TOXICOLOGY AND APPLIED PHARMACOLOGY 116, 1-9 (1992)

Synergistic Interaction of Nitrogen Dioxide and Ozone on Rat Lungs: Acute Responses

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Received January 30, 1992; accepted April 7, 1992

Synergistic Interaction of Nitrogen Dioxide and Ozone on Rat Lungs: Acute Responses. GELZLEICHTER, T. R., WITSCHI, H., AND LAST, J. A. (1992). *Toxicol. Appl. Pharmacol.* 116, 1-9.

Rats were exposed for 6 hr per day to either ozone alone (0.2-0.8 ppm), nitrogen dioxide (NO_2) alone (3.6-14.4 ppm), or to combinations of these two oxidant air pollutants. Their response was quantified by changes in the total protein content of lung lavage supernatants or by changes in the content of specific cell types in the lung lavage pellets. A concentration-dependent synergistic response was observed when rats were exposed to the combination of ozone and NO_2. Apparent threshold concentrations for the observation of synergistic interaction between ozone and NO_2 were assay specific, with epithelial cell content of lung lavage fluid being the most sensitive parameter evaluated, showing positive interaction (greater than additive response) at the lowest concentrations tested. Concurrent exposure to ozone and NO_2 was necessary to elicit greater than additive responses; no such interactions were seen upon sequential exposure to ozone or NO_2 in either order of presentation. Based upon apparent disappearance rates of ozone in the chambers during exposure of rats to ozone and NO_2, we modelled the predicted outcomes based upon the assumption that the two oxidant gases were reacting to form nitrogen pentoxide (N_2O_5) in the chambers. Agreement between predicted concentrations of ozone and NO_2 and those actually observed was excellent. Based upon such modelling estimates and our acute toxicological data, we conclude that synergistic toxicologic interactions between ozone and NO_2 are found only at concentrations very much higher than would be encountered in environmental or occupational settings. It remains to be determined whether there are any chronic toxicological responses to exposure to combinations of ozone and NO_2 at concentrations below the thresholds for observing acute responses.

Among the most important threats to human and animal health in photochemical smogs are the strong oxidant gases ozone and nitrogen dioxide. These arise as ambient air pollutants mainly as a consequence of oxidation of atmospheric nitrogen to nitrogen oxides (NO_x) during high temperature combustion of fossil fuels, such as occurs in automobile engines, in industrial furnaces, and in electricity generation from coal, oil, or natural gas. Concentrations of O_3 and of NO_2 attained in photochemical smogs are interdependent; NO_2 is a precursor of most of the O_3 formed, via a complex series of free radical reactions occurring in the polluted atmosphere (Committee on Medical and Biologic Effects of Environmental Pollutants, 1977a).

The toxicologic actions of O_3 and NO_2 have been attributed to their high oxidative capacity, and their effects on health have been extensively studied (Committee on Medical and Biologic Effects of Environmental Pollutants, 1977a, b; Menzel, 1984; Morrow, 1984). However, relatively little is known concerning whether there are significant toxicologic interactions between these two gaseous oxidants at environmentally relevant concentrations. Interactions of mixtures of toxicants can take several forms: additive, less than additive ("antagonism"), or more than additive ("synergistic") toxicity. In the ambient air, we breathe mixtures of pollutants; therefore, potential interactions between inhaled toxicants should be an area of concern for setting of ambient air quality standards by regulatory agencies. However, the experimental data to predict quantitatively (or often even qualitatively) toxicant interactions of potential regulatory significance are generally lacking. Thus, ambient air quality standards for some of our most common and prevalent pollutants, for example ozone, carbon monoxide, and nitrogen dioxide, are based upon estimated no-observable-effect levels for the pure pollutant, with no allowance for potential interactions with other toxicants inhaled concurrently. For the important oxidant air pollutants of photochemical smog, ozone, and nitrogen dioxide, we have recently observed an apparent synergistic interaction between these two agents when rats were simultaneously exposed to relatively high concentrations of each gas (Gelzleichter *et al.*, 1992). Our previous results identified a synergistic interaction, as defined by several assays of lung lavage fluid and cells, occurring between ozone and NO_2 during concurrent exposures to these oxidant gases at a fixed amount and ratio of concentration $\times$ time (C $\times$ T) product. The present study was designed to explore the mechanistic basis for this interaction to better understand the potential significance of this binary interaction for the proper risk assessment of these agents at the concentrations occurring in ambient air.

