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Effect of Chromium and Manganese Particles on the Interferon System

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ABSTRACT

Mammalian (LLC-MK_2) cell monolayers pretreated with either chromium or manganese particles depressed viral induction of IFN by approximately 50% but the presence of metal particles did not prevent exogenous IFN from conferring antiviral cellular resistance. Manganese particles were more detrimental to viral IFN induction than chromium particles in that almost tenfold less of the former achieved a comparable magnitude of IFN inhibition. Although rates of influenza virus multiplication in either chromium or manganese-treated and control cell cultures were similar, virus attained a level of growth almost twofold higher in metal-treated cells than in controls. This was related to suppression of viral IFN induction by metal particles. Neuraminidase treatment of cell surface salioglycoproteins or pretreatment of chromium or manganese particles with sialic acid abrogated the adverse activity of metal particles on viral IFN induction. These findings suggest that the receptivity and interaction of cell membrane-bound sialic acid residues with metal particles are involved in the altered cellular protective response described.

INTRODUCTION

THE role and toxicity of metal dusts and compounds associated with occupational-related human diseases and their potential health hazard as environmental pollutants have been cogently established and documented.[1-3] With increased attention focused on primary mechanisms by which metal elements affect biological systems, evidence now exists to indicate that certain metal compounds can affect adversely the efficiency of host defense mechanisms. Exposure to salts of cadmium, nickel, chromium, vanadium, manganese, gold, lead, or cobalt can alter host defense functions; i.e., decrease rate of respiratory tract clearance, reduce the number of alveolar macrophages and their phagocytic and enzymatic activities, and change significantly host immune status; i.e., suppression of antibody-mediated immunity.[5,7-12] The increased severity and mortality noted in experimental infections, and potentiation of host susceptibility to infectious agents; viral,[13-18] fungal,[19,20] and bacterial[16,21-24] may be a reflection of these alterations as well as other conjoint events modified by metal substances.

The interferon (IFN) system, an important component of the host's nonimmunologic defense mechanisms, is regarded as a primary protective determinant activated against viral infections. Additionally, IFNs may regulate or modulate various aspects of the immune response and influence the

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proliferation of both normal and malignant cells as well as various intracellular biochemical activities and products.[25] Numerous in vitro and in vivo studies have been carried out with a variety of metal salts, i.e., cadmium, nickel, zinc, mercury, lead, and cobalt which indicate that both components of the IFN system, induction and antiviral activity, may be adversely affected.[26-31] These findings suggested that as a consequence of depressed IFN synthesis by metal compounds, virus multiplication or host mortality was increased. Although these observations have been noted more often with the use of metal salts, similar findings were described with respirable metal particles of aluminum, copper, and nickel.[32]

In this reported study we investigated further the possible modification of IFN activities in the presence of metal particles of chromium and manganese. The factors affecting the response of mammalian cell cultures treated with these metal particles in relation to viral IFN induction, IFN-mediated antiviral cellular resistance, and influenza virus multiplication are described.

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, MD. Stock virus pools of each strain were prepared from embryonated chicken eggs in the manner described previously.[33] Influenza and Sendai virus pools contained 5×10^7 and 1×10^9 cell-infecting units of virus per ml, respectively, when assayed by the immunofluorescent cell-counting procedure.[33]

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 IFN, respectively. Cell lines were propagated in plastic tissue culture flasks (75 cm^2) with Eagle minimum essential medium fortified with 100 X Essential Vitamin Mixture (10 ml/l), 200 m*M* solution L-glutamine (10 ml/l) to which was added sodium bicarbonate (2.2 g/l), and 10% fetal bovine serum. Cells were maintained with the aforementioned medium containing only 0.5% fetal bovine serum.

Metals and reagents

Chromium (2 μm) and manganese (10 μm) particles, obtained from ALFA Products, Danners, MA, were made into w/v suspensions in phosphate-buffered saline (PBS), pH 7.1, and sterilized in an autoclave at 20 lb/in^2 pressure (126°C) for 15 min. Neuraminidase (*Vibrio cholerae*) and sialic acid (N-acetylneuraminic acid) were obtained from Grand Island Biological Company, Grand Island, NY, and Calbiochem-Behring Corporation, La Jolla, CA, respectively.

