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EFFECT OF EXPOSURE TO PAN AND OZONE ON SUSCEPTIBILITY TO CHRONIC BACTERIAL INFECTION

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report describes the results of an *in vivo* investigation designed to determine the effects of peroxyacetyl nitrate and ozone on susceptibility of mice and guinea pigs to chronic and acute respiratory infection. The agent used for the acute infectious disease was *Streptococcus pyogenes* whereas *Mycobacterium tuberculosis* served as the agent for the chronic respiratory infection. In these studies the relative comparison of the effects of these two pollutants on the resistance to laboratory induced bacterial infections of the lung could be obtained. As appropriate to the specific experiments, the parameters measured were mortality, survival time, retention or growth of inhaled microorganisms in the lung, histopathology of the respiratory tract tissue, pulmonary cellular defense system, and immunological response.

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ABSTRACT

The effects of peroxyacetyl nitrate (PAN) and ozone (O_3) on susceptibility of mice and guinea pigs to chronic and acute respiratory infections were studied. The agent used for the acute infectious disease was *Streptococcus* sp. whereas *Mycobacterium tuberculosis* served as the agent for chronic respiratory infection.

A significant increase in mortality due to streptococcal pneumonia was seen upon a single 3-hr exposure to PAN in concentrations ranging from 14.8 to 28.4 mg/m^3. The excess mortalities ranged from 8 to 39% and reduction in the survival time from 2.4 to 7.9 days. Within this concentration range of PAN a close relationship was present between the duration of exposure and concentration. Multiple daily exposures to 4.9 or 7.4 mg/m^3 PAN 3 hr/day, 5 days/week for up to 3 weeks had no effect on mortality, survival rates, or ability to clear inhaled *Streptococcus* sp. from the lungs.

Daily 3-hr exposures to 25.0 mg/m^3 PAN did not produce any marked changes in the chronic infection as measured by *M. tuberculosis* titers in their lungs. The diameter of erythemas, expressing the cutaneous delayed hypersensitivity reaction were persistently smaller in guinea pigs exposed to PAN than those exposed to air. Multiple exposures to 19.8 mg/m^3 PAN resulted in initial elevation of antibody titers, but depression of titers during the later (12 to 15 week) observation period. A single exposure to the same concentration of PAN resulted in a significant increase in total number of cells lavaged from their lungs but somewhat decreased levels of adenosine triphosphate (ATP). Exposure to 7.4 mg/m3 PAN 3 hr/day, 5 days/week for 2 weeks resulted in reduced total cell counts and a significant reduction of ATP levels in alveolar macrophages.

Scanning electron microscopic observations of the respiratory tract showed that the nonciliated cells of the nasal cavities and tracheas of mice exposed to PAN were raised and sloughing and excess mucus was present. In older mice lung congestion was enhanced by PAN exposure.

Exposures to ozone resulted in increased titers of *M. tuberculosis* in the lungs, depression of hypersensitivity reaction and elevation in serum antibody titers.

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SECTION 1

INTRODUCTION

Peroxyacetyl nitrate (PAN) is the first member of a series of peroxyacetyl nitrates ($R\text{-}C''\text{-}OONO_2$) that are important constituents of photochemical air pollution. PAN is a highly oxidized, unstable organic nitrogen compound that results from ultraviolet irradiation in air of certain unsaturated hydrocarbons in the presence of oxides of nitrogen. PAN was the first pure compound recognized as causing plant damage and eye irritation (1). A number of studies conducted to determine effects on animals indicate that the toxicity of PAN for mice is greater than that of sulfur dioxide (SO_2), similar to nitrogen dioxide (NO_2) and lower than that of ozone (O_3).

