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Interactions of Chrysotile and Benzopyrene in a Human Cell Culture Systems

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The risk of lung cancer related to asbestos exposure has been shown to increase disproportionately by cigarette smoking, suggesting a synergistic effect. Differing lengths of NIEHS chrysotile with benzopyrene [B(a)P, B(e)P] (organic by-products of combustion) were applied on normal human fibroblasts (cell line CI) to test for cytotoxicity (survival determined by colony-forming efficiency), binding of benzopyrene to DNA, and the production of benzopyrene metabolites.

At concentrations of 100 μg/mL, NIEHS short chrysotile was more cytotoxic than NIEHS intermediate chrysotile (3% and 17% survival, respectively); B(a)P and B(e)P concentrations up to and including 10μM were not cytotoxic. Simultaneous application of NIEHS short chrysotile with B(a)P or B(e)P did not decrease survival synergistically. On the contrary, application of B(a)P simultaneously with NIEHS intermediate chrysotile resulted in increased survival over that of intermediate chrysotile alone (25% and 17% survival, respectively). There were low levels of B(a)P bound to DNA in the presence of NIEHS short chrysotile or NIEHS intermediate chrysotile. Measurable levels of B(a)P-DNA adducts were formed both in the absence and in the presence of each size of NIEHS chrysotile. However, there was no strong indication of a perturbation of the level of DNA-B(a)P binding following simultaneous administration of increasing levels of asbestos in addition to 1 μM hydrocarbon. The asbestos had no demonstrable influence on the level of B(a)P metabolism during the 24-hr period following simultaneous exposure of asbestos and hyrdocarbons. In addition, the B(a)P-deoxynucleoside adducts formed both in the presence of 1 mg/mL short chrysotile and in its absence, as determined by co-chromatography on HPLC with standards formed by the reaction of the (+)*anti*-benzo(a)pyrenediol epoxide at the exocyclic amino group of deoxyriboguanosine (N^2).

Introduction

Human exposure to fibrous silicates is associated with the development of asbestosis (*1-3*) in a large percentage of asbestos workers (*4*). The risk of lung cancer related to asbestos exposure has been shown to increase disproportionately due to cigarette smoking, suggesting a synergistic effect. In one reported 5-year span, malignancies are 1.5 times greater and lung cancer 2.6 times more prevalent in those exposed to asbestos than nonexposed individuals (*2*). Asbestos workers who smoke cigarettes run an even higher risk of developing lung cancer, more than 90 times the normal rate of the general nonexposed, nonsmoking population and 30 times greater than asbestos workers who do not smoke (*5*). A similar study by McMillan et al. (*6*) in England also showed a significantly higher prevalence of asbestos-related lesions in asbestos workers who smoked or were ex-smokers, than in nonsmokers.

Idiopathic interstitial lung disease can be due to environmental toxins, i.e., chemicals and/or particulates. However, the primary consequences of asbestos exposure is fibrosis (*7, 8*). End-stage interstitial lung disease is frequently manifested as pulmonary fibrosis. Since pulmonary fibrosis is the characteristic lesion associated with asbestosis, we investi-

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gated the effects of asbestos with and without benzopyrene in a normal human dermal fibroblast (CI) cell culture system.

Chrysotile, a serpentine asbestos, was chosen for the study since it is the most common type of asbestos used in the United States. Two sizes of chrysotile, short and intermediate, mean length and width of 6.8 μm by 0.16 μm and 21.0 μm by 0.17 μm, respectively, as determined by scanning electron microscopy (*9*) were supplied by the National Institute of Environmental Health Sciences (NIEHS). We investigated the effects of the chrysotile using the following parameters: (1) cytotoxicity with and without simultaneous application of B(a)P or B(e)P; (2) metabolism of B(a)P in the presence and absence of chrysotile; and (3) binding of the B(a)P to DNA in the presence and absence of the chrysotile.

