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Project Summary

Effects of Pollutants on Human Viral Respiratory Disease

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Many epidemiologic studies have shown excessive respiratory disease morbidity in areas of high atmospheric pollution. This study was designed to develop and characterize an animal model and to investigate the possible interactive effects of infection and particulate air pollutants using small laboratory animals. Models of human parainfluenza virus type 3 disease were established by aerosol inoculation of hamsters and cotton rats. The temporal course of the following were examined: lung virus titers; pulmonary histopathology; alveolar macrophage function; changes in pulmonary mechanics; serum antibody development; and upper respiratory tract histopathology. Animals were exposed acutely (2 hours) to ammonium nitrate or lead oxide respirable aerosols before or following viral inoculation. Exposures ranging from 59-66 mg/m³ but not 0.76 mg/m³ of the nitrate resulted in a one-day extension of viral replication and concommitant retardation of peribronchial lymphocytic infiltration. Lead oxide exposures at levels greater than 2,350 μgm/M³ increased lung virus titers and serum antibody titers. The models developed in these studies may be useful for future work on chronic exposure to the same or other pollutants and on the pathogenesis of virus/pollutant interactions.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The observed excess of acute respiratory disease morbidity in areas of high atmospheric pollution lacks a clear explanation at present. It is known that the same infectious agents produce infections in both urban and rural settings, suggesting that pollutants may interact in some way with either agent or host to the disadvantage of the host. Since a complete study of the problem is not feasible in the normal human population, animal models can be used to obtain detailed information which can then suggest hypotheses and limited studies that could be accomplished in people.

Since most acute respiratory disease in humans is caused by virus infections, small laboratory animal models of the common types of infections would be useful to assess the added effects of inhaled toxicants. The full report describes studies in which hamsters and cotton rats infected with human parainfluenza virus type 3 (PV3) are exposed to two different particulate pollutants. The development of methods, pollutant exposure results, and conclusions are presented.

Discussion of Results

Model Development

The usefulness of the hamster as a model of human PV3 infection was described in 1964. Since this model had been established in our laboratories for other studies, it was used initially in the current work. An important early goal was the establishment of aerosol inoculation capability in our laboratories. The equipment used allows "nose-only" inoc-

ulation of 8 animals held in individual ports. A glass nebulizer generates the aerosol, and passage through a glass drying column yields a product with mainly respirable particles. In evaluating the generator for use with PV3, it was found that minimal foaming was obtained if virus was grown in a medium with only 1% fetal bovine serum. Air flow to the drying column had to be reduced to prevent loss of virus viability. Reproducible infection of hamsters and maximal virus yield from lung tissue was achieved by delivery of $10^{7.9}$ $TCID_{50}$ of virus into the chamber over a 20-30 minute period.

After standardizing the aerosol exposure conditions, it was possible to study the time course of the experimental infection. No virus could be found in the lungs 30 minutes after the aerosol exposure. On day 1, titers increased to $10^{5.8}$ $TCID_{50}$/gm lung, peaked at $10^{6.8}$ on day 2, then declined gradually with disappearance by day 6 or 7. Pathologic changes in the lung were most marked on day 5 or 6, and consisted of interstitial and peribronchial lymphocytic infiltration and marked proliferation of the bronchial epithelium. These findings were very similar to our earlier results obtained by intranasal instillation of PV3. A problem arose as work continued, in that the infection and pathology on additional aerosol exposures became unpredictable. It was suspected that the hamsters were becoming infected with PV3 or a related virus (i.e., Sendai or murine parainfluenza type 1) at the suppliers and thereby were resistant to the inoculations. A survey of suppliers revealed none who would certify freedom of their colonies from PV3 or Sendai virus.

