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The Effects of Ozone on the Respiratory Epithelium and Alveolar Macrophages of Mice. I. Interferon Production[1] (39423)

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The effects of inhaled ozone on the functions of the respiratory tract are of continuing interest due to the increasing importance of this air pollutant. Prolonged and repeated exposure of mice to ozone was found to cause alteration in the synthesis of DNA, RNA, and protein by lung tissue, together with proliferative and degenerative changes in the tracheobronchial mucosa (1, 2). It was observed by Miller and Ehrlich (3) that animals exposed to high levels of ozone became highly susceptible to infections with aerosolized bacteria.

Interferon is a protein synthesized and released by cells when induced by viruses or polynucleotides; the molecule has a wide spectrum of antiviral activity (4). Preparations of interferon have been used in various animal experiments to study resistance against both local and systemic virus infections. Merigan *et al.* (5) reported that the onset of influenza virus infection would be delayed when interferon was sprayed onto the upper respiratory tract. Respiratory disease following rhinovirus exposure was prevented by the procedure. In other studies interferon inducers, both viral and nonviral, have been used to stimulate the endogenous production of interferon. However, information is needed regarding the effects of ozone on interferon production both *in vitro* and *in vivo*. Interferon release by lung cells from ozone exposed mice was studied here because it is a sensitive indicator of altered cellular function and the release of this product also has implications for resistance of the host to respiratory viral infections. The capacity of the respiratory epithelium and alveolar macrophages to produce interferon *in vitro* is shown. In addition, evidence of an inhibitory effect on interferon synthesis by respiratory epithelium following *in vivo* exposure to ozone is presented.

Materials and Methods. Viruses. The California 11914 strain of Newcastle disease virus (NDV) was used as an interferon inducer. The Indiana strain of vesicular stomatitis virus (VSV) was employed in interferon assays.

Polynucleotide. Poly(I:C) (inosine:cytidine) was purchased from Biopolymers, Chagrin Falls, Ohio.

Mice. Ten week old specific pathogen-free male mice of the Swiss Webster strain (Hilltop Lab. Animals, Inc., Scottsdale, Penn.) were used. The mice were housed with cage-filter tops in an isolated room with 14 air exchanges per hour at a temperature of approximately 23°.

Ozone exposure. Mice were exposed to ozone according to the method of Plopper *et al.* (6). The mice were housed in an ozone chamber with 30 air exchanges per hour and exposed for 14 or 21 days at a 0.8 ppm concentration of ozone. At the end of the exposure period the mice were removed to ambient air and kept there for another 24 or 10 days. Control mice were held in a similar chamber and exposed to ambient air. Air was introduced into ozone chambers and ambient chambers through a CBR (chemical, bacteriological, radiological) filter.

Organ cultures. Organ cultures were prepared according to the technique described by Hoorn and Tyrrell (7). Tracheal explants from two mice were placed in a sterile 35 mm × 10 mm plastic culture dish to which 5 ml of maintenance medium (medium 199) containing 50 μg/ml gentamicin and 0.3% bovine serum albumin was added. The cultures were incubated at 35° in a humid atmosphere of 5% CO_2 in air.

Macrophage cultures. Alveolar macrophages were obtained from the mice using a slight modification of the technique described by Medin *et al.* (8). Lung lavages

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from 18 mice were pooled and the cells were sedimented from lavage fluid by centrifugation at 1000 rpm for 30 min at 5°. The supernatant was decanted and the cells were resuspended in 4 ml of MEM containing 10% fetal calf serum (FCS) and 50 μg/ml gentamicin. The cell suspension was then dispensed equally into 10 × 35-mm plastic culture dishes in 1-ml amounts. The macrophages were given time to adhere to the plastic surface during a 2-hr period at 37° in a 5% CO_2 atmosphere. Unattached cells were removed by aspirating the medium and the adherent cells were washed three times with 2 ml of Hank's solution. Average cell counting from 20 petri dishes indicated that the culture dish contained approximately 4.0×10^5 ($2.5\text{–}5.0 \times 10^5$) cells.