Materials and Methods

Cell Culture Techniques

A normal human dermal fibroblast cell line (CI) was established in the laboratory of Dr. R. W. Hart (Department of Radiology, The Ohio State University). This cell line was derived from explants of normal white male neonate foreskin. Tissue was minced while being digested with 0.01% trypsin (Worthington), then incubated in a 37°C water bath for 30 min. The trypsin was inactivated by adding 10 times the volume of Eagle's Minimum Essential Medium (EMEM) supplemented with 1 *mM* sodium pyruvate, 2 *mM* glutamine, 1.5× nonessential amino acids and 1.5× BEM vitamins (GIBCO), 10% fetal bovine serum (FBS) (FLOW) plus 100 U/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL fungizone (Squibb). Explants were plated into Corning 60 mm tissue culture dishes and incubated in a humidified, 5% CO_2 atmosphere at 37°C until cells covered at least 80% of the bottom of the dish.

Stock cultures were maintained in supplemented EMEM without antibiotics or fungizone. All cultures were routinely checked for bacteria, fungi, virus and Mycoplasma sp. by the Department of Pathology's Clinical Microbiology Laboratory (The Ohio State University) and/or The Ohio Department of Health Laboratories.

Stock cultures were serially (1:2) passaged at 95% confluence. Cultures were washed twice with phosphate-buffered saline (PBS) to remove residual EMEM. Trypsin (0.01%) was added to the cultures and allowed to act at room temperature (27°C) until the cells were detached from the substrate (5-15 min). Supplemented EMEM was then added at ten times the volume of the trypsin to inactivate the trypsin's proteolytic activity. This mixture was triturated three times to insure a monodispersed cell suspension. A 0.5 mL portion of cell suspension was mixed with 0.5 mL of trypan blue (0.4% trypan blue solution in normal saline, GIBCO) and counted on a hemocytometer, to determine cell viability and cell concentration. The remainder of the cell suspension was plated in Corning tissue culture flasks and supplemented EMEM was added. Cultures were incubated in the same manner as detailed above; cells were fed every third day until 95% confluent (approximately 1 week).

Cytotoxicity Methods

Normal human dermal fibroblasts (CI) were plated at a density of 500 cells/100 mm Corning disposable tissue culture Petri dish in supplemented EMEM. Cells were allowed to attach for 6 hr before application of the asbestos and/or the benzopyrene. Asbestos concentrations ranged from 0.1 μg/mL to 1 mg/mL (concentrations were made by serial dilution) and the benzopyrene concentrations ranged from 0.1 *μM* to 10 *μM*. Particulates were applied in the EMEM and the benzopyrene was applied in dimethyl sulfoxide-1 methanol (4:1, v/v). Twenty replicas were used for each test concentration, and each experiment was repeated three times. After 24 hr of exposure (incubation at 37°C and in a humidified 5% CO_2/95% air environment) the dishes were washed twice with 10 mL of PBS, and 10 mL fresh supplemented EMEM was added to each plate. A 5 mL portion of supplemented EMEM was added to the experimental cultures weekly until control colonies were at least 5 mm in diameter. Colonies were then fixed with Modified Carnoy's Fixative (methanol-glacial acetic acid, 3:1 v/v), rinsed three times with tap water and allowed to dry overnight. After drying, colonies were stained with saturated Crystal Violet. Colonies were counted electronically by using an Artek Model 880 dark-field counter.

Binding and Metabolism of Benzo(a)pyrene

Culture Techniques. Normal human fibroblasts (CI) were plated at 3.3×10^4 cells/cm^2 in EMEM plus 10% fetal bovine serum (FBS). Cells were fed with EMEM plus 10% FBS every third day until they reached confluence. At confluence, varying concentrations of NIEHS short chrysotile were applied simultaneously with 1 *μM* ^{3}H-B(a)P previously purified to greater than 98% radiochemical purity. EMEM plus 10% FBS was incubated with ^{3}H-B(a)P without cells as an oxidized control or was frozen immediately as a nonoxidized control.