IFN induction

Duplicate experiments were performed, and the procedure generally used to study the effects of chromium and manganese particles on IFN induction was carried out as follows: 5.0 mg chromium or 0.5 mg manganese particles suspended in 10 ml volume of maintenance medium was added to 75 cm^2 plastic flasks containing complete LLC-MK_2 cell monolayers which were then incubated at 35°C for 24 h. 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 h. 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 h, supernatant fluid was decanted and centrifuged at $100{,}000 \times g$ for 1 h and dialyzed against HCl-KCl buffer, pH 2.0, at 4°C for 24 h. Dialysis was con-

tinued against two changes of PBS, pH 7.1, at 4°C for 24 h. Fluids were passed through Millex filters GV (0.22 μm) (Millipore Corporation, Bedford, MA) to obtain sterile preparations. Samples were stored at −80°C until they were assayed for IFN activity. Preparations with antiviral activity possessed the biological and physical properties ascribed to viral IFNs.[34] Controls consisting of cell monolayers which were not treated with metal particles were handled exactly as described above.

IFN assay

An immunofluorescent cell-counting assay of IFN that has been described previously[35] was used to determine the IFN potency of test samples. IFN-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 IFN dilution that reduced the number of infected cells to 50% of the control served as the measure of IFN activity, i.e., 50% infected cell-depressing dilution ($ICDD_{50}$). With this assay, 0.89 IFN unit corresponds to 1.0 unit of National Institute of Health reference standard HuIFN-β (G-023-902-527).

Virus growth determination

Influenza virus replication concomitant with IFN production was measured in both untreated and chromium (3 mg) or manganese (0.3 mg)-treated LLC-MK_2 cells (3×10^6) maintained in 25 cm^2 plastic flasks. Following adsorption of virus to cells at 35°C for 1 h, 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 h, 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 IFN assessment in the described manner.

RESULTS

Metal particle concentration and cell viability

The cytocidal effect of different concentrations of both chromium and manganese particles was determined because the viability of LLC-MK_2 cells in the presence of these metals was a prerequisite to succeeding IFN induction experiments. Monolayers of nondividing cells (2×10^7) seeded with 10 ml suspensions of metal particles ranging in quantity from 0.01 to 2.0 mg/ml were incubated at 35°C for 24 h. Cell viability was determined by the trypan blue dye-exclusion procedure. Under the specified conditions, manganese was more cytocidal than chromium particles. Cells were able to tolerate a ten-fold higher quantity of chromium (0.5 mg/ml) than manganese (0.05 mg/ml)/3×10^7 cells without appreciable loss of viability (Table 1). Generally, these quantities of metal particles that minimally affected cell viability were used in subsequent experiments. Microscopic examination of cells exposed to metal particles, represented by chromium (Fig. 1), revealed adherence of particles to cell surfaces with possible intracytoplasmic localization.

Metal particles and viral IFN induction

Different quantities of chromium and manganese particles, ranging from 0.005 to 0.5 mg/ml in 10 ml volume, were tested for their effect on IFN induction by influenza virus in LLC-MK_2 cell monolayers. Results (Table 2) reveal that viral IFN induction was inhibited by approximately 50% in the presence of either chromium or manganese particles with quantities of 0.5 mg/ml and 0.1 mg/ml, respectively. The similar magnitude of inhibition by almost ten-fold less manganese than chromium particles indicates that the former was more toxic to the viral IFN induction process. With decreased

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF CHROMIUM AND MANGANESE PARTICLES ON VIABILITY OF LLC-MK_2 CELLS.

Metal conc.[a] *(mg/ml)*	*Surviving fraction of cells (± SEM)*[b]	
	Chromium	*Manganese*
2.0	0.478 (±0.020)	0.390 (±0.064)
1.0	0.729 (±0.128)	0.517 (±0.048)
0.5	1.000 (±0.000)	0.537 (±0.008)
0.2	1.000 (±0.000)	0.723 (±0.010)
0.1	1.000 (±0.000)	0.851 (±0.015)
0.05	ND[c]	0.969 (±0.031)
0.01	ND	1.000 (±0.000)
0 (Control)	1.000	1.000

[a]Cell monolayers were treated at 35°C for 24 h with 10 ml of metal particle suspension.

[b]Results are expressed as surviving fraction of cells ± standard error of mean which was computed by dividing the number of living cells (trypan blue dye-exclusion) in metal-treated cell monolayers by number of living cells in control (3×10^7).

[c]Not determined.

concentrations of either metal particles, this inhibitory activity on IFN production progressively diminished.

