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Effect of Nitrogen Dioxide on Influenza Virus Infection in Hamster Trachea Organ Culture[1] (39977)

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Initial studies (1-3) from our laboratories showed that acute and chronic exposure to nitrogen dioxide (NO_2) reduced the resistance of rodents to infection with *Klebsiella pneumoniae*. These studies were extended to demonstrate that squirrel monkeys exposed to NO_2 increased the susceptibility to bacterial and viral infection (4-7). In one study (5) all monkeys infected with influenza virus 24 hr before exposure to 10 parts per million (ppm) NO_2 died within 2 or 3 days after infectious challenge. Cigarette smoke has been shown to have similar effects on the resistance of mice to influenza infection (8).

A large body of evidence indicates that health hazards from air pollution must be concerned mainly with chronic (continuous) rather than acute (single) exposures. Fenters *et al.* (9) continuously exposed squirrel monkeys to 1 ppm NO_2 for approximately 16 months and challenged them five times with monkey-adapted influenza virus. This resulted in an earlier and greater serum neutralization antibody response than in control monkeys exposed to filtered air and challenged with virus. Chronic airways and parenchymal lesions were observed. On the basis of these findings, it was suggested that exposure to low NO_2 concentrations could possibly have an effect on respiratory cells, whereby the receptor sites could be altered to permit the virus to multiply without influencing normal functions. Since gaseous air pollutants are of interest from the standpoint of acute respiratory disease, study of NO_2 and its interaction with tracheobronchial epithelium is relevant to an understanding of resistance to respiratory infection.

Whole hamster trachea and tracheal ring organ cultures previously described (10) have provided a convenient and sensitive controlled experimental model for the study of influenza virus infections. Since NO_2 would most likely be in contact with the tracheal epithelium during inhalation of the gaseous pollutant, the hamster trachea organ culture model was selected for these studies. These short-term exposures to NO_2 induced subtle physiological responses in ciliated epithelium of hamster trachea in organ culture that accelerated alterations in ciliary activity and viral replication after influenza A/PR/8 virus infection.

Material and methods. Organ culture preparation. Organ cultures were prepared from the tracheas of 2-week-old Syrian golden hamsters of the CrRGH(SYR) strain by previously described methods (10). Tracheas were excised by sterile technique, washed in Hanks salt solution, and cut with a sharp razor blade into rings to include 2- to 3-mm-wide cartilage rings per explant. The explants were incubated in groups of five in 35-mm petri dishes containing Eagle's minimal essential medium (E-MEM) with Earle's base, and supplemented with a final concentration of 0.2% bovine albumin, 2 m*M* glutamine, 250 U/ml of penicillin, 250 μg/ml of streptomycin, and 100 U/ml of mycostatin. Explants were maintained in a water-saturated atmosphere of 5% CO_2 in air at 37°.

Virus and infection of organ cultures. Hamster-adapted influenza virus strain A/PR/8/34 (HON1) and the preparation of virus stock have been described previously (10).

Tracheal rings were maintained in culture for 24-48 hr before inoculation. Culture medium was withdrawn and 0.2 ml of inoculum containing 10^2HAD_{50} was added to each explant. After 2 hr of adsorption at 37°, each explant was washed three times with E-MEM, fresh medium added, and incubated at 37° in a humidified 5% CO_2-95% air mixture. Corresponding control cultures were inoculated with a noninfective

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diluent similar to that used to suspend the virus.

Harvesting of virus. At 24 hr and thereafter every 48 hr, culture medium was removed from three infected and control dishes and replaced with equal amounts of fresh medium. Medium removed was stored at −70° and titered simultaneously on completion of the experiment.

Virus titration. Infectivity assay was carried out by inoculating serial 10-fold dilutions of each virus sample into 4 tubes/dilution of African green monkey kidney (Vero) cells. After incubation at 37° for 4 days, culture medium was removed and tested for hemadsorption with 0.4% guinea pig erythrocyte suspension (11). Titers were calculated according to the method of Reed and Muench (12) and expressed as the 50% hemadsorption (HAD_{50}) dose.

Exposure techniques. Explants were exposed to NO_2 in a water-saturated gas-tight Plexiglas chamber (Bellco Glass Co., Vineland, N.J.) at 37°. Culture medium was removed from the dishes, leaving enough medium to keep the bottom of the explants moist and still permitting full exposure of the epithelium to NO_2. The NO_2 atmosphere was introduced from a cylinder of 1% NO_2 gas mixture in balanced air and diluted to the proper concentration with filtered air in a glass mixing chamber. The NO_2 was metered so it passed at a rate of 125 ml/min into the chamber. The NO_2 concentration of the chamber was determined by the Saltzman method (13). Chamber concentrations of NO_2 were monitored every 30 min during exposure. The pH of the medium following exposure to NO_2 resulted in final values of 6.3 to 6.7. After exposure, tissues were washed twice with E-MEM and the dishes were filled with 0.7 ml of fresh medium.