0041-008X/92 $5.00

METHODS

Ten- to 12-week-old male, chronic respiratory disease free, Sprague–Dawley rats (Banton Kingman, Gilroy, CA or Charles River, Portage, MI) were used throughout these experiments. All rats were housed in 4.2-m^3 glass and stainless-steel exposure chambers with flows adjusted to 30 changes of atmosphere per hour (except where otherwise noted in text). Rats were housed in wire mesh cages with food (Purina Rat Chow, Ralston–Purina, St. Louis, MO) and water freely available. Rats were kept on a 12-hr light (7:00 AM–7:00 PM) and 12-hr dark (7:00 PM–7:00 AM) cycle, with 6-hr exposure periods beginning at 1:00 AM and ending at 7:00 AM (except where otherwise noted). Ozone and NO_2 were generated, delivered, and monitored as previously reported (Gelzleichter *et al.*, 1992). NO_2 was generated by passing N_2 through a tank of the pure liquid dimer, nitrogen tetroxide (N_2O_4), and delivered via stainless-steel lines to the exposure chamber mixing inlets. NO_2 concentrations were monitored with a chemiluminescent oxides of nitrogen monitor (Dasibi Corp., Glendale, CA, Model 2108) calibrated against a Dasibi gas calibrator Model 1005-CE-2. Ozone was generated by passing medical-grade oxygen through a silent arc discharge ozonizer (Erwin Sander Corp., Giessen, Germany) and delivered to the exposure chamber mixing inlets via Teflon lines. Ozone concentrations were determined by uv photometric ozone monitors (Dasibi Corp., Model 1003-AH) calibrated against a standard reference photometer (Serial No. 4) at the California Air Resources Board Quality Assurance Laboratory. Both the ozone and the NO_2 monitors quantified steady-state oxidant concentrations at cage level in the chambers. Actual ozone and NO_2 concentrations are given in the figure legends, while nominal concentrations are indicated in the tables, figures, and text otherwise.

Exposures. Rats were exposed for 3 consecutive days, 6 hr per day, to 0.8 ppm O_3 alone, to 14.4 ppm NO_2 alone, or to their combination. Combined exposures were either concurrent (both at night between 1:00 AM and 7:00 AM) or sequential (one of the gases during the day from 1:00 PM to 7:00 PM, and the other during the night from 1:00 AM to 7 AM). To determine if the synergistic toxicologic effect observed upon exposure to mixtures of ozone and NO_2 was concentration dependent, we exposed rats to various concentrations of ozone alone, of NO_2 alone, or of their mixtures. Rats were exposed for 6 hr per night (1:00 AM–7:00 AM) for 3 consecutive nights according to the following regimen: 0.2 ppm O_3 and/or 3.6 ppm NO_2; 0.4 ppm O_3 and/or 7.2 ppm NO_2; 0.6 ppm O_3 and/or 10.8 ppm NO_2; or 0.8 ppm O_3 and/or 14.4 ppm NO_2. Data from the highest exposure levels (0.8 ppm O_3 and/or 14.4 ppm NO_2) have been reported elsewhere (Gelzleichter *et al.*, 1992).

Lung lavage analysis. Analytical methods for lung lavage constituents have been described previously (Gelzleichter *et al.*, 1992). Briefly, after termination of rats with pentobarbital their lungs were lavaged with 6 × 5 ml of phosphate-buffered saline (PBS), pH 7.0. The lavagate was centrifuged (300*g* × 30 min) and the supernatant and cell pellet were separated. A portion of the supernatant was analyzed colorimetrically for protein content using the Pierce Micro-BCA protein analysis kit (Pierce, Rockford, IL). After the lavage pellet was resuspended in PBS, a portion of the suspension was smeared on a glass slide and stained with Wright-Leishman stain; a total of 400 cells were counted per slide and were scored as a percentage of the total cells enumerated for (a) epithelial cells, (b) PMNs, (c) monocytes/macrophages, and (d) other cells. Cell identities were determined based on the following characteristics: (1) Macrophage/monocyte; nucleus stains dark purple, is highly textured and convoluted, and is usually lobular in shape. The cytoplasm appears foamy and often contains vacuoles. (2) Epithelial cell; nucleus stains purple, but has a smoother texture than do monocytes or macrophages. The cytoplasm has a reddish tinge and cilia can often be identified on the cell surface. (3) PMNs; have a distinct, multilobed nucleus, which stains very dark, while the cytoplasm stains very lightly. Total cell number was determined by quantifying the DNA content of the lavage pellet. An aliquot of the lavage pellet was lysed with 10% Trition X-100 (Sigma, St. Louis, MO) and DNA was fluorimetrically detected with ethidium bromide as the chromophore (Gelzleichter *et al.*, 1992). Standard curves were constructed using known amounts of salmon testes DNA (Sigma).

Statistical analysis. For analysis of protein content of lung lavage fluid, data were expressed as a percentage of the matched controls. As there were small variations from different batches of animals, control values from different experiments were not pooled, but each data set was compared only with its matched control group. As the numbers of epithelial cells and polymorphonuclear leukocytes found in lung lavagate of control rats were relatively small, the resultant data are expressed as increased number of cells above control values.