Samples were incubated for 24 hr as previously described. Following incubation, the medium was aspirated and saved for the metabolite study. Cells

were then washed three times with PBS to remove remaining radioactivity. A 5 mL portion of lysis solution (8*M* urea, 0.1*M* PBS, 1% SDS and 0.01*M* EDTA) was applied and allowed to stand for 5 min, dishes were scraped and the lysate aspirated. PBS (5 mL) was used to remove residual lysate and pooled.

Samples were sealed in serum bottles and frozen at −20°C until analysis.

DNA Isolation. The DNA was isolated by hydroxyapatite chromatography as described in detail previously (*10*). Briefly, the lysates were adsorbed onto 0.5 mg of Bio-Rad HTP-DNA grade hydroxyapatite and the gel was washed three times with 10 mL of 8 *M* urea plus 0.01 *M* sodium phosphate (pH 6.8) followed by 10 washes of 10 mL of 0.12 *M* sodium phosphate buffer (pH 6.8). The DNA was eluted from the gel by two washes with 1.5 mL of 0.48 *M* sodium phosphate (pH 6.8). The pooled DNA washes were extracted four times with equal volumes of water-saturated *n*-butanol to assure that all free, noncovalently bound radioactivity was removed. Subsequently, an aliquot of the DNA preparation was removed for hydrocarbon-DNA binding determination by analysis of radioactivity (liquid scintillation counting following acid hydrolysis, in 6 mL of Amersham PCS II on an LKB model 1216 Rack-Beta) and DNA quantitation via the 3,5-diaminobenzoic acid fluorescence reaction (*11*).

B(a)P-DNA Adduct Analysis. The purified DNA samples were hydrolyzed to the deoxyribonucleoside level enzymatically as described previously in detail (*10*). The resulting B(a)P-DNA adducts were analyzed on Zorbax-ODS (4.6 × 250 mm) columns employing a 50 to 80% methanol-in-water linear gradient over 30 min at a flow rate of 1 mL/min on a Varian Model 5000 instrument. Sixty 0.5-mL fractions were collected with an ISCO model 1800 fraction collector. The effluent was monitored at 330 nm with a Vari-chrom UV detector and for fluorescence (excitation = 330 nm, emission > 389 nm) with a Schoeffel model GM 970. Each fraction was analyzed by liquid scintillation counting following addition of 5 mL Amersham PCS II. The vials were analyzed and the data plotted with the LKB model 1216 in the DPM-PLOT mode.

Metabolism Analysis. The extent of B(a)P metabolism was estimated by the method of DePierre et al. (*12*), modified as indicated. Briefly, 1 mL of medium was treated with equal volumes of 80% ethanol and 0.5 *N* NaOH; the resulting mixture was extracted and 2.5 mL of cyclohexane. The unmetabolized B(a)P in the organic layer was analyzed by liquid scintillation counting following removal of water by treatment with 1 g of anhydrous Na_2SO_4.

Results

NIEHS short chrysotile was more toxic to normal human dermal fibroblasts than NIEHS intermediate chrysotile at concentrations greater than 100 μg/mL (Fig. 1). Concentrations less than or equal to 10 μg/mL of both the short and intermediate chrysotile resulted in similar minimal effects.

Application of either B(a)P or B(e)P [a noncarcinogenic analog of B(a)P] produced no cytotoxic effect (Fig. 2). Application of NIEHS short chrysotile simultaneously with either B(a)P (Fig. 3) or B(e)P (Fig. 4) did not enhance the cytotoxic effects of the asbestos alone. However, application of NIEHS intermediate chrysotile simultaneously with B(a)P (Fig. 3) or B(e)P (Fig. 4) seemed to diminish the cytotoxic effects of the fiber.

Disregarding the benzopyrenes and pooling all the data by concentration (Table 1), at concentrations of 1 μg/mL or less there were no significant differences between the cytotoxicity of the short or intermediate chrysotile, whereas at concentrations of 10 μg/mL and 100 μg/mL there was a significant difference between the two types of chrysotile. A dose of 1000 μg/mL of either type of chrysotile is extremely toxic (Figs. 1, 3 and 4), and there is no significant difference (Table 1).

