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Effects of Lignite Fly Ash Particulates and Soluble Components on the Interferon System

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Induction of interferon by influenza virus was depressed by approximately 50% when mammalian (LLC-MK_2) cell monolayers were pretreated with lignite fly ash. The presence of fly ash, however, did not impair the ability of exogenous interferon to confer antiviral cellular resistance. Influenza virus multiplication in cell monolayers pretreated with fly ash attained a twofold higher level of growth than that noted in normal cell monolayers. This was related to suppression of viral interferon induction by fly ash. Whereas aqueous extracts of fly ash had no adverse effect on interferon induction, extractions of fly ash by either polar or nonpolar solvents, by horse serum with or without EDTA (a metal chelator), and fractionation of serum extracts yielded corresponding compounds, most likely organic and inorganic, that were antagonistic to viral interferon induction. Residual fly ash particulates after extraction by horse serum with EDTA were still capable of inhibiting viral induction of interferon. These findings indicate that several soluble components inherent to lignite fly ash and the particulate matrix per se may modify, independently or in concert, cellular defense behavior. Neither polar, nonpolar, nor horse serum extracts of lignite fly ash, however, showed mutagenic activity as determined by the *Salmonella* histidine reversion assay. Removal of cell-membrane-bound sialic acid (*N*-acetylneuraminic acid) by neuraminidase or pretreatment of lignite fly ash with sialic acid abolished the adverse activity of fly ash on viral interferon induction. This suggests that the interaction of cell-membrane-bound sialic acid residue with fly ash particulates may be involved in the altered state of cellular behavior described in response to viral induction of interferon.

INTRODUCTION

One of the major atmospheric pollutants to which appreciable segments of the population are being chronically exposed is fly ash, a by-product of coal combustion from electric-generation plants (EPA, 1973; Anonymous, 1978). More than a million metric tons of coal fly ash was estimated to have been released into the atmosphere of the United States during the year 1974 (ERDA, 1976) and this atmospheric burden is predicted to increase with the exploitation of innovative coal combustion technologies for electric-energy production. That coal fly ash may be potentially hazardous to human health has been insinuated from studies undertaken to chemically characterize these particulates (Natusch *et al.*, 1975; Bencko and Symon, 1977; Fisher *et al.*, 1978; Coles *et al.*, 1979). It is now well established that coal fly ash contains substantial quantities of many carcinogenic and potentially carcinogenic compounds (Natusch, 1978). With the identification of many of these substances, assessments of their effects in association with fly ash particulates have been carried out in an attempt to enlarge our un-

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derstanding of potential biological consequences. Filtrates of coal fly ash, tested for mutagenic activity using the Ames bacterial histidine reversion assay (Ames *et al.*, 1975) have been demonstrated to contain both organic and inorganic mutagens (Chrisp *et al.*, 1978). The significance of these findings relative to coal fly ash lies in the reported high positive correlation between carcinogenicity of substances for animals or man and mutagenicity (McCann *et al.*, 1975).

In recent experimental studies concerned with the immunological consequences to inhaled coal fly ash, a decrease in murine splenic antibody response was observed (Eskew *et al.*, 1982) as well as reduced phagocytic activity of macrophages for bacteria (Zarkower *et al.*, 1982). Burning of coal of high beryllium content has been reported to cause immunological changes in humans which may be associated with signs of beryllium exposure (Bencko *et al.*, 1980). Coal fly ash has been also demonstrated to activate immune complement which may trigger the different processes implicated in inflammation (Hill *et al.*, 1982).

The possible effects of coal fly ash on another host defense mechanism, the interferon system, was investigated in an effort to enhance further our knowledge of the biological activities of these particulates. The interferon system is an important component of the nonimmunological defense mechanisms of the body and it is generally believed that interferon plays a role in recovery from acute primary viral infections (Baron, 1973). In addition to mediating antiviral defenses, it is recognized that interferon may inhibit proliferation of normal and malignant cells, modulate a variety of host immune responses, induce cell-surface alterations, and stimulate or inhibit intracellular enzymes (Baron and Dianzani, 1978). Mineral particulates of public health concern, i.e., coal dust (Hahon, 1974), asbestos fibers (Hahon and Eckert, 1976), and metals (Hahon *et al.*, 1980), as well as mutagens (Hahon *et al.*, 1979), carcinogens (DeMaeyer and DeMaeyer-Guignard, 1967; Sonnenfeld *et al.*, 1980), and diesel-engine emission particulates (Hahon *et al.*, 1982) have been shown to affect adversely interferon production.