To assess the possible magnitude of Sendai virus infection resulting in PV3 resistance, some cross-challenge experiments were done using cotton rats from our breeding colony. Groups of animals were inoculated with Sendai virus and allowed to recover (30% mortality was noted). Survivors were then exposed to varying amounts of PV3 by intranasal instillation, and the doses of virus required to infect 50% (ID_{50}) and produce pneumonia (PD_{50}) were calculated. Rats previously infected with Sendai required 100-fold more PV3 to achieve an infection rate of 50% compared to controls. The amount of PV3 needed to produce pneumonia was reduced by a like amount. The effect on the ID_{50} indicates cross-reacting immunity from the prior Sendai infection, and the reduced PD_{50} is thought to reflect the same thing. It has been noted in other models that challenge infection with the same agent may exaggerate and accelerate pneumonia changes, suggesting that many of the changes seen are the histologic reflection of immune responsiveness. Thus, freedom from Sendai virus infection is an important prerequisite for studies of PV3 in hamsters. Since this cannot be guaranteed currently by any known supplier, animals must either be bred in isolation or prescreened serologically before use. For this reason, subsequent work was performed primarily in cotton rats.

The cotton rat is known to be susceptible to infection with *Mycoplasma pneumoniae* and with respiratory syncytial virus. With evidence that it was also susceptible to human PV3, a complete study of this model was made and subsequently published. The course of infection and pulmonary histopathology were very similar to those described for the hamster, both being quite similar to the natural human disease. Another advantage of the cotton rat is that they show greater immune responsiveness than hamsters; the chief disadvantage is that they are not available commercially at the present time.

Macrophage Studies

A prime defense mechanism in the lung is the alveolar macrophage. Since inhaled toxicants could adversely affect this "first line of defense," as can several viral respiratory diseases, evaluation of macrophage function could be a sensitive indicator of interaction between pollutants and infectious agents. Studies were undertaken of cotton rat alveolar macrophage function during the course of PV3 infection. The functional state measured was oxidative metabolism as reflected by chemiluminescence of zymosan-stimulated cells.

Bronchopulmonary cells were collected by endotracheal lavage at intervals during experimental PV3 infection. At each time period, cells were suspended in a luminol solution, and background light emission was measured in an ATP spectrophotometer. Zymosan was then added, and the stimulated chemiluminescence of cells from infected animals was assessed with matched controls. By day 2 of infection, chemiluminescence was decreased to 44% of control ($p = < .025$) and on day 4 to 28% ($p = < .001$). Suppression was 65% of control on day 7, and 84% on day 10, neither of these being statistically significant.

Exploring the cause of reduced macrophage oxidative metabolism suggested that the cells were undergoing a non proliferative infection with PV3. Macrophages cultured *in vitro* could not b infected with the virus. However, three fourths of the cells collected from infecte animals contained viral antigen as show with the indirect immunofluorescenc method. To prove that the cells wer infected, as opposed to containing phago cytosed dead virus, hemadsorption wa done of cell preparations using guinea pi erythrocytes. Approximately one-fifth o the cells were hemadsorption positive indicating expression of viral proteins or the external macrophage membrane. Attempts to verify this by electron microscopy were unsuccessful.

Upper Respiratory Tract Models

For some studies of inhaled pollutants it may be of interest to investigate the upper respiratory tract, since considerable deposition of inspired materials takes place there. In small laboratory animals it is difficult to obtain accurate samples for quantitative study from the nasal passages, and the cartilage and bone of the skull interfere with histological studies. Some methods were developed which could be useful in future work.

A 3 mm instrument cleaning brush was tested for quantitative sampling of the nasal passages. The brush tip is identical to those used to collect specimens during bronchoscopy, and has been used in other work in our laboratories for nasal biopsies in adults and children. In either hamsters or cotton rats, insertion and twirling of the brush in the nasal passages removed an epithelial tissue sample which packs the area among the brush bristles (often 3-4 mg of material). The collected cells can then be shaken from the brush by vibration, for purposes such as virus titration, or solubilized for assays such as heavy metal quantitation.

