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EFFECTS OF REPEATED EXPOSURE TO NITRIC ACID VAPOR AND OZONE ON RESPIRATORY TRACT CLEARANCE IN THE RAT

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This study assessed the effects of repeated exposure to environmentally realistic levels of two urban air pollutants—nitric acid vapor (HNO_3) and ozone (O_3)—on tracer particle clearance from the rat respiratory tract. The measurement of particle clearance efficiency is important because failure of this function could contribute to a buildup of foreign matter in the respiratory tract. Separate groups of rats (30 rats per group) were exposed nose-only for 4 h/day, 3 days/wk (a surrogate for an air pollution episode) for 40 wk to 4 atmospheres: purified air; 0.15 ppm O_3; 50 $\mu g/m^3$ HNO_3; and 0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3. At 4 wk prior to the end of the exposure, the rats inhaled radiolabeled tracer particles nose-only for 30 min. A clearance measurement protocol was then followed to estimate the rates of early and late clearance. Early (presumably upper respiratory tract) clearance was monitored during the 48 h following tracer deposition by the analysis of radioactivity excreted in the feces, while late (presumably deep lung) clearance was characterized by a combination of chest counting and 30 day postexposure sacrifice radioactivity analyses. The exposure to O_3, both alone and in combination with HNO_3, produced a more than 30% (statistically significant) delay during the initial phase of upper respiratory tract clearance, and all of the pollutant exposures may have stimulated deep lung clearance. The directions of these effects were the same as those observed previously when rats were acutely exposed to O_3-containing atmospheres.

Historically, the concentrations of various air pollutants in the South Coast Air Basin of southern California have often exceeded state and federal ambient air quality standards. For example, data from Azusa, CA, summarized by the South Coast Air Quality Management District, indicated that in 1986 the state standard of 50 $\mu g/m^3$ for PM-10 (airborne particulate mass with aerodynamic diameters less than 10 μm) was exceeded in about 80% of the 24-h samples, and the state ozone (O_3) standard of 0.09 ppm was exceeded on 45% of the days. Nitrogen dioxide (NO_2) peak 1-h concentrations were on the

order of 0.2 ppm, and maximum 24-h average nitric acid (HNO_3) concentrations were about 20 µg/m^3 in a nearby community. Russel et al. (1985) reported that HNO_3 and peroxyacetylnitrate (PAN) are the major end products of nitrogen oxide (NO_x) emissions over a 24-h period. During daylight, HNO_3 formation is driven by the chain of photochemical reactions that forms O_3; hence, the HNO_3 concentration patterns during the daylight hours are similar to those of O_3. At night, however, HNO_3 can continue to form via the reaction of O_3 with residual NO_2 (Russel et al., 1985).

Clinical studies have not convincingly demonstrated significant differences between the effects of exposures to O_3-alone and exposures to mixtures of O_3 and acid sulfate aerosols (Kleinman et al., 1981; Kulle et al., 1982; Stacy et al., 1983; Horvath et al., 1987; Aris et al., 1990, 1991). These clinical exposures were of short duration (about 2 h), and effects were assessed immediately postexposure. Some effects may have been noted if subjects were exposed for longer durations, or if responses were followed for longer periods. In contrast, the results of some laboratory animal studies and epidemiological field studies have suggested that inhaled acids can amplify the effects of O_3 (Last et al., 1986; Amdur, 1989; Lippmann, 1989). However, other toxicological studies have demonstrated antagonistic effects of acids on the effects of O_3 (Nadziejko et al., 1992).

The measurement of inhaled particle clearance efficiency is potentially useful in toxicological evaluations. Such measurements evaluate the ability of the respiratory tract to rid itself of insoluble radiolabeled tracer particles during and/or following exposures to test agents. This type of measurement is important because failure of this self-cleaning mechanism could contribute to a buildup of foreign matter in the respiratory tract. Therefore, particle clearance was measured in the present study as a means of evaluating the potential health effects of a lengthy (40 wk) series of exposures of rats to two air pollutants—an oxidant (O_3), and an acid (HNO_3)—both separately, and in combination. The study was novel for our laboratory in that (a) a chronic exposure regimen was used, in contrast to earlier subchronic or acute exposure scenarios, and (b) a simplified clearance measurement protocol was followed.