An investigation was made to determine whether there was a sequence and time relationship between treatment of cell cultures with metal particles and the introduction of the viral inducer. Results (Table 3) show that both chromium and manganese particles depressed IFN production within the same time periods and in the same sequence. Prior treatment of cell cultures with either metal particles for as little as 4 h before the inducer agent, or the simultaneous addition of metal particles with

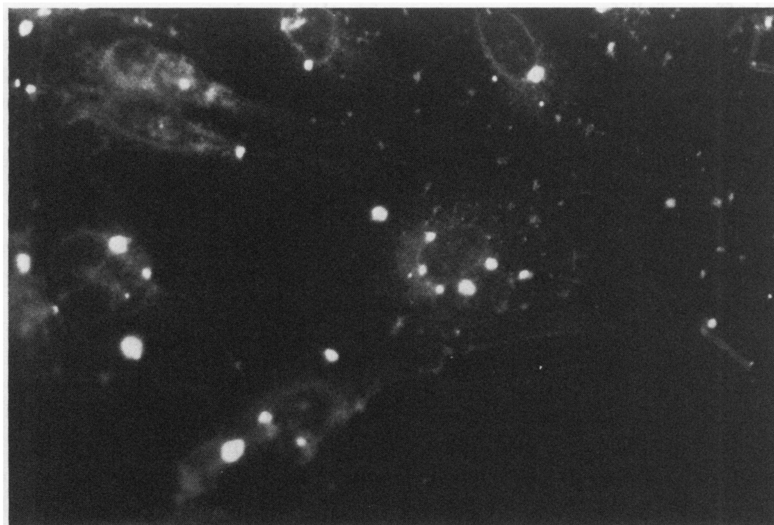

FIG. 1. Adherence of chromium particles to LLC-MK_2 cells × 200.

TABLE 2. EFFECT OF CHROMIUM AND MANGANESE PARTICLE CONCENTRATIONS ON IFN INDUCTION BY INFLUENZA VIRUS.

Metal conc. (mg/ml)[a]	*Chromium*		*Manganese*	
	IFN ($ICDD_{50}$/ml)[b]	*% IFN inhibition (± SEM)*	*IFN ($ICDD_{50}$/ml)*	*% IFN inhibition (± SEM)*
0.5	85	51.5(±3.8)	ND	
0.2	95	45.8(±4.4)	ND	
0.1	133	24.0(±9.1)	72	54.2(±1.4)
0.05	173	1.2(±4.7)	94	40.2(±2.6)
0.01	ND[c]		116	26.2(±7.1)
0.005	ND		160	0.0(±0.0)
0 (Control)	175	0.0	157	0.0

[a]LLC-MK_2 cell monolayers were treated with 10 ml of metal suspension.
[b]Reciprocal of 50% infected cell-depressing dilution.
[c]Not determined.

the viral inducer depressed viral IFN induction by approximately 50%. Protraction of exposure time of cells to particles for as long as 20 h did not increase the 50% inhibitory effect of metal particles on IFN production. When the sequence was changed with the viral inducer added 4 h prior to metal particles, comparable inhibition of IFN production was still evident. Thereafter, the introduction of either chromium or manganese particles at times greater than 4 h after the inducer agent had no appreciable effect on IFN production.

An experiment was performed to ascertain whether a soluble complex in suspensions of metal particles was present to account for the phenomenon noted. Metal particle suspensions, corresponding supernatant fluids obtained after sedimentation of particles and resuspended metal particles were examined for their effect on viral IFN induction. Results (Table 4) show that supernatant fluids from corresponding metal particle suspensions did not significantly affect viral induction of IFN indicating the absence of a soluble IFN inhibitor. Both initial metal particle suspensions and resuspended particles comparably depressed viral IFN production.

TABLE 3. TIME AND SEQUENCE RELATIONSHIP BETWEEN ADDITION OF CHROMIUM AND MANGANESE PARTICLES AND INFLUENZA VIRUS ONTO LLC-MK_2 CELL MONOLAYERS AND IFN INDUCTION.

Relationship between particles and addition of virus at zero time (h)[a]	*Chromium*		*Manganese*	
	IFN ($ICDD_{50}$/ml)[b]	*% IFN inhibition*	*IFN ($ICDD_{50}$/ml)*	*% IFN inhibition*
−24	100	54.6	98	51.0
−7	96	56.4	100	50.0
−4	90	59.1	100	50.0
0	92	58.2	79	60.0
+4	100	54.6	100	50.0
+7	210	4.6	200	0.0
+16	215	2.3	210	0.0
(Control)	220	0.0	200	0.0

[a]Particles (Cr 5.0 mg, Mn 0.5 mg) suspended in 10 ml maintenance medium and added to cell monolayers at designated hours prior to or after the addition of virus at 0 time.
[b]Reciprocal of 50% infected cell-depressing dilution.