To determine which main effects and interactions were significant a factorial analysis of variance was performed on the cytotoxicity data. The main effects determined to be significant at $p < 0.01$ were length (short versus intermediate chrysotile), group (i.e., short, intermediate, short plus B(e)P, etc.) and concentration. Interactions of length versus group and length versus concentration also proved to be significant at $p < 0.01$ (Table 2). An aftertest, Newman-Keules, was used to determine the level of significance of specific interactions (Tables 1 and 3). Specific groups versus length were analyzed (Table 3). It was determined that the following groups were significantly different from each other at $p < 0.05$: intermediate chrysotile versus intermediate plus B(a)P or intermediate plus B(e)P, short chrysotile plus B(a)P versus intermediate plus B(a)P, and short plus B(e)P versus intermediate plus B(e)P.

Table 4 details the effect of varying doses of NIEHS short chrysotile on B(a)P-DNA binding. No clear pattern of enhanced binding with increasing levels of fiber emerged in these studies. Less than 10% of the benzo(a)pyrene was metabolized by the fibroblasts at all asbestos concentrations.

The B(a)P-deoxyribonucleoside adducts from the control (0 μg/mL chrysotile) and highest dose (1000 μg/mL) groups are compared in Figure 5. In both cases the major B(a)P-DNA adduct, fractions 27-32, cochromatographs with the standard prepared from

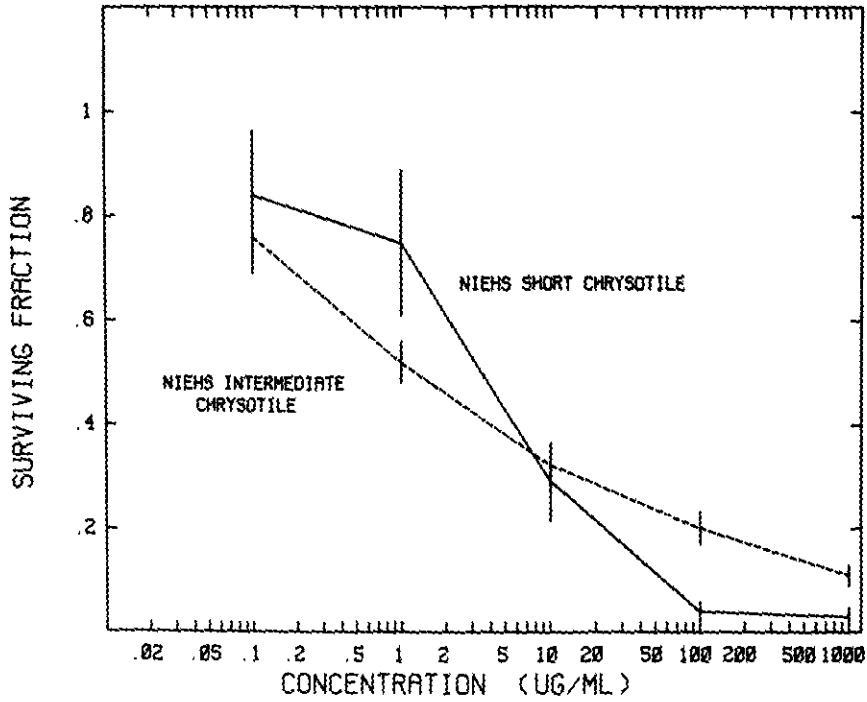

FIGURE 1. Cytotoxicity on 24-hr treatment of CI with NIEHS chrysotile.

