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Effect of Coal Rank on the Interferon System

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Studies on the induction of interferon by influenza virus in monkey kidney (LLC-MK_2) cell monolayers pretreated with coal dust revealed that the inhibitory activity of the mineral on this process was related to coal rank. Maximal inhibition of viral interferon induction was noted with high rank coal and the degression of this activity was related to coal's position in the carboniferous series; anthracite, bituminous, subbituminous, lignite, and peat. The cytocidal activity of each rank of coal, however, was comparable in relation to the corresponding quantities of coal dust that were tested indicating that findings related to interferon production are not explicable on the basis of remaining viable cells. Coal dust, irrespective of rank, also did not affect the ability of exogenous interferon to confer antiviral cellular protection. An inverse relationship mediated by coal of different rank occurred between influenza virus growth and interferon levels which suggested that increased virus growth reflected the ability of higher rank coal to affect adversely interferon production.

INTRODUCTION

"Rank" is a term customarily used to refer to the position of coal in the carboniferous series and encompasses certain physical, chemical, and technological properties inherent to the different coals, i.e., calorific value, carbon, and volatile matter contents. Beginning with coal of the highest rank, the position of different coals in decreasing order follows: anthracite, bituminous, subbituminous, and lignite (brown coal) of which the latter is only slightly more formed than peat. Coal rank has assumed importance as the focus of numerous studies since it was first proposed that rank of coal may be a factor to account for regional differences in the prevalence of coal workers' pneumoconiosis (Hart and Aslett, 1942). Although other factors undoubtedly influence the disease process, accumulated evidence has confirmed that a relationship exists between coal rank and the risk of developing pneumoconiosis (Hicks *et al.*, 1961; Morgan, 1968; Key, 1971; Reisner and Robock, 1977; Bennett *et al.*, 1979). Areas that mine the highest-ranked coal appear to have the highest prevalence of the disease (Morgan, 1975).

Interferons are genetically inducible proteins of cellular origin in response to viral, nonviral microorganisms, and other diverse substances (Stewart, 1979). Interferons from different cellular origins may vary in their molecular weight, degree of species specificity, and certain physical properties, however, antiviral activity is a common attribute. In addition to their importance as a component of the host's nonimmunologic defense mechanism, interferons may regulate or modulate numerous aspects of the immune response and influence the proliferation and biochemical activities of both normal and malignant cells (Taylor-

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Papadimitriou, 1980). Previously, we demonstrated that anthracite and bituminous coal dust depressed interferon induction by influenza virus in mammalian cell cultures (Hahon, 1974). It was of interest, therefore, to determine whether the inhibitory action of coal dust on viral interferon induction may be related to coal rank.

This report describes an *in vitro* study on the influence of different ranks of coal dust on (1) cytocidicity, (2) viral induction of interferon, (3) multiplication of influenza virus, and (4) interferon-mediated antiviral cellular resistance.

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, Rockville, Maryland. Stock virus pools of each strain were prepared from embryonated chicken eggs in the manner described previously (Hahon *et al.*, 1973). Influenza and Sendai virus pools contained 3×10^7 and 1×10^9 cell-infecting units of virus per milliliter, respectively, when assayed by the immunofluorescent cell counting procedure (Hahon *et al.*, 1973).

Cell cultures. Rhesus monkey kidney (LLC-MK_2) and human Chang conjunctival (clone 1-5c-4) cell lines obtained from the American Type Culture Collection were used for induction and assay of interferon, respectively. Cell lines were propagated in plastic tissue culture flasks (75 cm^3) with Eagle's minimum essential medium 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.

Coal. Coal dusts in decreasing order of rank and their respective mean diameters (μm), as determined with a Coulter Counter (Coulter Electronics Inc., (Hialeah, Fla.), were examined: anthracite, Central Pennsylvania (2.55); bituminous, Pittsburgh, Pennsylvania (2.52); subbituminous, New Mexico (2.58); lignite, Texas (2.57); and peat, North Carolina (2.60). Suspensions of the dusts (w/v) were prepared in phosphate-buffered saline (PBS), pH 7.1, and sterilized in an autoclave at pressure of 20 lb/$in.^2$ (126°C) for 15 min.

Interferon induction. Duplicate experiments were performed, and the procedure generally used to study the effects of coal dusts on interferon induction was carried out as follows; a 5-mg suspension of coal dust in a 10-ml volume of maintenance medium was added to 75-cm^3 plastic flasks containing complete LLC-MK_2 cell monolayers 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 wavelength of 253.7 nm, was added onto cell monolayers that were then incubated at 35°C for 2 hr. The multiplicity of infection 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 filters (0.22 μm) (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 interferon (Lockhart, 1973). Controls consisting of cell monolayers which were not treated with coal dust were handled exactly as described above.