This report describes an *in vitro* study on the influence of lignite fly ash particulates on (1) viral interferon induction (2) interferon-mediated antiviral cellular resistance, (3) multiplication of influenza virus, and (4) an evaluation of the mutagenic activity and effect on interferon production of the soluble components of fly ash.

MATERIALS AND METHODS

Viruses. The Ao/PR/8/34 influenza and parainfluenza (Sendai) virus strains employed in this study were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. Stock pools of influenza and Sendai viruses, prepared from chick embryonated eggs, contained 2.0×10^8 and 1.0×10^9 cell-infecting units (CIU) of virus per milliliter, respectively, when assayed by the immunofluorescence cell-counting procedure (Hahon *et al.*, 1973).

Cell cultures. Rhesus monkey kidney (LLC-MK_2) and human Chang conjunctiva (clone 1-5c-4) cell lines obtained from ATCC were used for induction and assay of interferon, respectively. Cell lines were propagated in plastic tissue-culture flasks (75 cm^2) with Eagle's minimum essential medium (MEM) fortified with 100 × essential vitamin mixture (10 ml/liter), 200 mM solution L-glutamine

(10 ml/liter) to which was added sodium bicarbonate (2.2 g/liter), and 10% fetal bovine serum. Cells were maintained with the aforementioned medium containing only 0.5% fetal bovine serum.

Fly ash and reagents. Bulk fly ash, collected at power plant stacks, was the combustible product of lignite coal used at the Texas Utility Generating Company, Sulphur Springs, Texas. Mineral analysis of the fly ash showed that predominately it consisted of SiO_2 (58%) and Al_2O_3 (20%) and small amounts of Fe_2O_3, CaO, MgO, SO_3, Na_2O, K_2O, and TiO_2. The mean diameter and volume of fly ash particulates was 2.51 μm and 8.28 μm^3, respectively. Fly ash particulates were made into w/v suspensions in phosphate-buffered saline (PBS), pH 7.1, and sterilized in an autoclave at 20 psi pressure (126°C) for 15 min. Neuraminidase (*Vibrio cholerae*) and sialic acid (*N*-acetylneuraminic acid) were obtained from GIBCO, Grand Island, New York, and Calbiochem-Behring Corporation, La Jolla, California, respectively.

Fly ash extractions. Fly ash (20 g) samples were extracted with either 250 ml of dichloromethane (DCM) or methanol by constant agitation at room temperature (22°C) for 16 hr. DCM and methanol extracts were collected separately by filtering through Whatman No. 4 filter paper, concentrated to dryness in a rotary evaporator, and then dissolved in 1.5 and 3 ml dimethyl sulfoxide (DMSO), respectively. To ensure minimal cytocidicity in subsequent experiments, optimal concentrations of extracts to be used were determined by the trypan blue dye-exclusion procedure.

Fly ash (50 mg) samples were extracted with 5-ml quantities of horse serum (GIBCO, Grand Island, N.Y.) by agitation at 35°C for 5 days. After centrifugation of mixtures at 2000g for 20 min, supernatant fluids (horse serum extracts) were filtered through Millex GS 0.45-μm filters (Millipore Corp., Bedford, Mass.). In equal volume, horse serum extract was mixed with 2 mM disodium ethylenediaminetetraacetic acid (EDTA) and stirred overnight at 4°C to chelate metals complexed with serum proteins. Horse serum extract with EDTA and normal horse serum (control) were mixed with either saline or EDTA, respectively. All mixtures were fractionated on Sephadex G-25M PD-10 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) by elution with 3 void volumes of double-distilled water. To ensure sterility, all mixtures and fractions were passed through Millex GS 0.22-μm filters. Initial horse serum mixtures and the three corresponding fractions from each mixture, in volumes of 2.0 ml, were used to assess their influence on viral interferon induction.

Interferon induction. Duplicate experiments were performed, and the procedure generally used to study the effect of fly ash particulates on interferon induction was carried out as follows: 5 mg suspension of particulates in a 10-ml volume of maintenance medium was added to 75-cm^2 plastic flasks containing complete LLC-MK_2 cell monolayers (2×10^7 cells) which were then incubated at 35°C for 24 hr. Residual medium was decanted and 2 ml of influenza virus, which had been inactivated by ultraviolet irradiation for 45 sec at a distance of 76.2 mm and a wavelength of 253.7 nm, was added onto cell monolayers that were then incubated at 35°C for 2 hr. The multiplicity of infection (MOI) was approximately 2.0. Inoculum was removed and 10 ml of maintenance medium