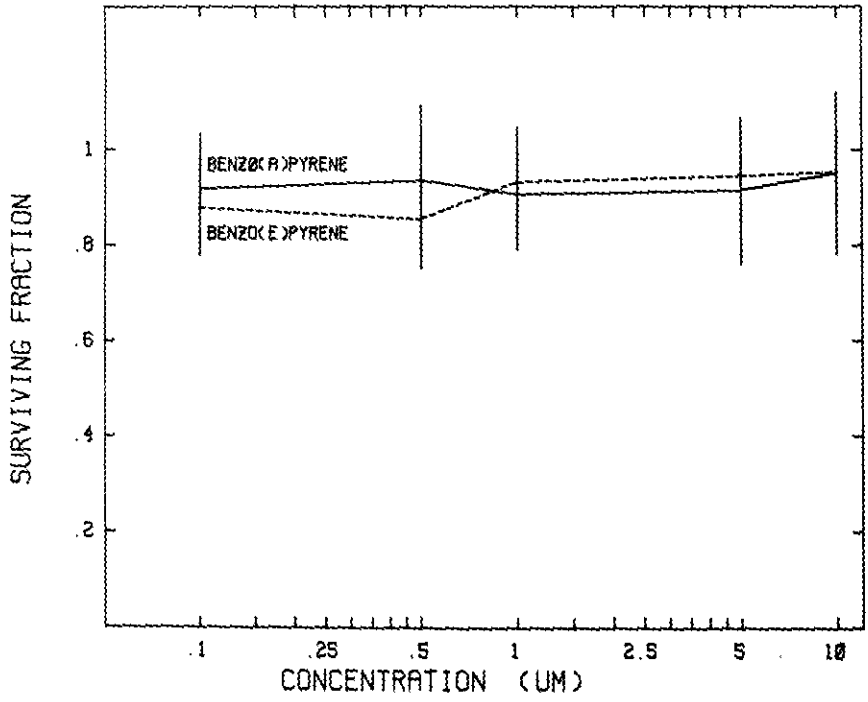

FIGURE 2. Cytotoxicity on 24-hr treatment of CI with benzopyrene.

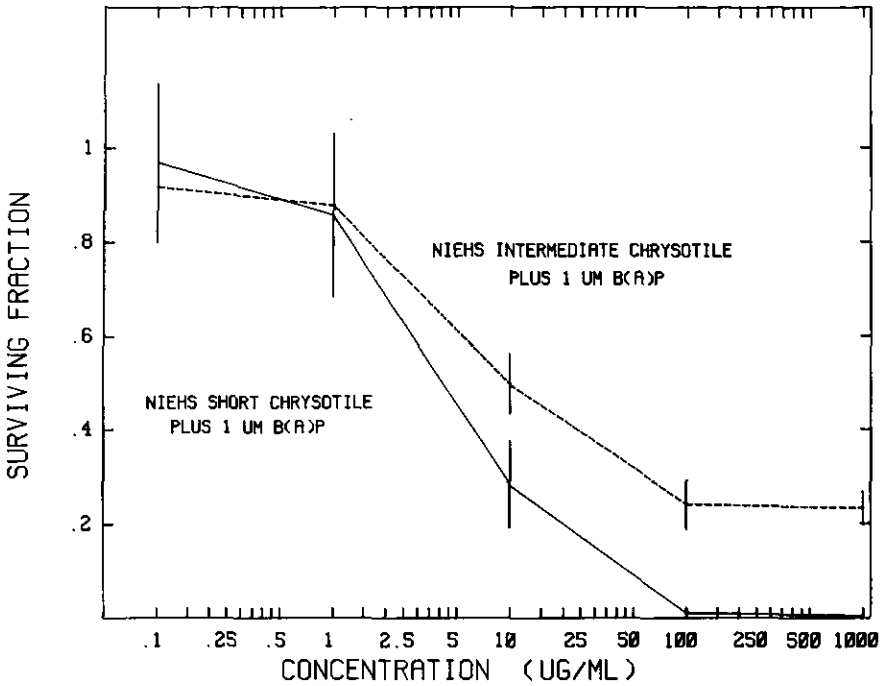

FIGURE 3. Cytotoxicity on 24-hr treatment of CI with simultaneous application of NIEHS chrysotile plus benzo(a)pyrene.