METHODS

Animals

The animals used were specific-pathogen-free Fischer 344 rats (Simonsen Laboratories, Inc., Gilroy, CA). Males weighing 200–300 g were shipped in filter-equipped boxes to reduce the exposure to particulate air pollutants during transit. The rats were housed in a gas/

vapor-scrubbing and particle-filtering laminar air-barrier caging system, in wire-bottom stainless steel cages (over dust-free sodium chloride litter). Animals were housed for about 1 wk prior to, and between, exposures. Serological assays, by Simonsen, indicated freedom from common respiratory-tract pathogens at the time of shipping. Freedom from lung disease was confirmed by quality-control histopathologic examinations at our laboratory. In addition, sentinel rats maintained in our vivarium with the exposed rats were assayed serologically and histopathologically (on an alternating monthly basis) during the 40-wk exposure to monitor for antigens to pathogens and respiratory diseases; none were observed. All personnel handling the rats wore disposable plastic gloves, clean laboratory coats, shoe covers, particle-filtering masks, and hair covers to minimize entry of pathogens into the exposure room and vivarium (which together comprised a barrier facility). Additionally, the bottoms of the shoe covers of personnel entering the barrier facility were cleaned by having lab personnel step on a tacky mat, followed by a mat (in a shallow tray) soaked with disinfectant. Drying the bottoms of the shoe covers on a carpet completed the process required for entry to the exposure/housing area.

Pollutant Generation, Characterization, and Exposure

Rats were exposed nose-only in plastic tubes (with aluminum nose cones) that were plugged into the walls of 1-m^3 University of Rochester-type hexagonal cross section stainless steel chambers. This exposure system has been previously described (Prasad et al., 1988). The system was designed to exclude rat-generated dusts from the exposure atmospheres (and the laboratory room) and to prevent the neutralization of HNO_3 atmospheres by ammonia (generated by bacterial action associated with the rats' excreta). The chambers were supplied with chemically purified particle-free throughput air at a controlled temperature of about 22°C and a controlled relative humidity of about 60%. Ozone was generated by passing filtered medical-grade oxygen through two electrical ozone generators (Sander Ozonizer, Type III; Osterberg, Germany) connected in parallel to dampen O_3 fluctuations. The O_3 was then injected through fluorocarbon tubing into an inlet port in a stainless steel spool piece at the top of the exposure chamber for dilution before distribution to ports that supplied each of the rats. O_3 was separately generated in this manner for the O_3-alone chamber and the O_3 + HNO_3 chamber. The O_3 concentration was monitored continuously at the breathing zone of the rats using fluorocarbon sampling tubing and a calibrated ultraviolet absorption monitor (model 1003-AH; Dasibi Environmental Corp., Glendale, CA). Nitric acid vapor was generated by passing purified dry air over an aqueous solution of HNO_3 (6 N), which was thermostatically maintained at 30°C in a glass vessel. The vapor was diluted with purified,

humidified air and equilibrated to 60% relative humidity. The nitric acid was metered into the chambers to achieve the target concentration of 50 µg/m^3. All materials used in the HNO$_3$ generation, delivery, and exposure systems were preconditioned to HNO$_3$ and were made of glass, fluorocarbon plastic, or stainless steel. Nitric acid vapor was collected from the rats' breathing zone onto nylon filters (Nylasorb; Gelman Sciences Inc., Ann Arbor, MI) downstream from fluorocarbon-coated quartz fiber filters (Pallflex TA1220; Pallflex Products Corp., Putnam, CT). Particulate nitrates (and other particles) were collected on the quartz filters, but the HNO$_3$ vapor passed through and was collected on nylon filters. Filters were extracted in a dilute buffer solution (0.03 M NaHCO$_3$ + 0.024 M NaCO$_3$; pH = 10.2) and analyzed for nitrate by ion chromatography. Two 2-h samples were collected during each 4-h exposure period and were averaged to obtain a daily concentration.