was added to each flask. After incubation at 35°C for 24 hr, supernatant fluid was decanted and centrifuged at 100,000*g* for 1 hr and dialyzed against HCl–KCl buffer, pH 2.0, at 4°C for 24 hr. Dialysis was continued against two changes of PBS, pH 7.1, at 4°C for 24 hr. Fluids were passed through Millex GV 0.22-μm filters (Millipore Corporation, Bedford, Mass.) to obtain sterile preparations. Samples were stored at −80°C until they were assayed for interferon activity. Preparations with antiviral activity possessed the biological and physical properties ascribed to viral interferons (Lockart, 1973). Controls consisting of cell monolayers which were not treated with fly ash particulates were handled exactly as described above.

Interferon assay. An immunofluorescence cell-counting assay of interferon that had been described previously (Hahon *et al.*, 1975) was used to determine the interferon potency of test samples. Interferon-treated cell monolayers were challenged with 10^4 cell-infecting units of Sendai virus, and infected cells were visualized by direct fluorescent antibody staining. The reciprocal of the interferon dilution that reduced the number of infected cells to 50% of the control served as the measure of interferon activity, i.e., 50% infected cell-depressing dilution ($ICDD_{50}$). With this assay system, 0.8 international reference human (69/19) interferon unit assayed as 1 U.

Virus multiplication. Influenza virus replication concomitant with interferon production was measured in fly ash-treated (3 mg) and untreated (control) LLC-MK_2 cell monolayers (3×10^6 cells) maintained in 25-cm^2 plastic flasks. Following adsorption of virus to cells at 35°C for 1 hr, MOI of 1.0, cell monolayers were rinsed with PBS and incubated at 35°C with 5 ml of maintenance medium. At designated time intervals from 0 to 48 hr, flasks were removed and stored at −80°C. Thereafter, each flask was thawed (25°C) and frozen (−80°C) twice to disrupt cells, and the fluid content of each flask was divided into aliquots. One aliquot was assayed for virus content and the other processed for interferon assessment in the described manner.

Mutagenesis assay. Horse serum, DCM, and methanol extracts of fly ash were assayed for mutagenic activity by detecting reverse mutation from histidine dependent to histidine independent of tester strains TA98 and TA100 of *Salmonella typhimurium*. A plate incorporation test with metabolic activation (liver S-9 homogenate, Wistar/Lewis rats) and without activation was used. The procedure for the histidine reversion test was in accord with the standard assay (Ames *et al.*, 1975).

RESULTS

Viral Induction of Interferon in the Presence of Fly Ash

A preliminary consideration of this study was to determine the maximal quantity of fly ash that LLC-MK_2 cells could tolerate without loss of viability. Monolayers of nondividing cells (2×10^7) were incubated at 35°C for 24 hr with fly ash that ranged in quantity from 0.5 to 30 mg. The trypan blue dye-exclusion procedure was used to estimate the viability of treated cells. Cell viability was unaffected by 5 mg fly ash, but in the presence of 10, 20, and 30 mg fly ash, cell

survival was reduced to 83, 46, and 23%, respectively. Generally, the quantity of fly ash used in succeeding experiments was 5 mg/2 $\times$ 10^7 cells.

The induction of interferon by influenza virus was investigated using LLC-MK_2 cell monolayers that had been pretreated for 24 hr with different quantities of lignite fly ash. Results (Table 1) show that viral interferon production was depressed by approximately 50% in the presence of 5 mg fly ash/2 $\times$ 10^7 cells. This inimical effect was diminished progressively with smaller amounts of fly ash until it was no longer evident with 0.5 mg fly ash.

The time and sequence of fly ash and viral-inducer administration onto cell monolayers in relation to the inhibition of interferon production was determined. Results (Table 2) indicate that while pretreatment of cells with fly ash for as little as 4 hr markedly depressed interferon production, this event was also pronounced to the same extent when fly ash and the viral inducer were simultaneously added to cells. Furthermore, when fly ash was added to cells 4 hr after the viral inducer, it was still capable of inhibiting interferon production by approximately 50%. Partial inhibitory activity by fly ash was also noted when it was added 8 hr post-virus administration.

To determine whether lignite coal, the source of the resultant fly ash tested in this study, may also adversely affect viral interferon induction, bulk samples of coal obtained at three different stages of the crusher process were assessed in the manner similar to that described for fly ash. Results revealed that all lignite coal samples (5 mg/2 $\times$ 10^7 cells) reduced interferon production on the average of only 6% as compared to 56% reduction by fly ash.