Interferon production in organ culture. Organ cultures were inoculated with interferon inducers on the fourth hour of incubation. The medium was removed and 0.2 ml of NDV suspension containing 5×10^5 plaque-forming units (PFU) or 0.2 ml of MEM containing 10 μg of poly(I:C) was dripped onto the tissue fragments. Control organ cultures received 0.2 ml of MEM. Adsorption of the inducers proceeded in the cultures for 1 hr while incubating at 35° in a 5% CO_2 environment. The cultures were then washed three times with 2 ml of medium 199 and replenished with 5 ml of medium 199 containing 0.3% bovine serum albumin and 50 μg/ml of gentamicin. Tracheal cultures were incubated for 16 hr at 35° in a humidified 5% CO_2 atmosphere. At the end of the incubation period, the culture fluid was collected by aspiration and stored at −20°. The tracheal fragments were also stored at −20°. Viability of the respiratory epithelium was observed by active ciliary movement. Control cultures retained this function through 7 days of incubation. Although cultures from mice exposed to ozone showed ciliary activity at the end of the incubation period for assay of interferon production, a decrease in the ciliary movement was noticed when compared with control cultures.

Interferon production in macrophage cultures. The monolayers of alveolar macrophages in culture dishes were inoculated with NDV at a multiplicity of infection of 10 or 1 ml of poly(I:C) containing 50 μg as interferon inducers. Control cultures received 1 ml of MEM. After adsorption for 1 hr, the cell cultures were washed three times with 2 ml of MEM and 5 ml of MEM containing 50 μg/ml of gentamicin was added. The cultures were incubated for 16 hr and the culture fluid was collected and stored as described for the tracheal cultures.

Interferon assay. Interferon from the organ cultures and the macrophage cultures was assayed by the plaque reduction technique using L-cells, with VSV as the challenge virus (9).

The interferon titer was expressed as the reciprocal of the highest dilution of the sample showing 50% plaque reduction. A standard reference mouse interferon (catalog number G002-90A-511) provided by National Institutes of Health was included in each assay. One unit of interferon in our assay was equivalent to two units of the reference interferon.

To assay for intracellular interferon from the organ culture the tracheal explants were three times frozen at −20° and thawed at room temperature. The explants were then ground in a Dounce homogenizer and the cellular components were suspended in 5 ml of MEM. The suspension was subjected to two more steps of freezing and thawing and then centrifuged at 15,000 rpm for 1 hr. The supernatant was collected and assayed for intracellular interferon.

The antiviral substance demonstrated in this study was considered to be mouse interferon for the following reasons. It was shown to be (i) stable at pH 2.0, (ii) nonsedimentable at 100,000*g* for 1 hr, (iii) inactivated by trypsin treatment, (iv) specific for host species, (v) inhibited by Actinomycin D, and (vi) inactivated at 56° for 1 hr (9, 10).

Results. The production of interferon from tracheal organ cultures of mice exposed to 0.8 ppm of ozone and mice exposed to ambient air is shown in Table I. The data was obtained from two experiments. It was apparent that exposure of mice to 0.8 ppm of ozone for a period of 10 days did not affect the capacity of the tracheal epithelial cells to produce interferon *in vitro*. Both NDV and poly(I:C) served as

TABLE I. INTERFERON PRODUCTION BY TRACHEAL ORGAN CULTURES FROM MICE[a]

Days[b]	Interferon inducer	Interferon titer (units/4ml)			
		Ozone exposed mice (0.8 PPM)		Mice maintained in ambient air	
		Expt I	Expt II	Expt I	Expt II
0	None			0	0
	NDV			128	128
	poly(I:C)			64	64
4	None	0			
	NDV	64			
	poly(I:C)	64			
6	None		0		
	NDV		64		
	poly(I:C)		64		
8	None	0	0		
	NDV	32	64		
	poly(I:C)	64	64		
9	None		0		
	NDV		128		
	poly(I:C)		64		
10	None		0		
	NDV		128		
	poly(I:C)		64		
11	None		0		
	NDV		0[c]		
	poly(I:C)		0		
12	None	0	0		
	NDV	0	0		
	poly(I:C)	0	0		
14	None			0	0
	NDV			32	64
	poly(I:C)			32	128

Days	Interferon inducer	Interferon titer (units/4ml)			
		Ozone exposed mice (0.8 PPM)		Mice maintained in ambient air	
		Expt I	Expt II	Expt I	Expt II
15	None	0			
	NDV	0			
	poly(I:C)	0			
15		Remove to Ambient air			
17	None		0		
	NDV		0		
	poly(I:C)		0		
20	None		0		
	NDV		0		
	poly(I:C)		0		
21			Remove to ambient air		
24	None		0		
	NDV		0		
	poly(I:C)		0		
31	None		0		
	NDV		8		
	poly(I:C)		8		
32	None				0
	NDV				32
	poly(I:C)				64
39	None	0			
	NDV	64			
	poly(I:C)	128			

[a] Tracheal organ cultures from two mice were inoculated with either 10 μg of poly(I:C) or 5×10^5 PFU of Newcastle disease virus. After incubation at 35° for 16 hr culture fluids were adjusted to pH 2.0 with 2*M* perchloric acid and kept at 4° for 4 days. The resulting precipitate was removed by centrifugation at 15,000 rpm for 1 hr and pH of supernatant was readjusted to 7.0 with 2*M* NaOH. Interferon assay on culture fluids was carried out on L-cells using VSV as the challenge virus. Interferon titer is expressed as the highest dilution showing 50% plaque reduction.