Histologic study of the nasal passages and paranasal structures was made of PV3-infected cotton rats. At various points during experimental disease at necropsy the head was removed, freed of surface tissue and muscle and fixed in 10% neutral formalin. After fixation the skull was placed in de-calcifying solution (primarily made of HCl) followed by thorough washing in water. A sharp blade was used to make sections across the head between the nose tip and orbits, through the orbits, and through the posterior plane containing the otic bullae; after histological processing these specimens provided frontal views of the nasal tubinets. Harderian glands and paranasal

sinuses and the middle ear cavity, respectively. In PV3 infection, marked nasal epithelial cell proliferation was noted, but no infiltration of the Harderian glands was seen. Cytopathic changes were presented in the ciliated epithelium lining the middle ear cavity, but there were no exudates in the lumen.

Studies of Pulmonary Mechanics in PV3-Infected Animals

Studies of pulmonary mechanics in young male hamsters infected with PV3 were conducted to determine the effects of the infection on lung function during the period of time when viral titers from lung homogenates and cellular reaction are greatest. One hundred gram animals were inoculated using the aerosol technique described above. Similar groups of animals were sham inoculated using viral carrier medium as the inoculum. Pulmonary mechanics were studied five days after inoculation using techniques previously described. Five separate experiments were conducted which included 24 control and 24 infected hamsters. Although the tidal volume was significantly decreased in the infected animals as compared to control, the minute ventilation was increased which resulted from an increase in respiratory frequency. There was a significant decrease in compliance in the infected hamsters when compared to control and an increase in expiratory resistance. Although inspiratory and average resistances tended to be greater in infected animals, they were not significantly different from controls. These data are consistent with findings from studies of humans with acute viral bronchiolitis and also with results anticipated after reviewing the histology of the lung from infected hamsters. Although the changes in mechanical and ventilatory parameters are small, using these techniques in this animal model of a very common human disease should prove to be very useful in evaluating therapeutic strategies.

Particulate Pollutant Exposures

A series of studies were performed in which animals were exposed acutely (2 hours) to particulate aerosols of ammonium nitrate or lead oxide before or during experimental infection with PV3. All pollutant exposures were performed and controlled by the engineers at the Health Effects Research Laboratory-Research Triangle Park (HERL-RTP); virus exposures were done in our laboratories as described before. As a minimum study, parameters in all experiments were lung histology and virus titration; additional examinations in some cases included measurement of pulmonary mechanics and viral serology. No natural mortality was observed with any of the combinations of exposures.

Ammonium Nitrate Exposures

A group of exposures of PV3-infected cotton rats to NH_4NO_3 were completed and analyzed. Two protocols were used. In the first experiment, NH_4NO_3 exposure was given initially followed by PV3 inoculation on the same day. The second protocol involved PV3 infection first, followed by NH_4NO_3 exposure on day 2-3 or day 4-5 of experimental disease, with sacrifice 24 hours later when maximum virus yield would be expected (early sacrifice) or at the time of peak pulmonary histopathologic change (later sacrifice). These experiments were done with a high-level exposure to NH_4NO_3 particles in air, 59-66 mg/M^3 for 2 hours. One experiment was performed with a much lower level of the pollutant, 0.76 mg/M^3 of air for 2 hours.

Animals given pollutant followed by virus had less pulmonary histopathologic change on day 3 of disease but more on day 6 than did rats given virus alone. However, less PV3 was recovered on day 6 from animals given NH_4NO_3 than from the infected controls. While the differences were not significant statistically, the result suggested that NH_4NO_3 had an effect on both virus and host in terms of virus peak yield and prolongation of pulmonary disease.

In the second type of experiment, the pollutant exposure was for 2 hours on either day 3 or day 5 of experimental disease with subsequent sacrifice points on days 6, 7 or 14. Again, it was found that the earlier pollutant exposure diminished virus yield from the lung tissue, but that pulmonary pathologic change was enhanced later. Differences in the groups did not persist during the recovery phase, as suggested by results from a few animals held for 14 days.

The effect of lesser amounts of NH_4NO_3 also was examined. Cotton rats were exposed to 0.76 mg/M^3 of air either the same day as PV3 inoculation or three days after infection was established. The results indicated no effect of the pollutant relative to control animals given PV3 alone. It can be concluded that either the NH_4NO_3 failed to affect the experimental model, or that the indicators used to measure the effects are insufficiently sensitive.