TABLE 4. INFLUENCE OF CHROMIUM AND MANGANESE PARTICLE SUSPENSIONS AND CORRESPONDING SUPERNATANT FLUIDS ON IFN INDUCTION BY INDUCTION OF INFLUENZA VIRUS.

	Chromium		*Manganese*	
Test preparation[a]	*IFN ($ICDD_{50}$/ml)*[b]	*% IFN inhibition (± SEM)*	*IFN ($ICDD_{50}$/ml)*	*% IFN inhibition (± SEM)*
Initial particle suspension	77	53.4(±2.1)	80	51.0(±2.8)
Supernatant fluid	160	3.1(±0.0)	155	6.1(±0.8)
Resuspended particles	85	48.5(±1.0)	80	51.0(±1.2)
Maintenance medium (control)	165	0.0	165	0.0

[a]Ten ml suspensions of chromium (0.5 mg/ml) and manganese (0.05 mg/ml) were centrifuged at 18,000 × g for 30 min. Supernatant fluids were decanted and sedimented metal particles were resuspended in 10 ml maintenance medium. LLC-MK_2 cell monolayers were pretreated with appropriate test preparations at 35°C for 24 h.
[b]Reciprocal of 50% infected cell-depressing dilution.

To obviate the circumstance that reduced IFN yields may be related to the adsorptive power of metal particles for IFN, 5 mg chromium and 0.5 mg manganese were mixed with 2 ml each of IFN of known potency. After incubation at 35°C for 20 h, the metal particle-IFN suspensions were clarified by centrifugation and the supernatant fluids were assayed for IFN activity. Results showed that IFN was not bound or adsorbed to either metal particles because the titers of IFN suspensions in the presence of either chromium or manganese, were 72 and 78 $ICDD_{50}$/ml, respectively. This was comparable to the control IFN preparation of 78 $ICDD_{50}$/ml.

To determine whether the ability of influenza virus to infect cells was influenced by the presence of metal particles, which could affect the efficacy of the viral IFN induction process, LLC-MK_2 cell monolayers (3×10^5 cells) on coverslips were treated at 35°C for 24 h with chromium (0.5 mg and 0.2 mg) or manganese (0.2 mg and 0.1 mg) particles. Virus was then adsorbed onto cell monolayers at 35°C for 2 h and, then, cell cultures were incubated at 35°C for 24 h. Control cell monolayers without metal particles were virus-infected in a similar manner. Results revealed that virus titers in cells treated with different quantities of either chromium or manganese particles ranged from 2.4×10^7 to 2.6×10^7 CIU/ml. This was comparable to that of the control, 2.3×10^7 CIU/ml. These data indicate that virus infectivity and associated primary virus–cell interactions (virus attachment and penetration) were not affected by the presence of metal particles.

Virus multiplication and IFN-mediated antiviral action in the presence of metals

Influenza virus growth and concomitant IFN production were studied in LLC-MK_2 cell monolayers that were pretreated for 24 h with either chromium or manganese particles and in untreated (control) cell cultures. The rates of virus multiplication in all metal treated and control groups of cell cultures were similar and plateaus of virus growth were reached in approximately 20–24 h (Fig. 2). However, virus growth in cell monolayers treated with either manganese or chromium attained a level that was almost two-fold higher than that noted in control cell cultures. While the maximal production of IFN that occurred in cell monolayers treated with metal particles was 25 $ICDD_{50}$/ml, the IFN yield in control cell cultures was approximately 100 $ICDD_{50}$/ml. These findings suggest that the slightly higher level of virus growth noted in cell cultures pretreated with either manganese or chromium particles than in normal cells may be a reflection of the inhibitory activity of metal particles on the IFN synthesis process.