[b] The day on which mice were sacrificed and tracheal organ cultures prepared.

[c] Probability that failure of interferon production was a result of chance less than 0.001. Calculated on the basis of the binomial distribution.

active interferon inducers. Also the tracheal cells were not releasing interferon at the time the animals were sacrificed since interferon was shown to be undetectable in the absence of an inducer. However, starting from the 11th day of exposure, there was an inhibition in the capacity of the organ cultures to produce interferon. This inhibition was detected in samples assayed on days 11, 12, 15, 17, and 20 from animals in the ozone environment. Assays performed on control samples and the macrophage samples during the period of inhibition indicated the specificity of the interferon depression for tracheal explants of ozone-exposed mice.

After the mice had been taken from the ozone chamber to ambient air, the impaired cellular function persisted for a time as shown in a sample taken after 3 days in the ambient air (Experiment II, Day 24). However, 10 days after the mice were removed to ambient air, the organ cultures had started to produce small amounts of interferon (Experiment II, Day 31). A sample studied after the animals had been in ambient air for 24 days revealed complete recovery to the normal level of interferon production (Experiment I, Day 39).

It is not possible to come to the conclusion that the ozone inhibited the production of interferon in organ cultures just by assay-

ing the extracellular interferon. It is feasible that interferon might be produced but ozone inhibits its release, since it is known that certain chemicals such as *p*-hydroxymercuribenzoate affect the release of interferon without decreasing its production (11). Intracellular interferon was assayed to determine whether the absence of extracellular interferon could be due to an accumulation of the unreleased product by the cells. No interferon could be detected on the days on which extracellular interferon was not detected indicating that there was no accumulation of intracellular interferon.

Results obtained with alveolar macrophages were in marked contrast to those obtained from tracheal epithelium (Table II). The alveolar macrophages of mice exposed to ozone produced interferon as efficiently as the alveolar macrophages of mice kept in ambient air. Ozone exposure of mice for 21 days had no effect on the capacity of the alveolar macrophages to produce interferon. In the presence of interferon inducers the macrophages released interferon at equivalent titers without regard to their origin from ozone treated lungs or those exposed to ambient air.

Discussion. The ability of tracheal epithelium to respond normally to interferon inducers was lost after approximately 10 days in an elevated ozone environment. In one experiment (Experiment II) the unresponsive state persisted for 10 days while the animals were inhaling 0.8 ppm of ozone and for at least 3 days after returning to ambient

TABLE II. INTERFERON PRODUCTION BY MACROPHAGE CULTURES FROM MICE[a]

		Interferon titers (units/4ml)			
		Ozone exposed mice (0.8 PPM)		Mice maintained in ambient air	
Days[b]	Interferon inducer	Expt I	Expt II	Expt I	Expt II
0	None			0	0
	NDV			512	256
	poly(I:C)			512	256
4	None	0			
	NDV	512			
	poly(I:C)	256			
6	None		0		
	NDV		256		
	poly(I:C)		256		
8	None	0	0		
	NDV	512	256		
	poly(I:C)	256	256		
12	None	0	0		
	NDV	512	256		
	poly(I:C)	256	256		
14	None			0	0
	NDV			1024	256
	poly(I:C)			256	256
15	None	0			
	NDV	1024			
	poly(I:C)	512			
15		Remove to ambient air			
17	None	0	0		
	NDV	512	256		
	poly(I:C)	512	256		

		Interferon titer (units/4ml)			
		Ozone exposed mice (0.8 PPM)		Mice maintained in ambient air	
Days	Interferon inducer	Expt I	Expt II	Expt I	Expt II
21	None		0		
	NDV		256		
	poly(I:C)		512		
21			Remove to ambient air		
24	None		0		
	NDV		256		
	poly(I:C)		256		
25	None	0			
	NDV	512			
	poly(I:C)	256			
31	None		0		
	NDV		256		
	poly(I:C)		256		
32	None	0			0
	NDV	512			512
	poly(I:C)	256			256
33	None			0	
	NDV			512	
	poly(I:C)			512	
39	None	0			
	NDV	512			
	poly(I:C)	512			

[a] Macrophage cultures were prepared from lung lavages of 18 mice. Macrophage cultures were inoculated with either 50 μg of poly(I:C) or Newcastle disease virus at a multiplicity of 10. Culture fluids were collected and processed for interferon titration as described for the tracheal organ cultures.