Nonadditive interactions were defined as a toxicologic response other than effect additive (Pöch, 1981), where predicted response due to a combination of agents is equal to the sum of responses observed when both agents are administered separately. A two-way factorial analysis of variance (two-way ANOVA) was conducted individually at each concentration level to detect toxicologic interactions. Toxicologic interactions were considered to be synergistic when (1) the response to combined exposures exceeded the sum of responses when each agent was given individually, and (2) two-way ANOVA indicated a toxicologic interaction at $p < 0.05$. Likewise, interactions were considered antagonistic when combined exposures resulted in a less than additive response and two-way ANOVA indicated a toxicologic interaction at $p < 0.05$. Data were analyzed for significance using the Superanova program on an Apple MacIntosh computer. The type III squares subprogram, which is essentially a χ^2 test with Yates' correction, was used throughout. In this manner data were assigned to one of four groups: ozone alone, NO_2 alone, both together, or neither. To examine whether observed greater than additive effects were concentration dependent, a three-factor ANOVA was set up assigning a third variable for relative concentration of ozone or NO_2. Concentration values of 1, 2, 3, and 4 were assigned to groups receiving 0.2, 0.4, 0.6, or 0.8 ppm of ozone, respectively, and 3.6, 7.2, 10.8, or 14.4 ppm of NO_2, respectively. Combined ozone plus NO_2 groups were assigned concentration values of 1, 2, 3, and 4. Each concentration level was then assigned to its appropriate matched controls to complete the 2 × 2 × 4 design. For all statistical analyses values of $p < 0.05$ were taken to indicate a significant difference.

RESULTS

Sequential vs Concurrent Exposures

The first, and simplest, mechanism that might explain the synergistic interaction between ozone and NO_2 would be a direct chemical reaction between the two substances to produce a more toxic species. We initially approached this issue by determining whether rats exposed sequentially to ozone and NO_2 responded in the same way as rats exposed concurrently to the two gases.

Results of assays for the total amount of lavageable protein are given in Fig. 1. It is evident that for sequential exposures, the increase in lavageable protein is approximately equal to the additive sum of the increases due to ozone alone plus NO_2 alone. This observation holds true for both the ozone night/NO_2 day group and the ozone day/NO_2 night group. However, when exposures to ozone and NO_2 were concurrent there was a dramatic increase in lavageable protein that was significantly greater than additive ($p < 0.001$). Likewise, as shown in Fig. 2, lavageable epithelial cells showed responses that were additive for the O_3 night/NO_2 day group, and antagonistic for the NO_2 night/O_3 day group ($p < 0.05$), but were greater than additive when the two gases were given concurrently ($p < 0.001$). Lavageable PMNs, as well, showed responses that were additive when ozone and NO_2 were given sequentially, but were greater than additive when both were

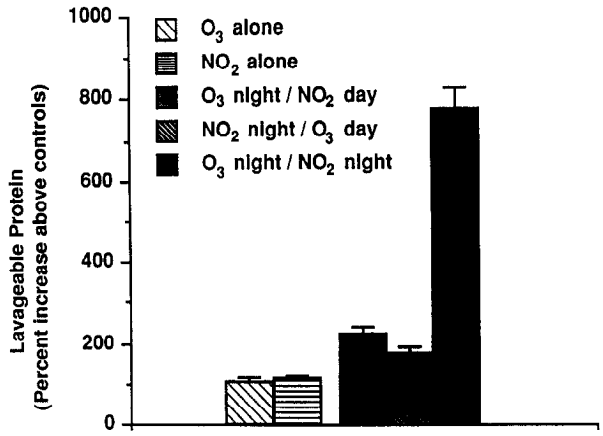

FIG. 1. Protein content of bronchoalveolar lavage fluid. Bars represent mean values ±1 SE. Rats were exposed for 6 hr per day for a total of 3 days to either 0.8 ppm of ozone, 14.4 ppm of NO_2, or their combination. Combined exposures were either intermittent (O_3 exposure 1:00 AM–7:00 AM plus NO_2 exposure 1:00 PM–7:00 PM, and vice versa) or concurrent (both exposures 1:00 AM–7:00 AM). Groups receiving ozone alone or NO_2 alone were exposed from 1:00 AM to 7:00 AM. Protein content for control rats exposed to filtered air was 1.31–2.97 mg protein. Actual chamber concentrations were as follows: data are expressed as mean concentrations (ppm) ±1 SD with *n*, the number of determinations in parentheses. O_3 day, NO_2 night: 0.78 ± 0.02 (255), 14.41 ± 0.21 (261). O_3 night, NO_2 day: 0.80 ± 0.02 (261), 14.31 ± 0.68 (262). O_3 and NO_2 together, night: 0.82 ± 0.02 (257), 13.76 ± 0.73 (257). O_3 alone: 0.80 ± 0.02 (879). NO_2 alone: 13.34 ± 1.05 (261).

given concurrently ($p < 0.001$). Therefore, synergistic responses were only observed when exposures to ozone and NO_2 were concurrent.

Concentration Dependence

Results of assays to quantify the total lung content of lavageable protein are shown in Fig. 3. A synergistic response, as determined by two-way analysis of variance, was observed at the highest concentrations of ozone plus NO_2 tested, 0.8 and 14.4 ppm, respectively. By three-way analysis of variance, this synergy between O_3 and NO_2 was found to be highly concentration dependent ($p < 0.001$).