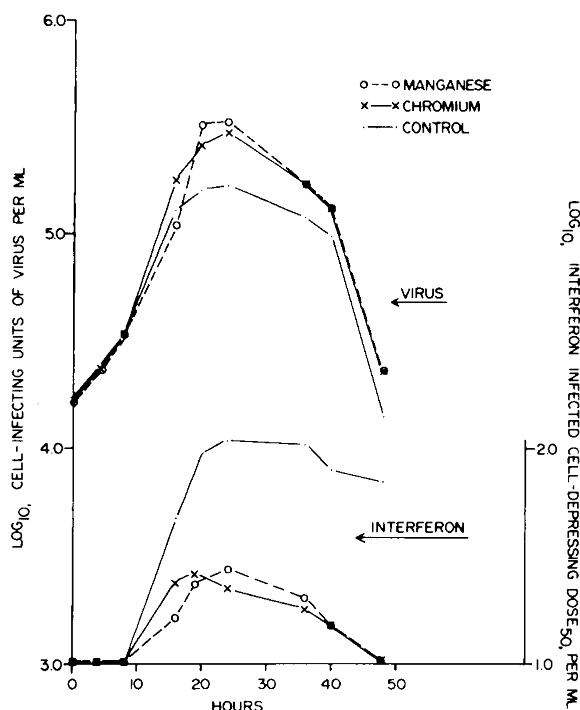

FIG. 2. Growth curves of influenza virus concomitant with IFN production in untreated (control) and chromium or manganese-treated LLC-MK_2 cell monolayers.

To determine whether metal particles may affect IFN-mediated resistance of cells to virus infection, 1-5c-4 cells were pretreated for 20 h with chromium or manganese particles or the appropriate control medium. Thereafter, an IFN preparation of known potency was assayed in the usual manner using these metal-pretreated cell cultures and the cells were challenged with Sendai virus. Results revealed that IFN titers in manganese- and chromium-treated cells were 98 and 100 $ICDD_{50}$ml, respectively, which was comparable to that of the control, 100 $ICDD_{50}$/ml. The presence of metal particles did not impair the ability of exogenous IFN to confer antiviral cellular protection.

Role of cell membrane-bound sialic acid

Glycoprotein-bound sialic acid is an integral component of mammalian cell surfaces. To study the role of cell membrane-bound sialic acid relative to the activity of manganese and chromium particles on viral IFN induction, cell cultures were treated with 100 units of neuraminidase at 35°C for 1 h to remove sialic acid residue. Thereafter, cell monolayers were exposed for 24 h to metal particles and viral inducer was added in the usual sequence and manner. Results (Table 5) indicate that removal of cell-bound sialic acid by neuraminidase completely negated the inhibitory activity of chromium and manganese particles on IFN induction. Abrogation of this detrimental activity of metal particles strongly suggests that sialic acid acts as a receptor which interacts with adventitious agents, in this case chromium and manganese particles, to initiate the process that culminates in altered cellular response, i.e., viral IFN induction.

To confirm further the role of sialic acid in this phenomenon, chromium and manganese particles were mixed and incubated at 35°C for 4 h with sialic acid (N-acetylneuraminic acid) in an attempt to mask the deleterious role of the metal particles on viral IFN induction. The particles were then sedi-

TABLE 5. EFFECT OF CHROMIUM AND MANGANESE PARTICLES ON INFLUENZA VIRUS IFN INDUCTION IN LLC-MK_2 CELL MONOLAYERS PRETREATED WITH NEURAMINIDASE.

Cell treatment	*IFN ($ICDD_{50}$/ml)*[a]	*% IFN inhibition (± SEM)*
Neuraminidase[b] + manganese	325	0.0(±0.0)
manganese	165	47.8(±6.7)
Neuraminidase + chromium	320	0.0(±0.0)
chromium	157	50.4(±6.2)
Neuraminidase	340	0.0(±1.8)
None	316	0.0

[a]Reciprocal of 50% infected cell-depressing dilutions. Mean of duplicate determinations.

[b]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. Cell monolayers pretreated with 100 units neuraminidase and metal suspensions containing 0.5 mg manganese or 5.0 mg chromium per 10 ml.

mented by centrifugation, washed in PBS, pH 7.1, and introduced onto cell monolayers in the usual manner. Results (Table 6) show that both chromium and manganese particles interacted with sialic acid as evidenced by the negation of the inhibitory activity of those particles on viral IFN induction. Metal particles that were not treated with sialic acid customarily depressed IFN production by approximately 50%. These findings indicate that a complex between metal particles and sialic acid was probably formed which negated the former's adverse activity on viral IFN induction and adds further support for a role of cell membrane-bound sialic acid in the described phenomenon.

TABLE 6. EFFECT OF CHROMIUM AND MANGANESE PARTICLES PRETREATED WITH SIALIC ACID (N-ACETYLNEURAMIC ACID) ON INFLUENZA VIRUS IFN INDUCTION IN LLC-MK_2 CELL MONOLAYERS.