Interferon assay. An immunofluorescent cell-counting assay of interferon that has 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 growth curve determinations. Influenza virus replication concomitant with interferon production was measured in both untreated and coal dust-treated (0.1 mg/ml) LLC-MK_2 cells (3×10^6) maintained in 25-cm^3 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.

RESULTS

A comparative determination was made of the cytocidal activity of coal dust of different rank for LLC-MK_2 cells because maximal cell viability of cultures in the presence of these minerals was a prerequisite in succeeding interferon experiments. Results (Table 1) show that cell survival was comparable with regard to each rank of coal and the corresponding quantities of coal dust that were tested. Cell survival in the presence of 2.0, 1.0, and 0.5 mg/ml; quantities of coal dust irrespective of rank, was approximately 40, 50, and 100%, respectively.

The effect of coal dust of different rank on interferon induction by influenza virus was determined in cell monolayers that had been pretreated for 2 hr with 0.5 mg/ml of coal dust. Results (Table 2) reveal that coal of high rank (anthracite, bituminous) depressed interferon production by approximately 66%. Coal of succeedingly lower rank was less adverse to this cellular defense process. The extent of coal dust's inhibitory activity on viral interferon induction appeared to be related to coal rank.

To determine whether coal of different rank may influence interferon-mediated antiviral resistance of cells, an interferon preparation of known potency was assayed in the usual manner on 1-5c-4 cell monolayers that had been treated earlier for 20 hr with either anthracite, bituminous, subbituminous, lignite, peat dust, or

TABLE 1
EFFECT OF COAL DUST OF DIFFERENT RANK AND QUANTITY ON SURVIVAL OF LLC-MK_2 CELLS

Quantity[a] (mg/ml)	Percentage of cells surviving (± SE)[b]				
	Anthracite	Bituminous	Subbituminous	Lignite	Peat
2.0	37.6 (±1.0)	42.0 (±2.0)	38.2 (±1.1)	39.2 (±0.2)	39.2 (±0.5)
1.0	54.9 (±1.3)	60.9 (±0.3)	50.8 (±2.0)	52.1 (±2.3)	50.3 (±0.5)
0.5	104.0 (±1.0)	102.0 (±3.0)	112.5 (±0.5)	93.1 (±0.2)	108.5 (±2.5)
0 (Control)	100	100	100	100	100

[a] Cell monolayers were incubated at 35°C for 24 hr with 10 ml of coal dust suspensions.
[b] Results were computed by dividing the number of living cells (trypan blue dye exclusion) in coal dust-treated cell monolayers by number of living cells in control (1.0×10^7) $\times 100$.

the appropriate control medium. In cell cultures that were pretreated with coal dust of different rank, interferon assay values ranged from 160 to 175 $ICDD_{50}$/ml. These values were comparable to that of the controls in which the mean interferon value was 163 $ICDD_{50}$/ml. The presence of coal dust regardless of rank did not impair the ability of interferon to confer antiviral cellular protection.

The multiplication of influenza virus concomitant with interferon production was followed in cell monolayers that had been exposed to coal dust of varied rank and in untreated (control) cells. Results depicted in Fig. 1 show that a comparable rate of virus growth occurred in coal dust-treated and control cell cultures. The highest levels of virus growth were achieved, however, in cell cultures that were treated with coal of higher rank (anthracite, bituminous). These levels were at least twofold higher than that noted in control cells. An inverse relationship mediated by coal of different rank occurred between virus growth and interferon levels. When cell cultures were treated with coal of exceedingly lower rank, interferon levels increased and virus growth levels progressively decreased. When cell cultures were treated with high-rank coal, interferon levels decreased and virus growth increased. These findings suggest that these differences in virus growth reflect the ability of different rank coal to affect interferon production.

TABLE 2
EFFECT OF COAL DUST OF DIFFERENT RANK ON INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS

Coal	Percentage interferon inhibition[a]		
	Test 1	Test 2	Mean (±SE)
Anthracite	71	62	66.5 (±4.5)
Bituminous	68	62	65.0 (±3.0)
Subbituminous	58	40	49.0 (±9.0)
Lignite	29	24	26.5 (±2.5)
Peat	20	14	17.0 (±3.0)

[a] Computed by dividing reciprocal of $ICDD_{50}$/ml interferon from coal dust (5 mg)-treated cells (1×10^7) by control $ICDD_{50}$/ml interferon minus 100. Control $ICDD_{50}$/ml for Test 1 and 2 were 100 and 50, respectively.