We also observed positive results with regard to concentration dependence of synergy for assays of lavageable epithelial cells and PMNs (Fig. 4), where again the degree of synergistic interaction was highly concentration dependent ($p < 0.001$ and $p < 0.001$ for epithelial cells and PMNs, respectively). Significant synergistic interaction was again observed at the highest concentrations of ozone and NO_2 tested, as well as at the intermediate concentrations, as evaluated by these assays.

Chemical Reactivity

Since it has been previously hypothesized that O_3 and NO_2 interact toxicologically due to the formation of N_2O_5 we directly examined whether a chemical reaction might be occurring between ozone and NO_2 in the exposure chambers by determining whether we could demonstrate depletion of ozone in the chambers when NO_2 flow was switched on, and vice versa. Figure 5 demonstrates the consumption of ozone as a function of the ozone and the NO_2 concentrations actually observed in our exposure chambers. These results indicate that there is substantial reaction occurring, and that the rate of reaction is in excellent agreement with the theoretical values calculated based upon pseudo-first-order kinetics (see Discussion). When the literature value for the rate constant of 0.0647 $ppm^{-1} \times min^{-1}$ is used, the equation yields calculated ozone reaction rates that are 17% higher than those actually observed. Recalculation of the apparent rate constant to fit our observed results yields a rate constant of 0.0555 $ppm^{-1} \times min^{-1}$. In Fig. 5, the points denote experimental data, while the lines denote the theoretical consumption as predicted by the rate equation for the reaction of NO_2 and ozone with NO_2 present in large excess with a rate constant value of 0.0555 $ppm^{-1} \times min^{-1}$. At the lowest set of nominal chamber concentrations (0.2 ppm of O_3 + 3.6 ppm of NO_2), the O_3 chamber concentration re-equilibrated to 0.29 ppm if ozone delivery was continued after NO_2 delivery ceased. This represents an apparent 30% loss of the O_3 in the chambers containing 3.6 ppm of NO_2. At

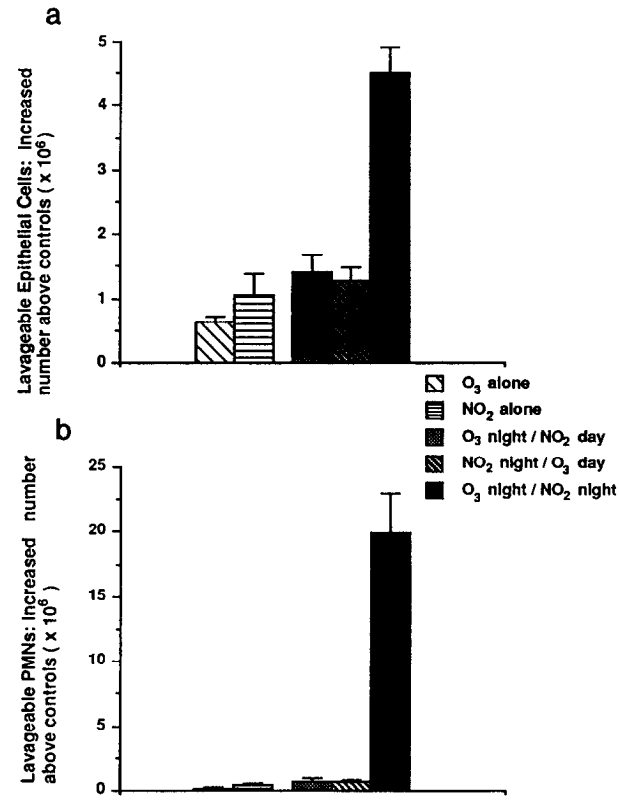

FIG. 2. Lavageable epithelial cells and polymorphonuclear leukocytes (PMNs). Values indicate the number of lavaged cells above control mean values ±1 SE. The average number of cells found in control rats exposed to filtered air were 0.595 ± 0.092 ($\times 10^6$) for epithelial cells and 0.002 ± 0.001 ($\times 10^6$) for polymorphonuclear leukocytes, respectively. The exposure regimen is described in the legend to Fig. 1.