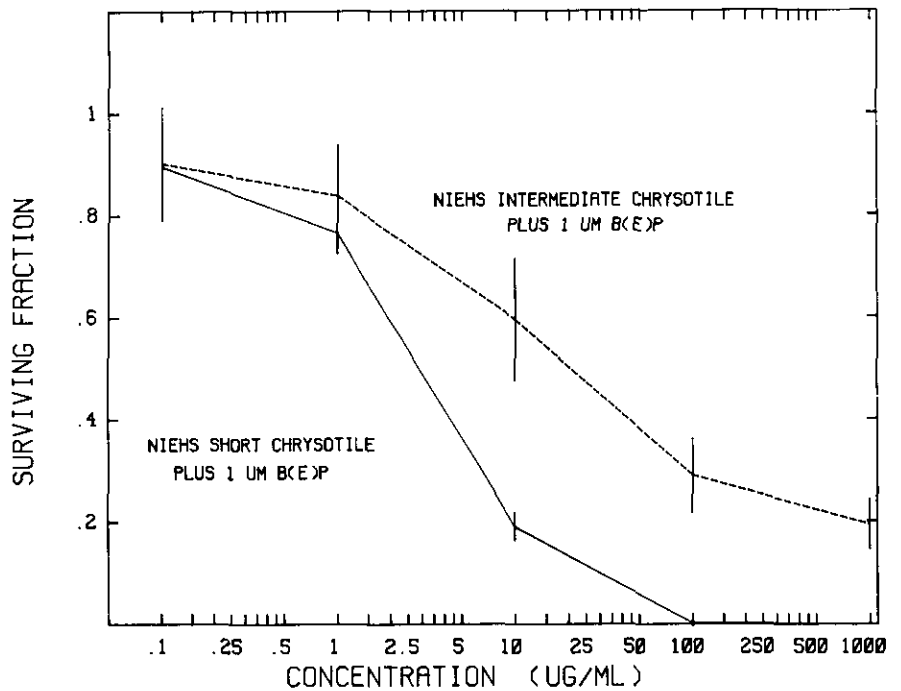

FIGURE 4. Cytotoxicity on 24-hr treatment of CI with simultaneous application of NIEHS chrysotile plus benzo(e)pyrene.

Table 1. Newman Keules: concentration versus length.[a]

Concentration, μg/mL	Mean survival fraction[b] Intermediate chrysotile	Short chrysotile	Difference	t Test
0.1	85.90	90.28	4.38	3.74
1.0	74.52	79.24	4.72	1.26
10.0	47.10	25.26	21.84	5.84*
100.0	23.32	1.62	21.70	5.80*
1000.0	13.96	1.18	12.78	3.42

[a]Relationship between the concentration and length of chrysotile as determined by cytotoxicity at $p < 0.01$.
[b]Mean of the survival fraction summed across groups (disregarding chemical applied with the chrysotile).
*Significant at $p < 0.01$.

Table 2. Factorial analysis of variance.

	Degree of freedom	F Test	Significance level p
Length	1	15.95	<0.001
Group	2	5.54	<0.01
Concentration	4	193.34	<0.001
Length × Group	2	5.91	<0.01
Length × Concentration	4	6.31	<0.001
Group × Concentration	8	1.27	N.S.
Length × Group × Concentration	8	0.61	N.S.

Table 3. Newman Keules: group versus length.[a]

	t test value (confidence interval)[b] Intermediate	Plus B(a)P	Plus B(e)P	Short	Plus B(a)P
Intermediate					
Plus B(a)P	4.79 (0.01)				
Plus B(e)P	6.20 (0.01)	1.41 (N.S.)			
Short	0.24 (N.S.)				
Plus B(a)P		3.37 (0.05)		1.18 (N.S)	
Plus B(e)P			6.64 (0.01)	0.69 (N.S.)	1.87 (N.S.)