Separate groups of 30 rats were exposed concurrently for 4 h/day, 3 days/wk for 40 wk to the following target atmospheres: purified air; 0.15 ppm O$_3$; 50 µg/m^3 HNO$_3$; 0.15 ppm O$_3$ + 50 µg/m^3 HNO$_3$. The measured pollutant concentrations (mean ± SD), based on weekly averages, were as follows: O$_3$ alone, 0.151 + 0.002 ppm; HNO$_3$ alone, 51.1 ± 3.9 µg/m^3; O$_3$ + HNO$_3$, 0.152 ± 0.002 ppm + 49.9 ± 3.6 µg/m^3. The concentrations of particulate-phase nitrate were negligible in comparison to the vapor-phase concentrations. A weekly exposure regimen of 4 h/day, 3 days/wk was selected for the study as a surrogate for an urban air pollution episode.

Tracer Microsphere Preparation and Deposition

The tracer microspheres were labeled at this laboratory with tightly bound ^{51}Cr, which had an in vivo leaching rate of less than 0.1%/day (Hinrichs et al., 1978). The starting particles were commercially available 1.1-µm-diameter monodisperse polystyrene latex microspheres (Duke Scientific Corp., Palo Alto, CA). Aerosols were generated from an aqueous suspension of the particles using a Lovelace-type compressed air nebulizer (Raabe, 1972) (In-Tox Products, Albuquerque, NM). The aerosolized particles were dried by heating and dilution with filtered air and electrically discharged using an [85]Kr discharger before entering the nose-only exposure chamber (Raabe et al., 1973). The labeled aerosol, sampled from the breathing zone of the rats using a calibrated Mercer-type seven-stage cascade impactor (Mercer et al., 1970) (In-Tox Products, Albuquerque, NM), had an activity median aerodynamic diameter of 1.8 µm and a geometric standard deviation (GSD) of less than 1.3. After 36 wk of pollutant or purified air (control) exposure, 12 rats at a time were exposed simultaneously to the radioactive aerosol in this system for 30 min. The rats were exposed to the tracer aerosol in plastic nose-only tubes with aluminum nose

cones (similar to the pollutant exposure tubes). The average deposition of ^{51}Cr was about 1 µCi/rat (about 4 × 10^4 Bq/rat), which was sufficient for the subsequent measurements.

Particle Clearance Measurements

Prior to this study a manpower-intensive rat clearance protocol had been followed (Phalen et al., 1975, 1980; Kenoyer et al., 1981; Mannix et al., 1982, 1983; Prasad et al., 1990). An examination of data from these prior studies indicated that nearly the same degree of information concerning the effects of pollutant atmospheres on clearance could be obtained using a simpler protocol. This new protocol (Table 1) is described next.

After the deposition of tracer particles, the rats' noses were washed with hypoallergenic "baby wipes" to reduce the externally deposited radioactivity. The rats were then placed in plastic counting tubes and positioned above a lead-shielded 3-in × 3-in cylindrical NaI(Tl) gamma-ray detector. The lead shielding was set up to favor radioactivity present in the thoracic region; however, other regions, including the head and portions of the gastrointestinal system, could also contribute to the counts. Each rat was counted with the lungs centered over the detector for 100 s to determine the initial total amount of radioactivity that was deposited in the respiratory tract. If a rat moved such that it was out of its proper position [with respect to the NaI(Tl) detector] for more than about 5 s during a count, then it was recounted. During the subsequent 2 days, 4 fecal collections from each rat were performed (at 8, 14, 24, and 48 h). The rate of early clearance was estimated by means of radioactivity excreted in feces because, due to the small size of a rat and because of the movement (limited as it was) of rats during counting, it would have been difficult to collimate so that gastrointestinal tract radioactivity would not interfere with counts of the respiratory tract. Fecal collections were terminated at 48 h because beyond this time, levels of fecal radioactivity were negligible (in numerous previous studies, less than 1% of the total fecal radioactivity was excreted after 48 h). One additional external count was performed for each rat at 48 h postdeposition. The data from this