In studies conducted by Campbell and coworkers (2) the LC_{50} for mice for a 2-hr exposure was 105 to 150 ppm PAN. The LC_{50} was closely related to the age of the animals and the environmental temperature maintained during the exposure. Mice, 4 to 6 month old were more susceptible than those 2 to 3 month old and increased susceptibility to PAN was seen at 90°F as compared to 80°F. The same investigators reported a depression of voluntary motor activity in mice during exposure to approximately 8 ppm PAN (3). Daily 6 hr exposure to 15 ppm PAN for 6 months caused 18% mortality, weight loss, and damage to the respiratory tract tissue in mice (4). The damage included tracheobronchitis, broncholotis, pneumonitis, mild emphysema, squamous metaplasia, and epithelial hyperplasia. In a study involving 21-year-old males exposed for 5 min to 0.3 ppm PAN and then exercised for 5 min a significant increase in oxygen uptake was reported when compared to those breathing normal air (5).

Tuberculosis infection in rodents has been described by various investigators (6,7,8,9). Many of the studies have been conducted to determine the effects of vaccination on the growth of the virulent human *Mycobacterium tuberculosis* strain H37Rv in the lungs and other organs of mice and guinea pigs (6,9). The attenuated *M. tuberculosis* strain R1Rv used in our studies has been grown in liver and spleen of rats (6). It is the most virulent of the attenuated strains and causes disease in silicotic animals (Personal communications, Mr. Logy, Trudeau Institute, Saranac Lake, NY). Immunological responses, especially cutaneous delayed hypersensitivity, to *M. tuberculosis* have been measured extensively in guinea pigs (10).

Studies of the effects of inhalation of air pollutants on the resistance to chronic respiratory infection, such as tuberculosis are very limited. Thienes and coworkers (11) investigated the effects of ozone on *M. tuberculosis* infection in mice. Exposure to 1.5 ppm O_3 4 hr/day, 5 days/week for 2 months did not alter the resistance to *M. tuberculosis* H37Rv nor the BCG vaccine strain. The authors noted that this infectious model was less sensitive to ozone than the acute disease model which used *Klebsiella pneumoniae* as the infectious agent.

The overall purpose of this program was to study the effects of PAN and ozone on the susceptibility of animals to acute and chronic respiratory infections. The acute disease was established in mice by respiratory challenge with *Streptococcus* sp., (Lancefield group C) aerosols. The chronic infection was induced in mice and guinea pigs by challenge with an aerosol of *M. tuberculosis* attenuated human strain R1Rv.

The various parameters measured in mice during the chronic disease studies were the *M. tuberculosis* titers in the lungs and spleens, number of tubercules in the lungs and histopathologic changes in lungs. The parameters determined in guinea pigs were serum hemagglutination antibody titers and cutaneous delayed hypersensitivity reactions to purified protein derivative (PDD). The parameters assayed in conjunction with the acute infection included mortality and survival time, clearance rate of inhaled bacteria from the lungs, and histopathologic alterations in the lungs, tracheas, and nasal cavities as observed by light and scanning electron microscopy. In a limited number of experiments the effects of exposure to the pollutants on the pulmonary cellular defense system were determined by examining the total and differential cell counts, viability, cell surface morphology and the phagocytic function of alveolar macrophages lavaged from lungs.

SECTION 2

CONCLUSIONS

Effects of single and multiple exposures to PAN on resistance to streptococcal pneumonia were determined. Single 2 to 3 hr exposures to PAN in concentrations ranging from 14.8 to 28.4 mg/m^3 significantly enhanced the susceptibility of mice to streptococcal pneumonia. A linear relationship was observed between CT index and percent increase in mortality. The average excess mortality among mice exposed to PAN was 20%. Multiple daily 3 hr exposures to 7.4 mg/m^3 PAN for 2 weeks or 4.9 mg/m^3 for 3 weeks had no effect on mortality, survival time or the rate of clearance of inhaled bacteria from the lungs.

Studies to determine the effects of PAN on the susceptibility of mice to *M. tuberculosis* were limited by the difficulty to produce large quantities of pure PAN. The highest concentration used was 25 mg/m^3 PAN given 3 hr daily for 6 days beginning 1 week after the challenge. This exposure caused only a minimal increase in the bacterial titers in the lungs, seen in the later stages of the infection.