Whether the ability of influenza virus to infect LLC-MK_2 cell monolayers was impeded by the presence of fly ash was determined by pretreating coverslip cell cultures with 0.1 and 0.2 mg fly ash for 24 hr. Virus was adsorbed onto cell monolayers at 35°C for 2 hr and, then, cell cultures were incubated at 35°C for 24 hr. Virus-infectivity titers were determined by the immunofluorescent cell-counting technique. Results showed that virus infectivity titers ranged from 6.4 $\times$ 10^7 to 7.3 $\times$ 10^7 CIU/ml in cell cultures that were pretreated with different

TABLE I

INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS PRETREATED WITH DIFFERENT QUANTITIES OF LIGNITE FLY ASH

Fly ash (mg)[a]	Interferon ($ICDD_{50}$)[b]	Interferon inhibition (% $\pm$ SE)
5	760	56.8($\pm$0.5)
2	950	46.0($\pm$1.2)
1	1060	39.8($\pm$1.2)
0.5	1750	0.6($\pm$1.2)
0(Control)	1760	0.0

[a] Fly ash/10 ml medium.

[b] Reciprocal of 50% infected cell-depressing dilution/10 ml from flasks with 2 $\times$ 10^7 cells. Means of duplicate determinations.

TABLE 2
TIME RELATIONSHIP BETWEEN ADDITION OF LIGNITE FLY ASH AND INFLUENZA VIRUS ONTO LLC-MK_2 CELL MONOLAYERS AND INTERFERON INDUCTION

Hourly relation between fly ash and addition of virus at zero time[a]	Interferon ($ICDD_{50}$)[b]	Interferon inhibition (% ISE)
−24	740	65.1(±3.6)
−16	770	63.7(±2.3)
−4	770	63.7(±2.3)
0	900	57.6(±0.2)
+4	1100	52.7(±4.0)
+8	1700[c]	27.7
+16	2120	0.0(±0.0)
Control (no fly ash)	2120	0.0

[a] Fly ash suspended in 10 ml maintenance medium added to cell monolayers at designated hours prior to or after the addition of virus at 0 time.
[b] Mean reciprocal values of two determinations of 50% cell-depressing dilution/10 ml medium.
[c] One determination.

quantities of fly ash. The titers were comparable to that obtained in untreated cell monolayers (6.8×10^7 CIU/ml). This comparability of virus infectivity indicates that associated primary virus–cell interactions (virus attachment and penetration) were not impaired by the presence of fly ash and precludes involvement of these factors in the observed depression of viral interferon induction.

An experiment was performed to determine whether the low yield of interferon may be related to the inactivation of interferon by fly ash. Quantities of fly ash, 5 and 10 mg, were suspended in a known interferon preparation and then incubated at 35°C for 24 hr. The same interferon preparation without fly ash served as the control. Subsequently, fly ash–interferon suspensions and the control were clarified by centrifugation and the supernatant fluids were assayed for interferon potency. Results indicated that in the presence 10 mg fly ash, approximately 15% of interferon was inactivated. However, with 5 mg fly ash, the quantity used throughout this experimental study, the loss of interferon potency was negligible (0.1%).

Effect of Fly Ash Extracts on Viral Interferon Induction

An experiment was designed to determine whether the supernatant fluid from aqueous fly ash suspensions contained soluble ingredients that may be responsible for the observed depression of viral interferon induction. Results (Table 3) show that viral interferon production in cells pretreated with supernatant fluid was comparable to that of untreated (control) cell cultures. Inhibition of interferon production by resuspended fly ash was comparable to that of the original fly ash preparation. It would appear, therefore, that lignite fly ash particulates per se were capable of initiating the aforementioned phenomenon to the exclusion of any aqueous soluble substances.

To investigate further the possibility that soluble chemical agents complexed

TABLE 3
INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS PRETREATED WITH LIGNITE FLY ASH SUSPENSION AND CORRESPONDING SUPERNATANT FLUID

Pretreatment of cell monolayer	Interferon ($ICDD_{50}$)[a]	Interferon inhibition (% ± SE)
Fly ash suspension	1150	49.8(±0.5)
Supernatant fluid	2150	6.2(±2.0)
Resuspended fly ash	1190	48.1(±0.1)
Maintenance medium (control)	2290	0.0

[a] Mean reciprocal values of two determinations of 50% cell-depressing dilution/10 ml medium.