TABLE 1. Modified clearance measurement protocol

Time postdeposition of labeled particles	Event	Purpose
−0.5 h to 0 h	Microsphere deposition	Introduce tracer
0 h and 48 h	Chest counts	Estimate particle deposition pattern
8, 14, 24, and 48 h	Fecal collections	Measure early clearance rate
30 days	Lung, trachea and larynx well count	Measure late clearance rate (normalized to 48-h chest count)

count (presuming no significant radioactivity in the gastrointestinal tract), in conjunction with the data obtained from the initial chest count, provided an estimate of the fraction of the initially deposited radioactivity that remained at the end of the early clearance phase. This approximates the fraction of radioactivity which initially deposited in the deep lung. The effect of exposure to the pollutant atmospheres on this fraction examines the possibility that the exposures may have affected the pattern of particle deposition.

Pollutant and purified air exposures of the radiolabeled rats were continued for 4 wk. The rats were then sacrificed and the residual radioactivity in their excised left lungs (with the larynx and trachea attached) was counted in a 3-in × 3-in cylindrical NaI(Tl) well counter; the rate of deep lung clearance was estimated using these data. The right lungs were used for other biological endpoints by other investigators. The results of our previous studies in which both the right and left lungs were counted had indicated that while a greater quantity of radioactivity deposited in the right lung (also reported by Raabe et al., 1977), the ratio of left lung radioactivity to right lung radioactivity was relatively constant, and not altered as a function of the exposure atmosphere. Some of these studies involved acute or subchronic exposures to O_3 and HNO_3. The larynx and trachea were included in the measurement since other investigators have reported long-term clearance components for these structures (Patrick & Stirling, 1977; Patrick, 1979). The net left lung activity for each rat was normalized by dividing by the net rat count result obtained for the same rat at 48 h postdeposition (when only deep lung radioactivity remained). The resulting value constituted an index of late clearance, termed the A_{30} (for activity remaining at 30 days postdeposition). The A_{30} is an index that characterizes deep lung clearance, since (a) based on measurements in our other studies, a negligible fraction of the total net counts observed was due to radioactivity remaining in the trachea and larynx, and (b) by 48 h postdeposition essentially all radioactivity deposited in the bronchial airways can be assumed to have been translocated from the lung region to the gastrointestinal tract and excreted.

It is apparent from the preceding description that early clearance was assayed after 36 wk of exposure to the test atmospheres, while the principal measure of late clearance was obtained after 40 wk of exposure. This procedure was necessary since the right lungs of the rats were needed for other endpoints soon after the 40 wk of exposure. Therefore, in order to allow 30 days postdeposition of tracer particles for late clearance to occur, it was necessary to perform the early clearance measurements after only 36 wk of exposure to the purified air and pollutant atmospheres.

The data from individual rats were grouped by exposure atmos-

phere. The exposure groups formed a balanced 2 × 2 design, and the results were analyzed using a two-way analysis of variance (ANOVA) for the effects of O_3, HNO_3, and the interaction of O_3 and HNO_3 on particle clearance. Years ago a decision was made to establish a two-tailed .1 significance level for clearance studies. This decision was based on computations performed to ensure that the probability of a type 2 error (the error in accepting a false null hypothesis) is maintained at a tolerable level, and so that the study could be conducted with a manageable number of animals. The results of our prior clearance studies that have been published (Phalen et al., 1980; Kenoyer et al., 1981; Frager et al., 1979; Mannix et al., 1982, 1983, 1991; Prasad et al., 1990) have been tested based on the $p < .1$ criterion.