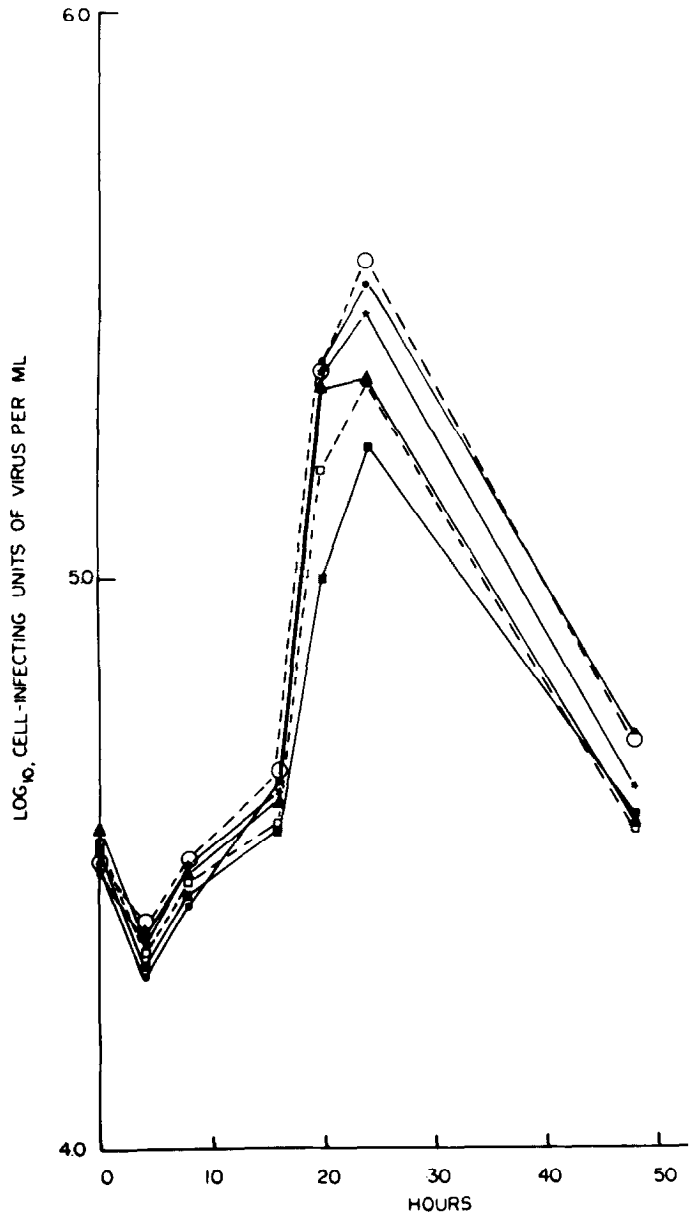

FIG. 1. Growth curves of influenza virus concomitant with interferon production in untreated (control) and different coal dust-treated LLC-MK_2 cell monolayers. Anthracite, ●—●; bituminous ○---○; subbituminous, ★——★; lignite, ▲—▲; peat, □---□; and control, ■—■.

DISCUSSION

The findings of this study demonstrate that the inhibitory activity of coal dust on viral interferon induction appeared to be related to coal rank. Beginning with coal of highest rank that maximally affected the depression of viral interferon induc-