All NO_2-treated tracheal explants used in the study were exposed to 2 ± 0.2 ppm (mean ± SD) for 1.5 hr, 5 days/week, for 1, 2, and 3 weeks. Control cultures were exposed to activated charcoal-filtered air.

Microscopic observations. Ciliary activity and the appearance of ciliated epithelium were determined with an inverted phase microscope at magnifications of 100 and 300×. The vigor of ciliary activity at the periphery of the explants was determined using an electronic stroboscope (General Radio, Concord, Mass.) as the light source. Culture dishes were placed in a 150 × 25-mm plastic dish with integral heat unit to maintain the temperature at 37° while determining the ciliary beat frequency. When the flash rate is set to the same speed as the ciliary beat, ciliary movement appears stopped. Ciliary beat frequency was measured at three separate areas on the lumen and their average was recorded as beats per minute.

Histopathology. After incubation, explants were fixed in neutral buffered formalin, dehydrated, cleared, and embedded in paraffin, and sections were stained with hematoxylin and eosin.

Results. Groups of tracheal explants were infected with influenza A virus immediately upon initiation of the NO_2 exposure, whereas other explants were first exposed to NO_2 for 1 and 2 weeks and then infected with the influenza virus. Explants infected immediately after initiation of the NO_2 exposure showed a pattern of ciliary activity similar to that of the infected controls exposed to filtered air (Fig. 1). A more rapid decrease in ciliary activity was seen in tracheal explants infected after 1- and 2-week NO_2 exposure than in controls held in filtered air. The decrease in ciliary activity was paralleled with epithelium that became uneven with a swollen appearance and progressed until all ciliated epithelial cells were shed. Noninfected control explants exposed to 2 ppm NO_2 first showed some decreasing ciliary activity after 14 days and no significant histological abnormalities. Ciliated cell loss was minimal with occasional vacuolation. Exposure to NO_2 during the 2-week period did not affect the number of mucus goblet cells. Occasional small areas of basal cell hyperplasia were observed in the explants. Tracheal glands showed no changes. After 3 and 4 weeks, the NO_2-exposed uninfected cultures showed a 25 and 63% decrease in ciliary activity with a minimal degree of damage to the epithelium. In uninoculated control cultures, the general outline of the epithelial surface remained sharp and the epithelium was pseudostratified columnar with well-preserved cilia that could be observed in H & E-stained sections. Mucus goblet cells were uncommon.

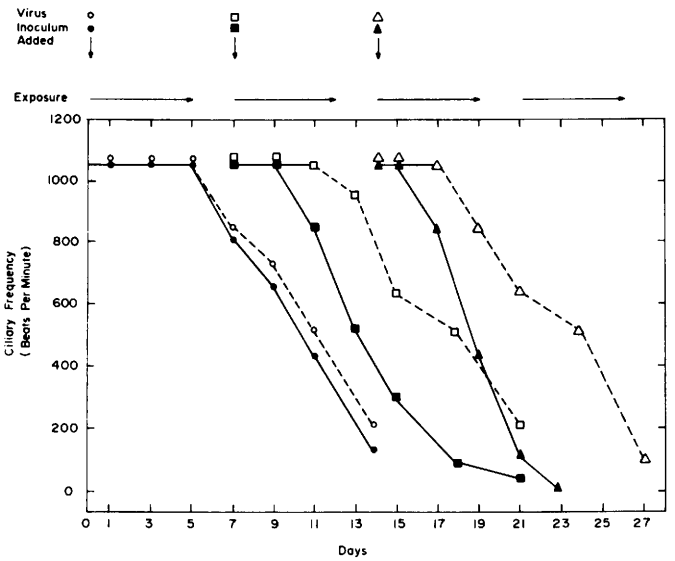

FIG. 1. Effects of 2 ppm NO_2 exposure and influenza A/PR/8 virus infection on ciliary function in hamster tracheal organ culture. The SD ranges which were <6% were omitted from the figure for clarity. The horizontal arrows represent period of NO_2 exposure and the vertical arrows indicate time virus inoculum was added. Controls: Open symbols indicate results from influenza-infected cultures exposed to filtered air. Tests: Closed circles represent explants infected immediately after initiation of NO_2, closed squares indicate results obtained from cultures exposed to NO_2 for 1 week and then infected with virus, and closed triangles represent explants infected 2 weeks after initiation of NO_2 exposure.