with lignite fly ash matrix may be involved in the observed depression of viral interferon induction, fly ash was extracted with solvents of different polarity, i.e., dichloromethane (DCM) and methanol. Lyophilized residues of fly ash extracts were dissolved in DMSO, concentrations were selected that caused minimal loss of cell viability, and, then, each extract was tested in the usual manner for its effect on viral interferon induction and for mutagenic activity. Results revealed that comparable quantities of both DCM and methanol extracts of fly ash depressed interferon synthesis by approximately 45 and 69%, respectively (Table 4). DMSO had no effect on interferon production. These findings indicated that lignite fly ash contained both polar and nonpolar chemical compounds that were capable of influencing the described phenomenon independent of fly ash particulate matter. However, neither DCM nor methanol extracts of lignite fly ash showed any mutagenic activity (Table 5) as determined by the *S. typhimurium* histidine reversion assay.

TABLE 4
INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS PRETREATED WITH SOLVENT EXTRACTS OF LIGNITE FLY ASH

Solvent	Extract (ml)[a]	Interferon ($ICCD_{50}$)[b]	Interferon Inhibition (%)
Methanol	1.0	360	69.3
	0.5	700	40.2
	0.2	1000	14.6
	0.1	1200	0.0
Dichloromethane	1.0	650	44.5
	0.5	750	35.9
	0.2	1000	14.6
	0.1	1200	0.0
Control	0.0	1170	0.0
DMSO	1.0	1150	1.8

[a] Extracts and DMSO diluted 1:10 to minimize cytocidal effect.
[b] Reciprocal of 50% infected cell-depressing dilution/10 ml medium.

TABLE 5
MUTAGENIC RESPONSE OF *Salmonella typhimurium* (TA98, TA100) IN HISTIDINE (His^+) REVERSION ASSAY TO EXTRACTS OF FLY ASH

Solvent	Extract (mg/plate)	Revertants (His^+)/plate			
		TA98		TA100	
		−S9	+S9[c]	−S9	+S9
Dichloromethane	62.5	27	32	148	188
	125	21	43	135	178
	250	26	28	136	163
	500	30	28	152	156
Methanol	62.5	30	33	142	195
	125	29	35	162	208
	250	30	30	136	186
	500	25	38	155	172
Positive control (2-AA)[a]		ND[d]	1490	ND	1744
Negative control (DMSO)[b]		24	32	160	176

[a] 2-Aminoanthracene, 2.5 μg/plate.
[b] Dimethyl sulfoxide, 0.1 ml/plate.
[c] Metabolic activator, rat liver S9 homogenate.
[d] Not determined.

Fly ash was extracted with horse serum in an attempt to determine further the nature of chemical agents complexed with lignite fly ash that manifest inhibitory activity towards viral interferon induction. A metal chelator, EDTA, was added to portions of horse serum extracts. These extracts and a suitable control were then fractionated as described under Materials and Methods. Serum extracts were assayed for inhibitory activity to interferon production and also tested for mutagenic activity. Results (Table 6) show that serum extracts (unfractionated) of fly ash with or without EDTA were comparable in their activity for depressing interferon production (54 vs 51% , respectively). Initial fractions of both these serum extracts were also comparable in activity to each other, indicating that serum proteins had formed soluble chemical complexes. Secondary fractions of serum extracts with or without EDTA, usually associated with low-molecular-weight components, showed no adverse activity toward viral interferon induction. The third fraction of serum with EDTA inhibited interferon production by approximately 50%; however, the third fraction of serum without EDTA had no effect on interferon synthesis. The presence of metals in the former may account for the findings noted because EDTA can act by chelating metals from serum proteins. The control preparation consisting of unfractionated and fractionated horse serum with EDTA showed no detrimental activity toward the interferon-induction process. Residue fly ash that had been subjected to extraction with horse serum and EDTA was effective in depressing interferon production com-

TABLE 6
FRACTIONATION OF HORSE SERUM EXTRACTS OF LIGNITE FLY ASH ON INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS

Horse serum extracts (HSE)	Interferon ($ICDD_{50}$)[c]	Interferon inhibition (% ± SE)
HSE + EDTA[a]	1120	54.3(±5.3)
Fraction 1[b]	1050	57.1(±9.2)
Fraction 2	2320	5.3(±6.6)
Fraction 3	1170	52.2(±1.4)
HSE + saline	1100	51.2(±6.5)
Fraction 1	1250	49.0(±11.6)
Fraction 2	2620	0.0(±1.3)
Fraction 3	2600	0.0(±1.3)
HS (control) + EDTA	2700	0.0(±0.0)
Fraction 1	2500	0.0(±0.0)
Fraction 2	2400	2.0(±0.5)
Fraction 3	2450	0.0(±0.0)
Fly ash (control)	1080	55.9(±5.2)
Fly ash after HSE	1300	46.9(±8.9)
Control (no fly ash)	2450	0.0