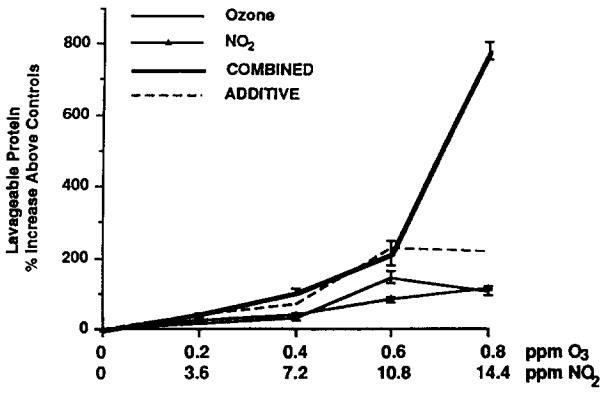

FIG. 3. Protein content of bronchoalveolar lavage fluid ±1 SE. Rats were exposed for 6 hr per day for 3 days to nominal concentrations of 0.2 ppm of O_3 and/or 3.6 ppm of NO_2, 0.4 ppm of O_3 and/or 7.2 ppm of NO_2, 0.6 ppm of O_3 and/or 10.8 ppm of NO_2, or 0.8 ppm of ozone and/or 14.4 ppm of NO_2. Actual concentrations of ozone and NO_2, respectively, were 0.2, 3.6: 0.22 ± 0.01 (727), 3.01 ± 0.12 (921); 0.4, 7.2: 0.41 ± 0.02 (960), 6.80 ± 0.89 (962); 0.6, 10.8: 0.62 ± 0.02 (632), 10.20 ± 1.34 (635); 0.8, 14.4: 0.82 ± 0.02 (257), 13.76 ± 0.73 (257). Actual concentrations of ozone alone were 0.2: 0.21 ± 0.01 (3980); 0.4: 0.41 ± 0.00 (923); 0.6: 0.60 ± 0.02 (676); 0.8: 0.80 ± 0.02 (879), while actual concentrations of NO_2 alone were 3.6: 4.82 ± 0.17 (1023); 7.2: 6.96 ± 0.48 (1029); 10.8: 10.35 ± 1.05 (705); 14.4: 13.34 ± 1.05 (261). The dotted line represents the calculated summation of the response to ozone alone plus the response to NO_2 alone.

the highest set of chamber concentrations (0.8 ppm of O_3 + 14.4 ppm of NO_2) the apparent amount of O_3 that reacted with NO_2 in the chambers was increased dramatically. The ozone concentration re-equilibrated to 2.09 ppm after NO_2 delivery ceased, indicating a 62% loss of the initial O_3. For the lowest concentrations the ratio of steady-state O_3 to reacted O_3 was 2:1; for the highest concentrations the ratio was 0.5:1. This represents a fourfold increase in the relative amounts of reactants from the lowest to the highest concentrations. It is evident from Fig. 5 that under conditions where significant toxicologic interactions occur we observe measurable chemical reactions occurring as well.

Figure 6 shows the calculated and the experimentally determined extent of reaction between ozone and NO_2 under conditions where the concentration of NO_2 is held constant at 14.4 ppm. Under this regimen, the rate of reaction is directly proportional to the ozone concentration and the fraction of ozone consumed should be constant over the entire concentration range. The three lines as drawn are theoretical and based upon the model; their agreement with the actual data points is excellent.

Figures 7a, 7b, and 7c show the toxicity data for this exposure regimen. It is clear from these results that the synergistic effect is highly concentration dependent and that the concentration at which a given toxic response begins to deviate from the additive model is highly assay specific. Changes in epithelial cell number appear to be the most sensitive parameter investigated, deviating significantly from the additive model at the lowest concentration of ozone tested (0.2 ppm O_3 + 14.4 ppm NO_2). Results from assays of total lavageable protein on the other hand, do not significantly deviate from the additive model until the highest concentration of ozone tested (0.8 ppm O_3 + 14.4 ppm NO_2).

To further examine whether the chemical reactivity of NO_2 could be the basis for the apparent reactive consumption of ozone observed in Figs. 5 and 6, we devised an experiment to create conditions where the O_3 and NO_2 concentrations could be kept constant while the concentrations of reaction products could be varied. To accomplish this, chamber gas residence times were increased, thereby allowing any putative chemical reaction occurring in the chambers to proceed. Since the rate constant in Equation 2 is expressed in units of $ppm^{-1} \times min^{-1}$, then in theory doubling the chamber residence time should double the amount of reaction products formed. Figure 8 shows the theoretical and actual levels of ozone disappearance in these experiments. Doubling the residence time led to an 88% increase in ozone disappearance. Although this is slightly lower than the 100% increase predicted by Equation 2 this result is in good agreement with theory.