RESULTS

There was no difference between the control group and the various exposure groups in the deposition fraction of tracer microspheres. The total respiratory tract deposition fraction (0.29 ± 0.03 SE) was consistent with the in vivo studies of Raabe et al. (1988) and the theoretical predictions for nasopharyngeal, tracheobronchial, and pulmonary deposition of Schum and Yeh (1980) . The effects of the pollutant atmospheres on the clearance of tracer microspheres are presented in Tables 2 and 3. The results of the analysis of data from the rat counts performed immediately postdeposition and after 48 h, which were used to determine the effects of the exposures on the pattern of tracer particle deposition, indicate that exposure to the various pollutant atmospheres did not affect the ratio of the radioactivity deposited in the deep lung region to that initially deposited in the entire respiratory tract, when compared with the ratio for the purified air-exposed group. For each of the 4 groups of rats, this ratio was on the order of 0.3 (i.e., 30%). These results were tightly grouped (group standard errors of 0.01), providing us with confidence in the design of the rat chest counter setup and the precision of its measurements. A two-way analysis of variance of the early (fecal) clearance data demonstrated a significant main effect of O_3 in delaying (by more than 30%) early clearance in the groups exposed to O_3 alone and to O_3 + HNO_3

TABLE 2. Fraction (Fr) of the total fecal radioactivity (0–48 h) excreted prior to 14 h postdeposition

	Purified air	$O_3{}^a$	HNO_3	$O_3 + HNO_3{}^a$
Fr	0.19	0.12	0.16	0.11
n	30	30	30	30
SE	0.03	0.02	0.03	0.03

[a]Significant main effect of O_3 (two-way ANOVA; $F = 3.9$, $p < .1$).

TABLE 3. A_{30} analysis for late clearance

	Purified air	O_3	HNO_3	$O_3 + HNO_3$
A_{30}	2.39	2.19	2.30	2.32
n^a	28	28	27	29
SE	0.08	0.08	0.10	0.06

Note. A_{30} = net count of the radioactivity in the excised left lung, trachea and larynx obtained using the well counter at 30 days postdeposition, normalized by the net count of the radioactivity remaining in the rat at 48 h postdeposition. Group means were not significantly different (two-way ANOVA).

[a]Outlier rats with A_{30} values more than two standard deviations from the mean of their group data were not included in the analysis.

(Table 2). The 14-h fecal radioactivity excretion results, expressed as the fraction of the total fecal radioactivity that was excreted by 14-h postdeposition, were considered to be the best measure of early clearance since they were not as behaviorally influenced as the 8-h data (many rats had not defecated by 8 h postdeposition), and since it was the collection nearest to the time point in previous studies at which 50% of the fecal activity was excreted. By 24 h postdeposition, there were no significant exposure-related effects produced by the pollutants (data not shown). The results of the data analysis for the late clearance measurement (A_{30}) are shown in Table 3. Although neither O_3 nor HNO_3 was observed to affect the mean A_{30} values in a statistically significant manner, there was a trend toward an acceleration in late clearance for groups exposed to these pollutants. Outlier rats (one or two per group) with A_{30} values that were more than two standard deviations from the mean values for their group were excluded from this analysis.

DISCUSSION AND CONCLUSIONS

Particle clearance studies involving exposures to O_3, HNO_3, and $O_3 + HNO_3$ have previously been performed and have been described in the literature. Prasad et al. (1990) performed a study in which rats were exposed nose-only 5 h/day for 5 days to a mixture of 350 μg/m³ HNO_3 vapor and 150 μg/m³ H_2SO_4 aerosol. The mixture significantly depressed phagocytic activity immediately postexposure, and the effects persisted for at least 4 days. Schlesinger et al. (1994) exposed rabbits to various concentrations of HNO_3 vapor (ranging from 50 to 450 μg/m³) for 4 h/day, 3 days/wk for 4 wk, and observed no effect of exposure on phagocytic activity. Acute, 4-h exposures of rats to 0.8 ppm O_3 have been found to delay early respiratory tract clearance, and accelerate late clearance (Phalen et al., 1980; Kenoyer et al., 1981). However, Schlesinger et al. (1992b)