A 3 hr exposure of guinea pigs to 29 mg/m^3 PAN or 2.9 mg/m^3 O_3 initiated within less than 3 hr after challenge with *M. tuberculosis* aerosol indicated that both pollutants had some effect on delayed hypersensitivity. The diameters of erythema were persistently smaller in animals exposed to PAN or ozone than those exposed to air. Exposure of guinea pigs to 19.8 mg/m^3 PAN or 0.98 mg/m^3 O_3 3 hr/day for 5 days, immediately after challenge resulted in antibody titers that were generally higher in guinea pigs exposed to ozone than those exposed to air. Titers in guinea pigs exposed to PAN were initially higher but later lower than those in guinea pigs exposed to air. Exposure to 0.98 or 1.96 mg/m^3 O_3 3 hr/day 5 days/week for 6 to 8 weeks beginning 1 to 3 weeks after challenge enhanced susceptibility of mice to tuberculosis. The effect was seen as marked increases in bacterial titers in the lungs.

Other parameters measured in conjunction with the exposure to PAN included damage to lungs, tracheas and nasal cavities observed by scanning electron microscopy. The single 3-hr exposure to 25 mg/m^3 PAN caused the nonciliated epithelial cells of the nasal cavity to rise up from the basal membrane and slough. Excess mucus was also present. A similar damage was seen upon daily 3 hr exposures for 2 weeks to 7.4 mg/m^3 PAN in young, 6 week old mice. In older mice (4 month old) exposed to PAN, congestion in the lungs was greater than in air controls. Daily 3 hr exposure for 3 weeks to 4.9 mg/m^3 PAN caused damage to nonciliated cells of the trachea similar to that observed in the nasal cavities.

A single 3-hr exposure to 19.8 mg/m^3 PAN did not alter the differential cell counts or the activity and viability of alveolar macrophages. However, there was a significant increase in total cell counts (macrophages and lymphocytes). Daily 3 hr exposure for 2 weeks to 7.4 mg/m^3 PAN caused a decrease in total cell counts and reduced cellular ATP levels in alveolar macrophages.

SECTION 3

RECOMMENDATIONS

The availability of sufficiently large quantities of pure PAN is mandatory to enable the continuation of health effects studies of this pollutant. This is esepcially important for the extension of multiple and chronic exposure studies to determine their effect on the resistance to respiratory infections.

Short-term exposures to PAN alone or in combination with other pollutants such as ozone can be investigated since the effects of acute exposures to PAN were in part determined in this study. Based on results obtained, such studies should include the determination of the effects of PAN on immune response. Specifically, the activity and viability of alveolar macrophages, serum antibody formation, macrophage migration inhibition and mitogen stimulation of lymphocytes in animals exposed to PAN should be defined.

SECTION 4

MATERIALS AND METHODS

ANIMALS

Sprague-Dawley CF_1 outbred female albino mice, 6 to 16 weeks old C57BL/6 black mice 6 weeks old and Murphy Breeding Laboratory CD_2F_1 hybrid brown mice, 7 to 10 weeks old, were used in these studies. The mice were housed in groups of 10 in stainless steel shoebox cages and provided food and water *ad libitum*. Hartley strain female guinea pigs (Murphy Breeding Laboratory) weighing 300-500 g were housed in groups of 2 or 3 and provided food and water *ad libitum*.

INFECTIOUS AEROSOLS

Mycobacterium tuberculosis

Mycobacterium tuberculosis R1Rv (Trudeau Institute #205) was grown in Proskauer and Beck broth in prescription bottles incubated horizontally for 4 to 8 weeks at 37°C until confluent pellicle growth was obtained. A loopful of the pellicle was transferred to 50 ml of Middlebrook 7H9 broth (Difco) containing 0.1% Tween 80 and incubated 7 to 10 days until the culture was turbid. The unwashed culture was homogenized using a teflon pestle and 50-ml homogenizing vessel. The titer was determined by serially diluting in 0.1% phosphate-gelatin (Bacto hemagglutination buffer (Difco) with 0.1% gelatin), plating 0.1 ml aliquots onto Dubos oleic acid albumin (OAA) agar containing 50 μ/ml penicillin G and 0.1% cycloheximide, and counting the colonies after 3 weeks incubation at 37°C. The cultures were frozen in 8-ml aliquots at -70°C.