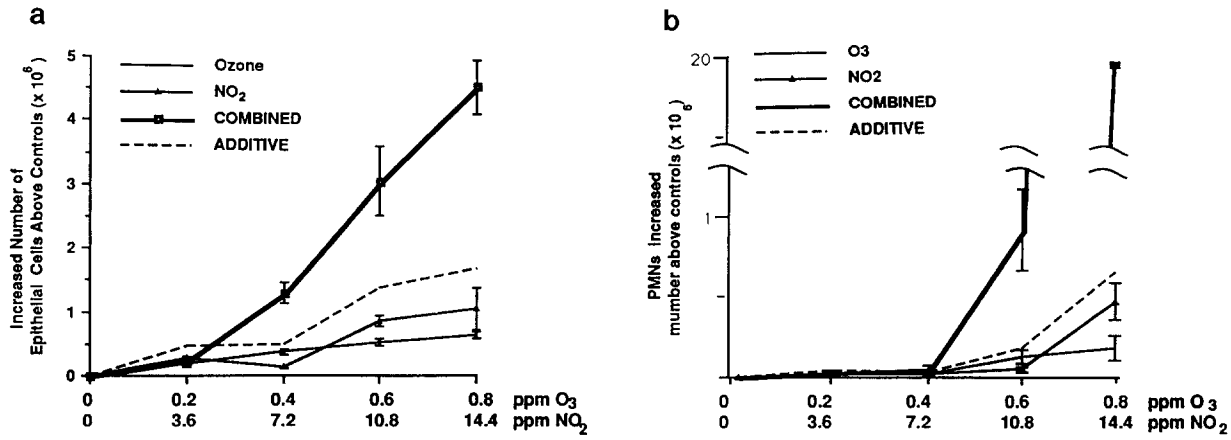

FIG. 4. (a) Lavageable epithelial cells and (b) polymorphonuclear leukocytes (PMNs). Values indicate numbers of lavaged cells above control mean values ±1 SE. Control values are as indicated in the legend to Fig. 2. The exposure regimen is indicated in Fig. 3.

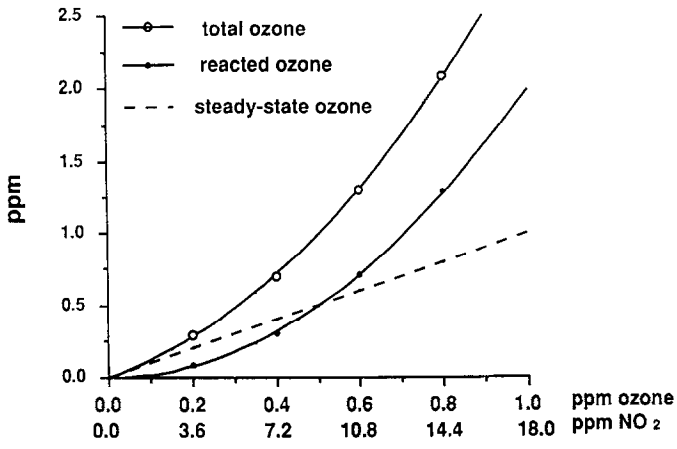

FIG. 5. Comparison of ozone concentrations as generated alone and in the exposure chambers after mixing with NO_2. Chamber ozone concentrations were analyzed under all of the exposure conditions discussed in the text, both in the presence and in the absence of NO_2. The dashed line labeled steady-state ozone is the line of identity obtained with ozone alone in the chambers when the concentrations of ozone indicated on the ordinate scale are added to the chambers. The open circles indicate the concentrations of ozone required at the generator to maintain the indicated concentrations of ozone in the chambers when NO_2 is simultaneously present in the chambers at the concentrations indicated on the ordinate scale. The curve fitted to these data points is calculated from the equation of $d[O_3]/dt = -k[O_3][NO_2]$, with $k = 0.0555$ ppm$^{-1} \times$ min^{-1} (see text). Finally, the solid circles are the calculated values of ozone reacted with NO_2 if we assume the difference between ozone concentrations with and without NO_2 present in the chambers to be equal to the reacted ozone. The curve fitted to these data points is calculated from the rate equation above, adjusted for the decreased concentrations of ozone observed experimentally.

DISCUSSION

Additive toxicologic interactions are often described as being dose additive (two or more agents target the same receptor with similar efficiencies, but not necessarily similar potencies) or effect additive (agents act on different targets to produce similar effects) (Pöch, 1981). Although the effects of ozone and NO_2 are both thought to be related to their oxidative properties, in a strict sense they clearly cannot share the same "mechanism of action." Whereas chronic ozone exposure leads to a diffuse fibrosis centered in the alveolar duct region (Schwartz *et al.*, 1976; Mellick *et al.*, 1977; Freeman *et al.*, 1974; Huang *et al.*, 1989; Chang *et al.*, 1991), chronic exposure to NO_2, on the other hand, produces a lesion that combines elements of fibrosis and emphysema in the lung parenchymal components (Freeman *et al.*, 1968, 1974). In addition, due to their different aqueous solubilities, lung deposition patterns for ozone differ from those for NO_2 (Morgan and Frank, 1977; Overton *et al.*, 1987), as does permeation through epithelial lining fluid (Postlethwait and Bidani, 1990; Postlethwait *et al.*, 1991). Therefore, specific molecular sites in the lung do not see the same "fractional dose" of these two oxidants. For these reasons we examined the data in this paper for effect additivity.

