLES OF PARTICULATES

Concentration of	Percentage inhibition of colony formation[a]				
	Duluth	Seattle	San Francisco	Taconite	Attapulgite

DISCUSSION

The cytotoxicity assay described previously (Reiss *et al.*, 1980) and applied in this study is a rapid and efficient test which is shown to be useful for the screening

TABLE 2

TOXICITY TO THE I-407 CELL LINE OF SAMPLES OF PARTICULATES

Concentration of sample tested (g/liter)	Percentage inhibition of colony formation[a]							
	Amosite control	Duluth drinking water (1)[b]	Seattle drinking water (2)	San Francisco drinking water (3)	Taconite tailings (4)	Attapulgite clay (5)	Amosite (8)
0.001	—	—	—	—	—	8	18
0.0025	—	—	—	—	—	—	—
0.005	—	21	—	—	—	9	5
0.0075	—	—	—	—	—	—	—
0.01	—	—	10	—	1	16	54 ± 13[c]
0.025	59 ± 15	21	—	—	0	—	72 ± 12
0.05	—	0	—	—	1	—	86
0.075	—	—	16	—	—	—	—
0.1	—	—	9	—	—	0	—
0.25	—	—	—	6	3	—	—
0.5	—	6	—	—	—	5	—
0.75	—	—	49	6	—	—	—
1.0	—	1	37[c]	96	2	3	—
2.5	—	30	73	100	33	35	—
5.0	—	88[c]	91	—	41	43	—

[a] These values represent the results of a total of seven experiments; many of these values are averages from duplicate experiments. Where more than two experiments are averaged, the standard deviation is included. Each value is the percentage inhibition of CFE of I-407 cells following exposure to water contamination samples. The average CFE for all experiments of I-407 cells not exposed to water contamination samples or amosite was 112.5 ± 41.2. An amosite standard at 0.025 g/liter was tested simultaneously with each experiment; the average percentage inhibition of CFE for all experiments of amosite-exposed cells was 59.2 ± 15.1. Chrysotile at 0.0025 g/liter exhibited approximately a 50% inhibition of CFE in I-407 cell cultures.

[b] Coded numbers used by the EPA to identify the six samples of particulates.

[c] A result widely divergent from this value was omitted because it was inconsistant with the trend of increasing concentration of the sample.

of samples of particulates from drinking water or other sources for the presence of asbestos. Although all of the samples tested were of similar densities, large differences in the toxicities of these samples were found using this assay. Furthermore, these toxicities correlated closely with the asbestos content described in the analysis of the samples provided by the EPA after the completion of the assays. The results of this investigation suggest that asbestos exerts its toxicity through specific physicochemical mechanisms and possesses a cytotoxic potential not found in nonasbestos samples composed primarily of attapulgite clay or taconite tailings.

This bioassay system developed for asbestos can be used as a rapid and accurate method for comparing the relative cytotoxicities of commercial fiber samples with fibers and particulates from relevant environmental samples. Among mineralogists there is considerable debate as to whether the amphibole fibers found in the water of Lake Superior should be called asbestos or asbestiform. Although the basic chemistries and crystal structure of the lake fibers are the same as a commerical form of asbestos, a number of mineralogists propose that there may be some critical differences which would change the hazard potential. Similar questions have been raised about the chrysotile associated with serpentinite quarry samples. The results of our assay suggest that the 4.2% fibers that are in Duluth drinking water (sample 1) have a toxic potential and behave like true asbestos; in contrast, the attapulgite clay containing 100% fibers displayed only minimal toxicity in this assay and is not asbestos-like. Thus, this *in vitro* assay, enables the screening of asbestos- and asbestiform-contaminated environmental samples for hazard potential, an undertaking that would be exceedingly difficult and require prolonged exposure *in vivo*.

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