[a]Relationship between the group and length of concentration as determined by cytotoxicity.
[b]t test value, with confidence interval in parentheses (i.e., $p<0.01$); N.S. = not significant.

the reaction of (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydroBP (BPDE I) and deoxyguanosine. This adduct has previously been confirmed as 7R-N²-(10β-[7β,8α,9α-trihydroxy-7,8,9,10-tetrahydroBP]yl)deoxyguanosine (7R-BPDE I-dG) (*11*). The enantiomeric 7S-N²-(10β-[7β,8α,9α-trihydroxy-7,8,9,10-tetrahydroBP]yl)deoxyguanosine (7S-BPDE I-dG) eluted slightly earlier (fractions 24-27) in this system and was observed only in the control cultures (Fig. 5*A*). This was interesting, since previous studies using cultured human bronchus and bladder explants without fiber have always produced the 7S-PBDE I-dG adduct (*11, 13*). The minor adduct eluting in fractions 21-24 in both control (Fig. 5*A*) and treated (Fig. 5*B*) cultures was unidentified, but by analogy with previous work (*14-16*) may be a BP-deoxycytidine adduct. Previous work with cultured human bronchus and bladder (*11, 13, 17*) and human colon (*18*) have been consistently given at least one deoxyguanosine adduct of the BPDE II ([+]-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydroBP) which eluted in fractions 33-35 in this system. This adduct may be present in small amounts in the controls but is not evident in the treated group. Finally, with these cultures, as with cultured human bronchus and blad-

Table 4. Benzopyrene binding to DNA in the presence of asbestos.

Short chrysotile concentrations, μg/mL	BP binding μmole/mole deoxyribonucleotide (± S.D.)[a]
0	1.73 ± 0.50
0.1	3.09 ± 0.68
1.0	1.55 ± 0.21
10.0	2.17 ± 0.28
100.0	1.92 ± 0.38
1000.0	2.40 ± 0.35

[a]$N = 3$ (the number of replicate samples).