Diggle and Gage (1955) were probably the first to propose that mixtures of O_3 and NO_2 could produce synergistic, or greater than additive, toxicologic lung damage. To test this hypothesis they exposed rats for 1 hr to various concentrations of ozone (0–17.7 ppm) and NO_2 (0–27 ppm). They showed that a mixture of the two produced greater acute effects (dyspnea, edema, and death) than would be expected from the sum of the toxicities of either gas alone. Their hypothesis was that the increased toxicity might be due to the formation of nitrogen pentoxide (N_2O_5), which is formed by the reaction $2\ NO_2 + O_3 \rightarrow N_2O_5 + O_2$. In previous studies they had shown N_2O_5 to be a potent lung toxicant (Diggle and Gage, 1954). However, the methodology used to produce gaseous nitrogen pentoxide in these older experiments included dissolving the N_2O_5 in chloroform and then nebulizing the mixture; therefore, the ambient atmosphere used in their experiments contained significant amounts of not only N_2O_5 but also chloroform (150 ppm), and substantial amounts of NO_2 as well. However, they concluded that nitrogen pentoxide was the likely toxicant since the toxicity of either chloroform or NO_2 when given individually could not account for the observed effects.

Other workers have exposed laboratory animals or cells under controlled conditions to mixtures of ozone and NO_2. However, different studies are difficult to compare directly as concentrations of the gases, their relative ratios, and the biological endpoints evaluated have differed in various laboratories. Goldstein (1976) exposed human erythrocytes to ozone, NO_2, or their mixtures at very high concentrations and measured various outcomes. In general, effects were found to be additive for most of the parameters evaluated. However, lipid peroxides were increased at a greater than additive level for low O_3 and NO_2 concentrations, but at a

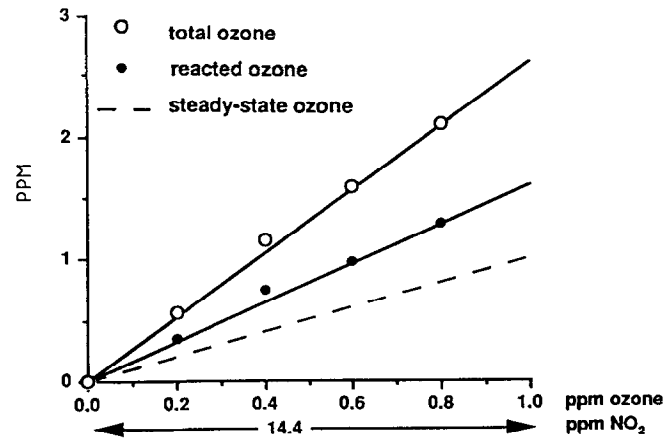

FIG. 6. Analysis of ozone concentrations as generated alone and in the exposure chambers after mixing with 14.4 ppm of NO_2. All experimental details are as in the legend to Fig. 5, except the NO_2 concentration was held constant at 14.4 ppm. The amount of ozone consumed (data points) was calculated as the difference between total ozone generated and ozone concentration as measured in the chambers. The solid lines are theoretical and based upon the rate equation given in the legend to Fig. 5.