Studies of Pulmonary Mechanics in PV3-Infected Animals Exposed to Ammonium Nitrate

Young male hamsters were inoculated with PV3 and then allowed to breathe an atmosphere containing 25 ppm NH_4NO_3 within 2 hours after inoculation with PV3. Pulmonary mechanical measurements were made five days after exposure to NH_4NO_3 when the effects of the PV3 infection were greatest. The results of studies of nine animals infected with PV3 (NH_4NO_3 unexposed) and nine animals infected with PV3 and exposed to NH_4NO_3 were analyzed. In addition to the usual mechanical ventilatory parameters studied, thoracic gas volume at functional residual volume (V_{TG}) was measured. No significant differences were found between the two groups for any of the mechanical or ventilatory parameters. The V_{TG} for the NH_4NO_3 exposed animals was significantly lower than for the unexposed group. Although the resistance values (R_i, R_e, R_A) tended to be higher in the exposed group, these were not significantly higher and the differences became even less when adjusted for V_{TG}. The data from these small groups of hamsters suggest that exposure to this level of NH_4NO_3 shortly after inoculation with PV3 does not markedly alter the acute course of PV3 infection. Additional studies are necessary to determine the effects of NH_4NO_3 exposure during different phases of the respiratory infection.

Lead Oxide Exposures

The effect of lead oxide exposure on the course of experimental PV3 disease was assessed using protocols similar to those in the ammonium nitrate studies. The initial experiment involved exposure of animals to lead oxide at a level of 2 mg/M^3 of air for 2 hours, followed by inoculation with PV3 on the same day. Animals were then sacrificed on either day 3 or day 5, when maximal virus replication and pulmonary pathology, respectively, would be expected The control animals were handled identically but not exposed to lead oxide. Analysis revealed no differences in either the amount of virus produced in the lung tissue or the quantity and quality of histopathologic changes.

In another experimental design, groups of cotton rats were inoculated with PV3,

and a portion of the animals were exposed to lead oxide aerosols at different concentrations for 2 hours on the third day of infection. Lead-exposed and unexposed animals were then sacrificed on day 5 of infection for lung virus quantitation and histopathology. A few representative animals from each group were saved to be bled at 3 and 6 weeks for PV3 antibody measurement. In Study A, in which a lead oxide exposure level of 3.136 mg/M^3 of air was used, the mean virus titers of lead-exposed animals were significantly higher on day 5 than among those receiving PV3 alone. The significance of this difference was supported by the serologic results. By the complement fixation method, animals receiving PV3 alone had an antibody titer of $2^{6.3}$ (geometric mean); with lead, the mean was $2^{8.75}$. The result suggests that lead-exposed animals dealt with a larger mass of viral antigen than did the other group.

In other lead level exposures, a trend toward similar results obtained with study A were seen at the lead oxide level of 2.350 mg/M^3 (study B), but the differences were not statistically significant. No effect of lower concentrations of lead oxide were demonstrated (study C).

Conclusions

Since the majority of human respiratory infections have a viral etiology, experimental models of some of the major ones would be useful in assessing interactive effects with atmospheric pollutants. In this project, hamsters and cotton rats were explored as models of a common human parainfluenza virus disease. The cotton rat provides the possibility for comparative studies of different etiologic agents in the same host, since they have been found susceptible not only to PV3 but to human adenovirus types 1-7, respiratory syncytial virus and *Mycoplasma pneumoniae*. These experimental models, which were used in acute exposures to two particulate pollutants, ammonium nitrate and lead oxide, provided some evidence of adverse effects on the disease. Effects included extension of pulmonary histopathology, and increase in the amount of virus in the lung tissue. The models may prove useful for chronic, or repeated acute, exposures to the pollutants tested in these studies and to other toxicants of interest. They also can be used to determine the pathogenesis of any interactive effects observed between pollutants and disease.

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The complete report, entitled "Effects of Pollutants on Human Viral Respiratory Disease," (Order No. PB 85-122 455; Cost: $8.50, subject to change) will be available only from:

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