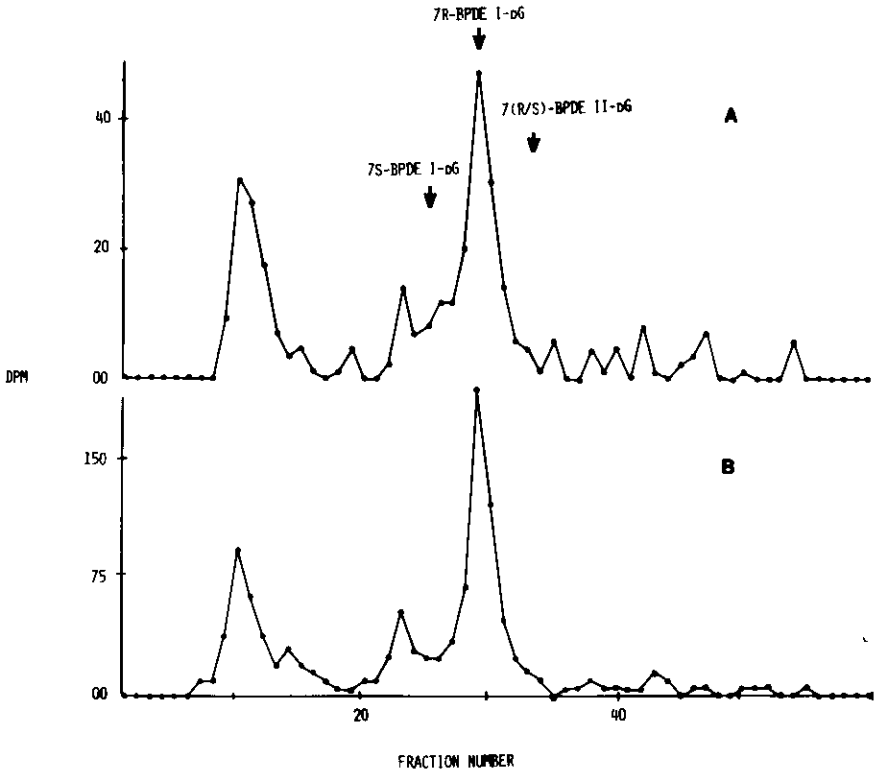

FIGURE 5. Benzo(a)pyrene adducts to DNA in the presence and absence of NIEHS short chrysotile as determined by HPLC: (*A*) control cultures, (*B*) treated cultures. 7R-BPDE I-dG = 7R-N^2-(10β-[7β,8α,9a-trihydroxy-7,8,9,10-tetrahydroBP]yl)deoxyguanosine; 7S-BPDE I-dG = 7S-N^2-(10β-[7β,8α,9α-trihydroxy-7,8,9,10-tetrahydroBP]yl)deoxyguanosine; 7(R/S)-BPDE II-dG = 7(R/S)-N^2-(10α-[7β,8α,9α-trihydroxy-7,8,9,10-tetrahydroBP)yl]deoxyguanosine.

der explants (*13*), there was no evidence of B(a)P-deoxyadenosine adducts which elute in later fractions (*38-43*). The early polar peak (fractions 8-12) is also unidentified at present. This peak was also found in incubations of human explant cultures with B(a)P (*11*).

Discussion

Most investigators studying the toxicity of asbestos have used nonhuman material (*19-24*) or nonfibroblast human cells (*25-29*). Reiss et al. (*28*) compared human embryonic intestine-derived cells (I-407) with adult rat liver-derived cells (ARL-6). Chrysotile was more toxic to the I-407 cells than the ARL-6 cells, and MCE-1 cells (mouse derived) were less sensitive than the ARL-6. In these systems they have demonstrated that fiber dimensions are correlated with toxicity (*20, 21, 30, 31*). In the human dermal fibroblast system, we have shown that the NIEHS short chrysotile is the most cytotoxic. NIEHS amosite has a higher aspect ratio than the short chrysotile and a lower aspect ratio than the intermediate chrysotile, but the amosite is far less toxic than either the intermediate or the short chrysotile (*32*).

Benzopyrene in our system does not show a synergism when applied simultaneously with either intermediate or short chrysotile. Also, benzopyrene in its unactivated state does not seem to be toxic to normal human fibroblasts. Human epithelial cells are more capable of metabolizing B(a)P to its ultimate carcinogenic form than are human fibroblasts (*33*). Our observations are in general agreement in that very low levels of BPDE adducts are formed by CI fibroblasts. Also, B(a)P-DNA (deoxyguanosine) formed in the hamster tracheal epithelial system chromatographed to the same place as the CI control peaks (*15*).

Thus, consistent with earlier results using NIEHS intermediate chrysotile (*34, 35*), the simultaneous addition of NIEHS short chrysotile and B(a)P did not enhance, in a dose-dependent manner, the metabolism and subsequent DNA binding of the hydrocarbon in human fibroblasts. However, when

the B(a)P-DNA adducts formed in the presence of 1 mg/mL NIEHS short chrysotile were compared by HPLC analysis with those generated in the absence of the fiber, the relative amounts of the various enantiomeric B(a)P-deoxyribonucleoside adducts appeared to be altered. Most notably, in the case of the adducts generated in the presence of the fiber, in marked contrast to the controls, the 7S-BPDE I-dG adduct was clearly missing. We are currently investigating this observation in more detail to see if this apparent perturbation of the stereochemistry of the B(a)P-activation-DNA-binding processes is altered in a dose-related manner. At present, the significance of this observation, if any, is unclear.

From our results a number of conclusions are suggested. (1) Available reactive surface area may play a major role in determining mineral cytotoxicity; for instance, on a weight/weight basis, short chrysotile has a greater surface area than intermediate chrysotile and therefore has more reactive sites than the intermediate chrysotile. (2) Masking of these reactive sites (such as Mg^{+2}) by certain organic compounds could possibly decrease the mineral's cytotoxicity. (3) The low levels of aryl hydrocarbon hydroxlase in the human fibroblasts could be responsible for the low levels of B(a)P metabolism (*33*).

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