[a] Disodium ethylenediaminetetraacetic acid.
[b] Horse serum extracts were fractionated on Sephadex, G-25M, PD-10 columns.
[c] Mean reciprocal values of two determinations of 50% infected cell-depressing dilution/10 ml from flasks containing 1×10^7 cells.

parable to that of untreated fly ash, indicating that the former may still contain inhibitory substances. When unfractionated horse serum extract and control horse serum were tested for mutagenic activity, results revealed no significant increase of *S. typhimurium* reverents (Table 7).

Interferon-Mediated Antiviral Cellular Resistance

To determine whether fly ash particulates may influence interferon-mediated resistance of cells to virus infection, an interferon preparation of known potency was assayed in the usual manner on clone 1-5c-4 cell monolayers. Cells (2×10^5) were pretreated for 24 hr with fly ash quantities of 0.1 or 0.2 mg or the appropriate control medium and then challenged with Sendai virus. Results revealed interferon titers for the control was 145 and 140 $ICDD_{50}$/ml each for both designated quantities of fly ash used to pretreat cells. The presence of fly ash particulates did not impair the ability of exogenous interferon to confer antiviral cellular protection.

Influenza Virus Growth in the Presence of Fly Ash

Influenza virus multiplication and concomitant interferon production were determined in normal LLC-MK_2 cell monolayers and in those pretreated 24 hr earlier with fly ash. Virus multiplication rates in normal and fly ash-treated cells

TABLE 7
MUTAGENIC RESPONSE OF *Salmonella typhimurium* (TA98, TA100) IN HISTIDINE (His^+ REVERSION ASSAY TO HORSE SERUM EXTRACTS OF LIGNITE FLY ASH

Solvent	Extract (mg/plate)	Revertants (His^+)/plate			
		TA98		TA100	
		−S9	+S9[c]	−S9	+S9
Horse serum	6.25	18	29	159	184
	12.5	21	31	149	190
	25.0	24	32	150	186
	50.0	21	33	161	167
Horse serum control	6.25	29	35	134	189
	12.5	23	28	152	177
	25.0	26	39	138	172
	50.0	31	33	140	178
Positive control (2-AA)[a]		ND[d]	2632	ND	1664
Negative control (DMSO)[b]		25	34	153	184

[a] 2-Aminoanthracene, 2.5 μg/plate.
[b] Dimethyl sulfoxide, 0.1 ml/plate.
[c] Metabolic activator, rat liver S9 homogenate.
[d] Not determined.

were similar to the extent that plateaus of virus growth were attained in 24 hr (Fig. 1). Virus growth in cell monolayers pretreated with fly ash, however, reached a level that was approximately twofold higher than noted in normal cell cultures. Whereas the rate of interferon production appeared similar during the first 16 hr in both cell cultures, at 24 hr, interferon production was almost threefold higher in control cell cultures than in fly ash-pretreated cells. This difference increased progressively with time. These findings suggest that the higher level of virus growth attained in cell monolayers pretreated with fly ash than in normal cell monolayers may be the consequence of partial suppression of interferon synthesis by fly ash.

Role of Sialic Acid

The activity of fly ash on viral induction of interferon was examined relative to sialic acid which is an integral component of mammalian cell membranes. Cell monolayers were first treated for 1 hr with different concentrations of neuraminidase prepared in sodium acetate buffer, pH 5.5, to remove membrane-bound sialic acid residues. Thereafter, cells were rinsed with PBS, pH 7.1, and exposed to 5 mg fly ash for 24 hr viral inducer was added in the usual sequence and manner. Results (Table 8) show that removal of cell-bound sialic acid by higher concentrations of neuraminidase (500 and 250 units) markedly abolished the inhibitory activity of fly ash on interferon induction. Cells treated with neuraminidase alone did not significantly affect the viral interferon-induction process. The