For aerosol dissemination, 6 ml of thawed culture was diluted approximately 1:10 in Middlebrook 7H9 broth to which a 10% solution of antifoam A (Dow Chemical, Midland, MI) was added. The bacterial suspension was disseminated at a rate of 0.75 ml/min from a DeVilbiss Model 841 continuous flow nebulizer using a primary filtered air flow of 8 liter/min into a 30x30x90-cm plexiglass chamber installed within a microbiological safety hood.

Groups of six guinea pigs were exposed to the aerosol for 2 min. Groups of 48 to 60 mice, housed in individual compartments of specially designed aluminum wire cages, were exposed for 20 min to the aerosol. The inhaled dose was determined by homogenizing lungs from three mice immediately after the challenge in 2 ml of 2% albumin and plating 0.2 ml of ten-fold phosphate-gelatin dilutions onto OAA agar. The plates were incubated for 4 weeks at 37°C. The number of bacteria in a total volume of 2.2 ml was considered as the number of *M. tuberculosis* per lung.

Streptococcus pyogenes

Streptococcus pyogenes group C (Lancefield strain) was passaged in mice, isolated from hearts, incubated for 18 hr at 37°C in Todd Hewitt broth and 1-ml aliquots frozen at -70°C. For aerosol dissemination, 1 ml of thawed culture was added to 100 ml of Todd Hewitt broth and incubated for 18 hr at 37°C. The culture was diluted approximately 1:10 in 0.1% peptone water and adjusted to 60% transmittance at 440 nm in a spectrophotometer (Bausch and Lomb Spectronic 20, Rochester, NY).

The bacteria were disseminated using a DeVilbiss Model 841 nebulizer by using a primary filtered air flow of 8 liter/min and a secondary humidified air flow of 28.3 liter/min into a 60x70x90-cm plexiglass chamber placed inside a microbiological safety hood.

Mice were exposed to the aerosol for 10 min. The inhaled dose was determined by homogenizing lungs from three mice in 0.1% peptone water and plating tenfold dilutions onto blood agar. The colonies were counted after 48-hr incubation at 37°C. The titer of the culture was determined by plating tenfold dilutions onto blood agar.

POLLUTANTS

Ozone (O_3)

Animals were exposed to O_3 in a 120x60x60-cm plexiglass chamber maintained at 24 $\pm$ 2°C and $\leq$40% relative humidity (RH). To prevent build-up of ammonia, deotized cage board (Upjohn Co., Kalamazoo, MI) was placed on the bottom of the chamber. A high voltage generator (IITRI) was used to convert filtered air to O_3. To provide the desired concentration, O_3 was mixed with filtered air in a glass mixing chamber and the mixture passed into the exposure chamber at a rate of 60 $\pm$ 5 liters/min. Concentration was monitored continuously with an O_3 Chemiluminescent Analyzer (Model OA 310, Meloy Laboratories, Springfield, VA), and was expressed in mg/m^3 (ppm x 1.95 = mg/m^3).

Peroxyacetyl Nitrate

Synthesis--

PAN was synthesized using procedures described by Kacmarek, et al. (12). Briefly, biacetyl was photolyzed in the presence of NO_x and oxygen. Biacetyl (1-1/2 torr) and NO_2 (4 torr) were loaded into a 3 liter bulb and then oxygen was introduced. The bulb was floated in an ice bath and cold water from the bath was circulated over the bulb. The chemicals were irradiated for 4 hr by UV light from an A-H6 lamp (Illumination Industries, Sunnyvale, CA) placed 35 cm from the bulb. Over 5 ml of the gas (equivalent at STP) produced from this photolysis yielded 80%.

$$(CH_3C(O)C(O)CH_3 + NO_2 \xrightarrow[h\nu]{O_2} CH_3C(O)OONO_2)$$