The lamina propria also appeared normal. Tracheal glands were present in rings obtained nearest the larynx; they possessed small mucus granule cells and serous cells.

The growth of the virus in explants infected immediately after initiation of NO_2 exposure was similar to that in controls held in filtered air (Fig. 2A). Cultures exposed to NO_2 for 1 week (Fig. 2B) and then infected with the virus reached a maximum titer 7 days after infection, 4 days earlier than the filtered air controls. The growth of virus in explants infected 2 weeks (Fig. 2C) after initiation of NO_2 exposure showed a peak titer 5 days after infection, whereas the maximum titer in the filtered air control was not reached until Day 9. In all three experimental conditions, the maximum virus titer was essentially the same, even though the time of peak virus production varied. In control dishes containing virus inoculum only, virus was not detected after 48 hr of incubation.

Discussion. The results of these studies demonstrate that pretreatment of hamster tracheal epithelium *in vitro* with NO_2 gas at 2 ppm for intermittent 1.5-hr periods prior to influenza A virus infection decreases ciliary activity when compared to infected explants exposed to filtered air. These *in vitro* effects have been recently validated when similar alterations in ciliary activity were observed in tracheal explants exposed to a solution of $NaNO_2$ equivalent to 2 ppm NO_2 gas (Schiff, unpublished observations). In addition, it was established that tracheal organ cultures exposed to NO_2 permit a more rapid production of virus than tracheas held in filtered air.

Nitrogen dioxide-treated tracheal ciliated epithelium fails to produce detectable changes in ciliary activity and epithelial architecture during the first 2 weeks of exposure. After 3 weeks in culture a noticeable decrease in the frequency of ciliary beating was observed, and by 4 weeks a marked decline was seen. A distinguishing characteristic of NO_2 is its relative lack of observable acute tissue response until a critical concentration is reached (14). The early effects of NO_2 on the virus-infected explants would therefore indicate a more subtle mechanism by which NO_2 affects ciliated epithelium. It has been suggested that the

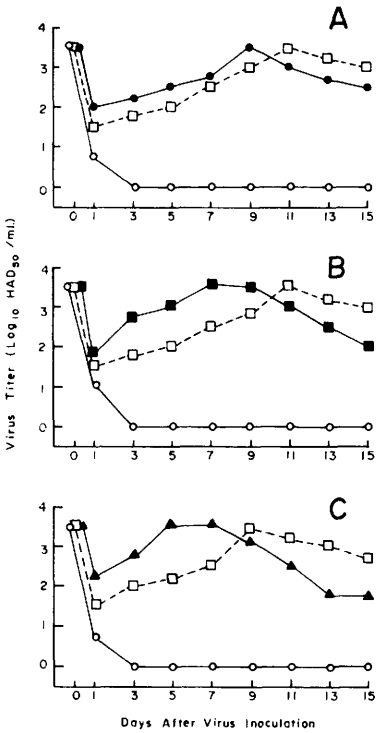

FIG. 2. Effects of 2 ppm NO_2 on replication of influenza A/PR/8 virus in hamster tracheal organ culture. Explants were infected with virus: (A) immediately after initiation of NO_2; (B) after exposure to NO_2 for 1 week; and (C) 2 weeks after initiation of NO_2 exposure. Values represent the results of experiments performed in duplicate. Culture fluid was harvested initially at 24 hr, then every 2 days, and assayed in tissue culture. Controls: Open circles represent values obtained during incubation of virus without explants and open squares represent values obtained from virus-infected explants exposed to filtered air. Tests: Solid circles represent results from explants infected immediately after initiation of NO_2, solid squares represent results from explants exposed to NO_2 for 1 week and then infected with virus, and solid triangles represent results from explants exposed to NO_2 for 2 weeks and then infected with virus.

site of interaction between NO_2 and the mucociliary apparatus is the energy source of the cilia. Moreover, evidence that NO_2 can affect lung homogenate enzymes linked to adenosine triphosphate formation has been reported (15).

Although the mechanism of NO_2-induced lesions remains to be elucidated, these data reflect a subtle but important physiological response to NO_2 that was unacommpanied by morphological evidence of tissue damage.

Summary. Pretreatment of hamster tracheal organ cultures with 2 ppm NO_2 for intermittent 1.5-hr periods prior to influenza A/PR/8 virus infection decreased ciliary activity, without significant changes in morphology, when compared to infected explants exposed to filtered air. Tracheal organ cultures exposed to NO_2 permitted a more rapid production of virus than tracheas held in filtered air. A mechanism for dissociation between morphological and functional events is suggested.

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