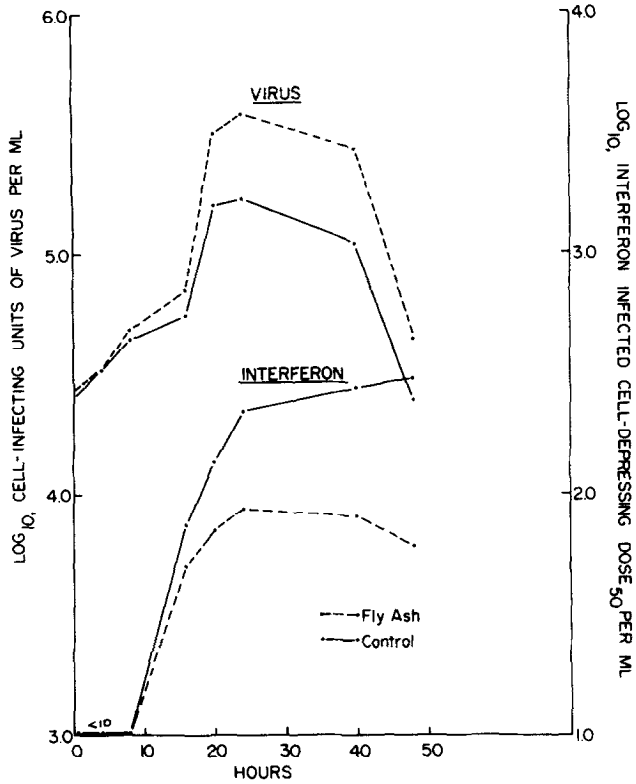

FIG. 1 Growth curves of influenza virus (Ao/PR/8/34) and concomitant interferon production in LLC-MK_2 cell monolayers treated 24 hr earlier with lignite fly ash.

TABLE 8

EFFECT OF LIGNITE FLY ASH ON INFLUENZA VIRUS INTERFERON INDUCTION IN LLC-MK_2 CELL MONOLAYERS PRETREATED WITH NEURAMINIDASE

Neuraminidase (units)[a]	Fly ash (mg)	Interferon ($ICDD_{50}$)[b]	Interferon inhibition (% ± SE)
500	5	1670	4.6 (±2.8)
250	5	1650	5.8 (±1.7)
100	5	1210	30.9 (±2.7)
50	5	1020	41.8 (±5.1)
0	5	940	46.3 (±5.5)
Controls			
500	0	1750	0.0
0	0	1750	0.0 (±2.0)

[a] One unit is defined as that amount of enzyme that releases 1 μM of *N*-acetylneuraminic acid from human acid d_1-glycoprotein/min at 37°C.

[b] Mean reciprocal values of two determinations of 50% cell-depressing dilution/10 ml medium.

findings strongly suggest that sialic acid may act as a receptor, interacting with fly ash, to affect the cellular response to a viral inducer of interferon.

To determine if reactive sites on fly ash particulates may interact or complex with sialic acid, suspensions of fly ash (5 mg) were mixed with different quantities of sialic acid (*N*-acetyneuraminic acid) and incubated at 35°C for 4 hr. Fly ash particulates were sedimented by centrifugation, rinsed, and introduced onto cell monolayers which were then exposed to viral inducer in the usual manner. Results (Table 9) show a marked reduction of the inhibitory activity of fly ash on viral interferon induction when the particulates were pretreated with 100 mg sialic acid. This ameliorating effect was progressively decreased with smaller amounts of sialic acid. That free sialic acid forms complexes with or masks the active components of fly ash, resulting in the abrogation of the latter's adverse activity on viral interferon induction, adds further support for a possible role of cell membrane-bound sialic acid in the described phenomenon.

DISCUSSION

The findings of this study demonstrated that viral induction of interferon was inhibited by approximately 50% when mammalian cell monolayers were pretreated with lignite fly ash. There was no evidence to indicate that this inhibitory phenomenon was attributed to either adsorption of exogenous interferon or impediment of primary virus–cell interactions (virus attachment and penetration) by the cellular presence of fly ash. With respect to the ability of interferon to confer antiviral cellular resistance, this aspect of interferon activity was unaffected by fly ash particulates. That the viral interferon-induction process was most sensitive and the mode of interferon's antiviral action was apparently insensitive to the cellular presence of fly ash is consistent with observations noted previously when other mineral and metal particulates were evaluated in the same *in vitro* system (Hahon *et al.*, 1980; Hahon *et al.*, 1982). The inhibitory activity of fly ash on viral interferon induction was pronounced when cells were either treated with fly ash before, added simultaneously with, or added 4 hr after the virus inducer.