[b] The day on which mice were sacrificed and macrophage cultures prepared.

air. When tested after 10 days in ambient air a partial ability to respond had returned. Experiment I indicated a complete return to normal interferon production upon induction in a test performed 24 days after return of the mice to ambient air. The impaired cellular function was shown for tracheal epithelium, but it is likely that the effect extended well into more distal airways.

The mechanism by which ozone inhibited the production of interferon by tracheal cells *in vitro* remains to be determined. It is possible that ozone inhibited interferon production at the level of transcription or translation. It has been reported by Werthamer *et al.* (2) that exposure of mice to 2.5 ppm daily for 2 hr resulted in a decrease of lung RNA synthesis with an increase in lung protein synthesis during the first 20 days of exposure. The increase in protein synthesis was thought to be associated with a replacement of lost structural (membrane) protein. Those events might in turn affect the formation of other proteins such as interferon. It is known that the synthesis of interferon messenger RNA and its subsequent translation is necessary for interferon production (12). Thus, any decrease in the synthesis of RNA may also affect the production of interferon. In the current experiment the epithelial cells were capable of normal interferon production upon induction for approximately 10 days in the high ozone environment before becoming unresponsive. The finding suggests exhaustion of a needed synthetic step or possibly an alteration in membrane structure on replacement epithelium in response to the oxidizing effect of ozone.

Macrophages washed from the lungs and manipulated *in vitro* for approximately 3 hr prior to contract with interferon inducers were found to be uniformly responsive. There was no significant difference in the titers of interferon from macrophage cultures of mice kept in ambient air and mice exposed to ozone. Valand *et al.* (13) reported that exposure of rabbits to 25 ppm NO_2 for 3 hr inhibited the capacity of the alveolar macrophages to produce interferon. They concluded that the inability of the NO_2 exposed macrophages to produce interferon was not due to a defect in adsorption, penetration, or uncoating of the virus, or to a loss of RNA synthesis (14). It is difficult to compare our results with those of Valand *et al.* (13, 14) as the inducer, method of interferon assay, and oxidant pollutant employed were all different.

Interferon has always been considered to play an important role in viral infection. During many viral infections interferon is present prior to antibody formation, and it has been suggested that interferon is the first host defense process to be mobilized. A number of viral vaccines and synthetic interferon inducers have been reported to have the potential of inducing interferon production. Panusaran *et al.* (15) demonstrated that instillation of an interferon inducer, CP20,961 *N,N*-Dioctadecyl-*N'*,*N*-bis-(2 hydroxyethyl) propanediamine, in the form of nose drops prevented the symptoms of rhinovirus infection and virus shedding in volunteers. The procedure was associated with the production of elevated interferon titers in the nasal washing. Administration of aerosolized poly(I:C) in mice was found to be effective against influenza infections (16). A number of viral vaccines have been investigated for potential use as interferon inducers in man (17, 18). In an experiment where volunteers received influenza A/Bethesda/68 (H_3N_2) wild-type virus, Murphy *et al.* (17) reported that six of seven volunteers had significant levels of interferon in their nasopharyngeal washings. In view of our findings, the environmental factors such as the level of atmospheric ozone may have to be taken into consideration if interferon inducers are to be used widely in the treatment and prophylaxis of viral infections.

Summary. The effects of 0.8 ppm ozone on the capacity of the tracheal epithelium and alveolar macrophages of mice to produce interferon *in vitro* was studied. Exposure of mice to ozone for a period of 11 days or more affected the capacity of the tracheal epithelial cells *in vitro* to produce interferon. The inability of the tracheal epithelium *in vitro* to produce interferon was not due to the inhibition in the release of intracellular interferon but to an inhibition in the production of interferon. There was a complete recovery of the ability of tracheal epithelium to respond to interferon inducers

after the mice were returned to ambient air 24 days post ozone exposure. However, ozone did not seem to have any affect on the capacity of the alveolar macrophages to produce interferon *in vitro*.

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