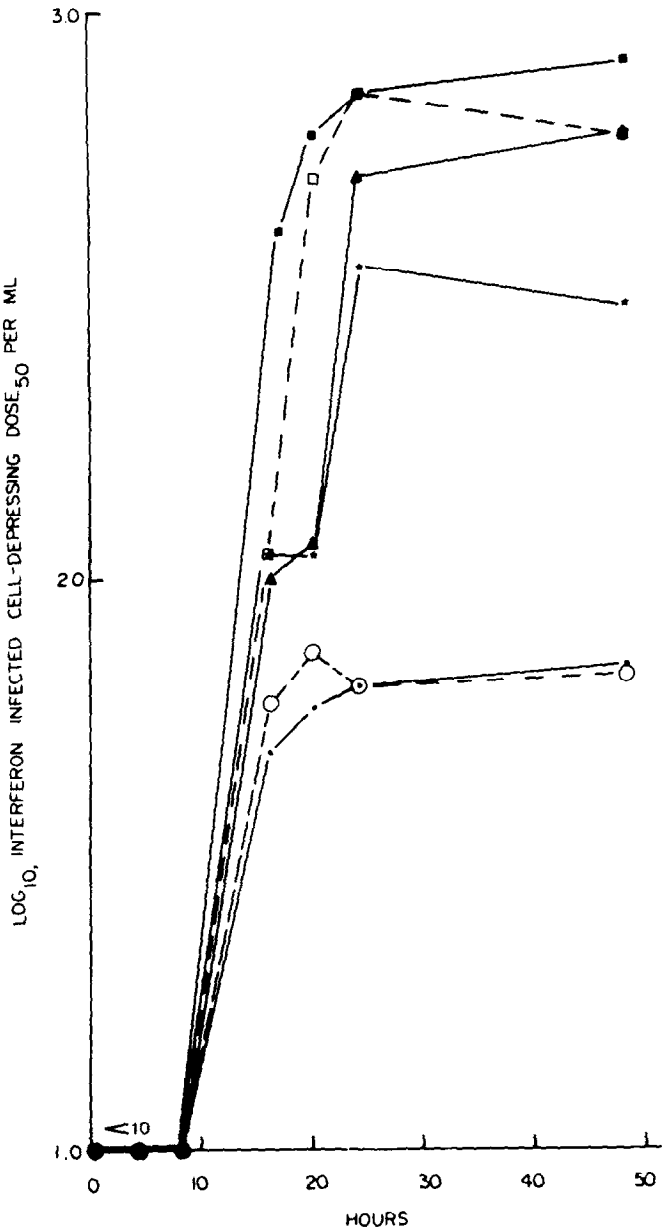

FIG. 1. (*Continued*)

tion, the order of coal's activity degressed as follows: anthracite, bituminous, subbituminous, lignite, and peat. It is difficult to interpret our findings of decreased viral interferon production and its relation to higher prevalence of pneumoconiosis in association with high-rank coal. At present, neither sufficient experimental nor epidemiologic data are available to assess the significance of interferon's part in this particular disease process. In certain respects, a similar

trend was noted with aflatoxins of varied carcinogenic activity (Hahon *et al.*, 1979). The degree of inhibition of interferon induction correlated with the proven carcinogenic potential of the aflatoxin forms. The cytocidal activity of each rank of coal was comparable, however, to the corresponding quantities of coal dust that were tested. This indicates that coal's inhibitory activity on interferon production is not explicable on the basis of remaining viable cells. Furthermore, the presence of coal dust regardless of rank did not impair the ability of exogenous interferon from conferring antiviral cellular protection. This implies that *de novo* cellular protein synthesis and other levels of molecular activities required for manifesting interferon's antiviral property (Stewart, 1979b) were unaltered by different ranks of coal dust.

An inverse relationship mediated by coal of different rank was apparent between influenza virus growth and interferon production. Interferon levels decreased and virus growth levels increased when cell cultures were exposed to high-rank coal. With coal progressively lower rank, interferon levels increased and virus growth levels decreased. These varied levels of virus growth appeared to be related to the ability of different ranks of coal to affect adversely the production of interferon. That this phenomenon was not due to impairment of virus integration (attachment and penetration) into cells by the presence of high rank coal was demonstrated previously (Hahon, 1974). Furthermore, the phenomenon was not accounted for by binding or adsorption of interferon to high rank coal, although coal irrespective of rank has been reported to be an excellent absorptive surface for proteins (Wagner, 1972).

Coal is a mineral of heterogeneous composition that may vary in its content of carbon, quantity and number of metallic trace elements, organic, inorganic, and volatile compounds, depending not only on rank but on the area from which it is mined. It had been demonstrated previously that high-rank coal particles (anthracite and bituminous) per se act on cell cultures to adversely affect viral interferon induction (Hahon, 1974) and that the cytotoxic effect of coal mine dust was also bound to particulate material (Seemayer and Manojlovic, 1981). Previous studies have shown, however, that coal contained the hydrocarbon 3:4 benzypyrene and natural oils that could be readily eluted by serum (Harington and Smith, 1964). Interferon synthesis may be inhibited by certain carcinogenic hydrocarbons (DeMaeyer and DeMaeyer-Guignard, 1967; Barnes *et al.*, 1981). Identity of the elements or compounds inherent to high-rank coal that are responsible for the phenomenon described in this study may aid in the understanding of the mechanisms by which interferon synthesis is affected. Because it is generally believed that interferon plays a role in recovery from acute primary viral infections (Baron, 1973) studies may be warranted to determine whether the rank of coal mined is a factor contributing to the risk and severity of viral respiratory infections among miners.

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