Treatment			
Metal[a] *(mg/ml)*	*Sialic acid (mg/ml)*	*IFN ($ICDD_{50}$/ml)*[b]	*% IFN inhibition (± SEM)*
Chromium-0.5 mg	100	199	0.0(±0.4)
Chromium-0.5 mg	0	86	56.6(±6.0)
Manganese-0.05 mg	100	207	0.0(±0.3)
Manganese-0.05 mg	0	85	57.1(±1.6)
Control (diluent)	0	198	0.0

[a]Cell monolayers were treated at 35°C for 24 h with 10 ml of metal suspension.

[b]Reciprocal of 50% infected cell-depressing dilution. Mean of duplicate determinations.

DISCUSSION

The findings of this study demonstrate that both chromium and manganese particles adversely affected an adaptive cellular response the production of viral-induced IFN but did not prevent exogenous IFN from conferring antiviral cellular resistance. The latter observation implies that de novo cellular protein synthesis and other levels of molecular activities required for manifesting IFN's antiviral property[36] were not impaired by the cellular presence of metal particles. The events described here were similar to that noted with other metal particles, copper, aluminum, and nickel,[32] and other metal salts.[29,30,38]

Manganese particles were more detrimental to viral induction of IFN than chromium particles in that almost ten-fold less of the former achieved a comparable magnitude of IFN inhibition. Cell cultures were also able to tolerate a tenfold higher concentration of chromium than manganese particles without appreciable loss of viability. In general, observations of the depression of IFN induction by chromium and manganese particles in relation to time and administration of the viral inducer were similar to that noted with other metal particles.[32] Prior treatment of cells with either chromium or manganese particles before the inducer agent, the simultaneous addition of particles and viral inducer, or reversing the sequence of adding the inducer 4 h prior to metal particles, still effectively inhibited IFN induction by approximately 50%. However, when metal particles were added more than 4 h after the inducer agent, the adverse effect of IFN production was completely negated. Within the time frame that virus generally induces the production of IFN (6–16 h), chromium and manganese particles introduced a few hours after the viral inducer were capable of subverting the IFN induction process at stages that may involve either the transcription of IFN mRNA, its translation, or post-transcription of IFN.

Preclusion of viral IFN induction by metal particles was not the consequence of impaired virus integration into cells. Virus infectivity in the presence of metal particles in the cellular environment was comparable to the control indicating that associated virus cell interactions, (virus attachment and penetration) were not impeded. Enhanced plaque formation of poliovirus, Semliki Forest, and West Nile viruses have been reported and attributed to a higher adsorption rate of virions to the cell surface in the presence of either copper, nickel, or cobalt salts.[38] These reported differences pertaining to the virus-cell adsorption process may be dependent on such factors as, virus strains, cell origin, concentration and form of metal preparations. The inimical effect on viral IFN induction was not related to the adsorptive power of chromium or manganese particles for IFN or to a soluble IFN inhibitor in suspensions of the particles.

Rates of influenza virus multiplication in either chromium or manganese-treated and control cell cultures were similar, however, virus growth reached a level almost twofold higher in metal-treated cells than in controls. It is suggested that this was a reflection of the inhibitory activity of metal particles on IFN synthesis. Increased influenza and Kilham (leucosis) virus yields concomitant with depressed viral-induced IFN synthesis with either cadmium chloride, copper, aluminum, or nickel-treated cells have been also noted.[28,32] The increased mortality of experimental hosts to virus infections, administered metal salts, has been attributed to reduced IFN synthesis or activity, or both.[13-16,29,31,39]

Because sialic acid occupies a terminal position in carbohydrate chains of mammalian glycoproteins, it may be expected that it would play an important role as a receptor at the cell surface.[40] Neuraminidase treatment of cell surface salioglycoproteins or the saturation of chromium and manganese particles with sialic acid, negated the detrimental activity of metal particles on viral IFN induction. This suggests that terminal sialic acid residues on cell surfaces act as receptors which may initially interact with certain adventitious agents leading to alteration of cellular protective responses. Results similar to that reported here have been noted with diesel engine emission particles[41] and with erthryocytes which became more resistant to hemolysis by asbestos fibers when cellular sialic acid was removed or fibers were treated with sialic acid.[42] Whether the interaction between cell membrane-bound sialic acid and metal particles and the resultant inhibition of viral IFN induction

involves the transmission of extracellular messages by salioglycoproteins to the intracellular environment,[43] a reflection of cell surface charges,[42] or other physico-chemical interactions, is not presently known.

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