found that a single 3-h exposure of rabbits to O_3 in concentrations above 0.1 ppm significantly depressed phagocytic activity, which could slow deep lung clearance. An exposure of rabbits to 0.1 ppm O_3 for 2 h/day, 5 days/wk for 1 yr indicated that while no changes from control values in tracheobronchial mucociliary clearance were observed during the exposure, tracheobronchial clearance times became progressively slower following the exposure (Schlesinger et al., 1992a). Nadziejko et al. (1992) exposed rats to 0.6 ppm O_3 + 1000 $\mu g/m^3$ HNO_3 vapor for 4 h, and other rats to 0.15 ppm O_3 + 250 $\mu g/m^3$ HNO_3 vapor for 4 h/day for 4 days. The results indicated that, in general, antagonistic interactions occurred for several macrophage function endpoints. Schlesinger et al. (1994) exposed rabbits to 50 $\mu g/m^3$ HNO_3 vapor + 0.15 ppm O_3 for 4 h/day, 3 days/wk for 4 wk. No effect of exposure on macrophage phagocytic activity was observed in that study.

The studies just described provide information concerning the effects of various durations of exposures to O_3, HNO_3, and O_3 + HNO_3 on respiratory tract defenses. The principal upper respiratory tract particle clearance mechanism (mucociliary clearance) and deep lung defense mechanisms (macrophage actions) have been found to be susceptible to O_3 exposure. Both O_3 and HNO_3 are known to deposit throughout the respiratory tract. Schlesinger et al. (1994) indicated that HNO_3 vapor deposits in substantial amounts in the conducting airways, but that it also reaches the respiratory airways. The distal airways and alveolar epithelial cells have been described as principal targets of oxidant pollutants (including O_3) (Warheit, 1989). But effects of acute exposure to O_3 on mucociliary (upper respiratory tract) clearance have also been observed (Frager et al., 1979; Phalen et al., 1980; Kenoyer et al., 1981), and morphological examinations have identified ciliated cells in the large airways as targets of O_3 damage (Schwartz et al., 1974; Frager et al., 1979).

In this study, the concentrations of O_3 and HNO_3 were on the order of environmentally realistic levels; thus, the findings have relevance to community exposures. The results indicate that the chronic exposure of rats to O_3 and to an O_3 + HNO_3 mixture produced small effects on respiratory tract clearance rates. The early clearance analysis demonstrated that O_3 significantly delayed particle clearance. For late clearance, there was a trend toward an acceleration for O_3-exposed rats, and perhaps for HNO_3-exposed rats as well. However, O_3 appeared to be the principal agent in eliciting this response. These results are consistent with results obtained in studies involving exposures of healthy human volunteers to HNO_3 and O_3, alone and in combination (Aris et al., 1993). Also, the directions of the O_3 effects (delay for upper respiratory tract clearance, trend toward an acceleration for the deep lung) were identical to those observed in much

shorter term (generally, 4 h acute) exposure studies involving higher concentrations of O_3 (on the order of 0.8 ppm), which suggests either a residual or small cumulative effect produced by exposure to O_3. Integration of the results of our study with the results of similar studies that are found in the literature also leads to the observation that O_3 is usually the more biologically active pollutant in such mixtures. However, our endpoints were specifically developed to study the effects of O_3, and they were later used to study mixtures. The copresence of HNO_3 (or other acids) in a mixture atmosphere with O_3 apparently may produce either greater than additive, additive, or antagonistic effects, depending upon other factors, including the region of the respiratory tract under consideration, the specific biological endpoint, the concentrations of the pollutants, and the duration of exposure.

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