TABLE 9
EFFECT OF LIGNITE FLY ASH PRETREATED WITH SIALIC ACID (N-ACETYLNEURAMINIC ACID) ON INFLUENZA VIRUS INTERFERON INDUCTION IN LLC-MK_2 CELL MONOLAYERS

Sialic acid (mg)	Fly ash (mg)	Interferon ($ICDD_{50}$)[a]	Interferon inhibition (% ± SE)
100	5	1750	7.9(±0.6)
50	5	1530	19.5(±1.3)
25	5	1290	32.1(±1.7)
10	5	1050	44.7(±3.8)
0	5	970	48.9(±1.0)
Control: 0	0	1900	0.0

[a] Mean reciprocal values of two determinations of 50% cell-depressing dilution/10 ml medium.

Apparently, fly ash was capable of adversely affecting the interferon-induction phase (inducer binding, uptake, and processing) which encompasses a period of approximately 6 hr (Stewart, 1981) even when this phase was already in progress. However, the subsequent addition of fly ash after the virus inducer, at 8 hr or later, partially affected and then had no effect on the interferon-production phase (transcription and translation of mRNA, and secretion of interferon).

Studies of influenza virus growth carried out in cell cultures pretreated with fly ash showed that the level of virus multiplication attained was approximately twofold higher than that noted in normal cell cultures. This was associated with the partial suppression of interferon synthesis by fly ash. Similar findings of increased influenza and Kilham (leucosis) virus yields concomitant with depressed viral-induced interferon synthesis from either coal dust, asbestos fiber, or metal-treated cell cultures have been reported (Hahon, 1974; Hahon and Eckert, 1976; Zasukhina *et al.*, 1977; Hahon *et al.*, 1980).

It has been established that several carcinogenic trace elements, i.e., As, Cd, Cr, Ni, including potential organic carcinogens, are highly concentrated on the surface of coal fly ash particulates (Linton *et al.*, 1976; Natusch, 1978). While approximately 2–3% of the mass of coal fly ash is soluble in water, the surface layer is apparently quite soluble. Serum proteins and other solvents of coal fly ash may form soluble complexes with polycyclic aromatic compounds and metals (Chrisp *et al.*, 1977). Supernatant fluid from aqueous lignite fly ash suspension had no adverse effect on the viral interferon-induction process. Fly ash particulates per se appeared to be solely responsible for the inhibitory activity. However, extractions of lignite fly ash by nonpolar (DCM) or polar (methanol) solvents, horse serum, or in combination with EDTA, and fractionation of serum extracts yielded corresponding compounds, most likely organic and inorganic, that depressed interferon induction comparable to that of initial fly ash particulates. After serum extractions, residual fly ash particulates were still capable of inhibiting interferon induction, indicating that the particulate matrix itself or other surface contaminants were responsible for the described phenomenon. It would appear, therefore, that lignite fly ash contains at least two or more components capable of adversely affecting interferon production. Whereas serum and solvent extractions of lignite fly ash were not mutagenically active, similar extractions of coal fly ash exhibited mutagenic activity (Chrisp *et al.*, 1977). These contrasting results may be a reflection of either geographic origin of coal, rank of coal, source of fly ash (Kubitschek and Venta, 1974), particle size, or the type of bacterial tester strains used in the Ames histidine reversion assay (Chrisp and Fisher, 1980). Several bulk samples of lignite coal, the source of resultant fly ash used in this study, minimally affected viral interferon induction. The biological inactivity of lignite coal in this regard has been noted previously and correlated with coal rank (Hahon, 1982). Nevertheless, our findings of depressed viral interferon induction by soluble products from lignite fly ash corresponded closely with those of mutagenic activity reported for soluble preparations obtained by similar extractions and fractionation of coal fly ash (Chrisp *et al.*, 1977). This may be an augury for the potential usefulness of the viral interferon-induction process for detecting mutagens not only in soluble but in particulate forms.

The removal of cell-membrane-bound sialic acid by neuraminidase or saturation of lignite fly ash particulates with sialic acid negated the adverse action of these particulates on the viral induction of interferon. Similar findings have been reported with diesel-engine emission particulates (Hahon *et al.*, 1982) and with erythrocytes becoming more resistant to hemolysis by asbestos fibers when cellular sialic acid was removed or the fibers were treated with sialic acid (Harington *et al.*, 1971). It may be expected that sialic acid would play an important role as a receptor at the cell surface because it occupies a terminal position in carbohydrate chains of mammalian glycoproteins (Jeanloz and Codington, 1976). Although the mechanisms by which messages are transmitted by surface glycoproteins are generally not well understood, sialic acids may form part of cell-membrane components that transmit extracellular stimuli to the intracellular environment (Roseman, 1974). The interaction between cell-surface sialic acid and lignite fly ash particulates and the resultant abrogation of viral interferon induction may involve this mode of transmission.

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