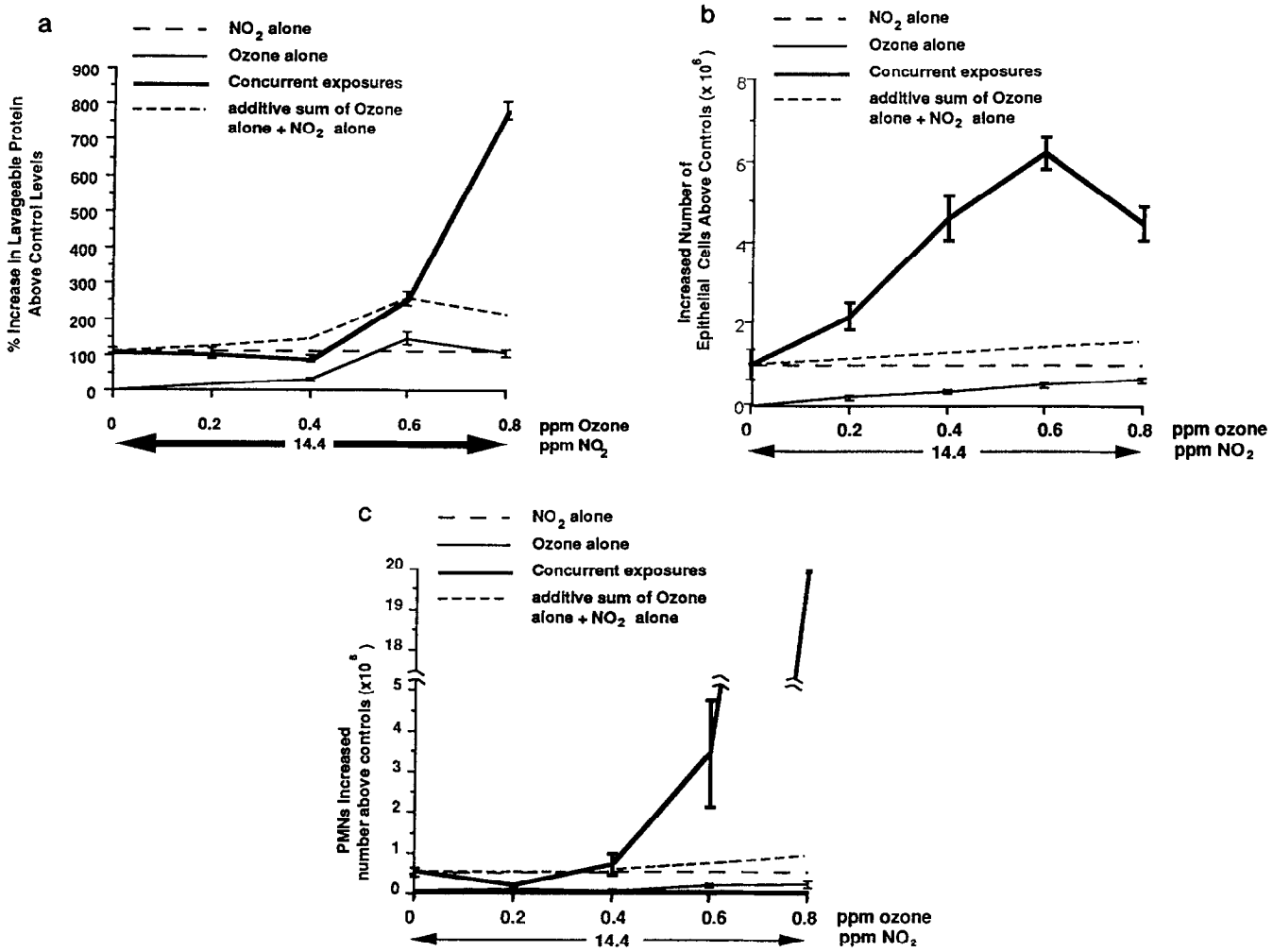

FIG. 7. (a) Percentage of increase in lavageable protein above control means, (b) increased number of epithelial cells, and of (c) polymorphonuclear leukocytes (PMNs), above control means. All data are expressed as mean values ±1 SE. Control mean values are as given in the legends to Figs. 1 and 2. Rats were exposed to 0.2, 0.4, 0.6, or 0.8 ppm of ozone in either the presence or the absence of 14.4 ppm of NO_2. The small, dashed lines give the calculated additive sum of the responses with O_3 or NO_2 alone, while the thick, solid lines give the actual responses observed with the mixture.

lesser than additive level at higher O_3 and NO_2 concentrations. Mustafa *et al.* (1984) exposed mice for 7 days to 4.8 ppm of NO_2, to 0.45 ppm of ozone, or to their combination and measured several parameters in their lungs. Greater than additive effects of the mixture were observed for some of the assays performed. Ichinose and Sagai (1989) exposed rats and guinea pigs to 0.4 ppm of NO_2, to 0.4 ppm of ozone, or to their combination for 2 weeks, and measured several lung constituents. Some of the assays indicated greater than additive effects for the mixture. Interestingly, synergistic responses were observed with both species, but by the criterion of different specific assays. Lee *et al.* (1990) have also observed synergistic responses of rats to mixtures of ozone and NO_2. Several groups of workers (Ehrlich *et al.*, 1977; Graham *et al.*, 1987) have reported either additive or greater than additive responses to mixtures of ozone and NO_2 when mortality of mice after exposure to aerosols of virulent bacteria was the criterion evaluated; synergy was often associated with higher ratios of NO_2 to ozone in these experiments. Freeman *et al.* (1974) reported morphological changes in lungs of rats exposed to 0.9 ppm of ozone plus 0.9 ppm of NO_2 that were more severe than those predicted from their results with exposure to either gas alone.

Although many different laboratories have observed synergistic toxicologic interactions between O_3 and NO_2, it is unclear what concentrations of either toxicant are needed to elicit this synergistic response. Comparisons of studies are confounded by the fact that apparent synergistic interactions are highly assay specific and are apparently species specific as well. In addition, different laboratory conditions can have profound effects on the extent of chemical reactivity between ozone and NO_2. The studies described herein were designed to identify correlations between concentrations of these two oxidants and/or reaction products with the degree of synergistic effect. It should be noted that the ratios of NO_2 to ozone in our studies are very high compared to most of the

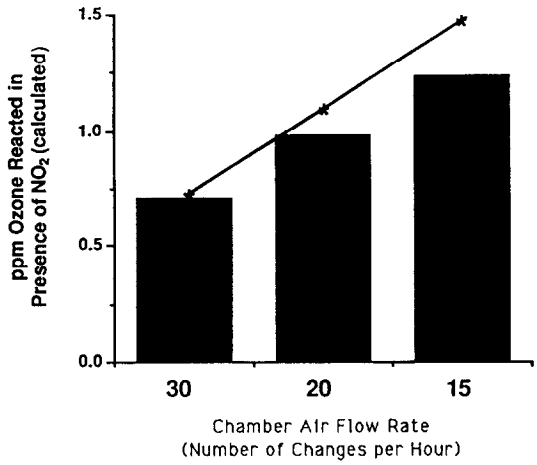

FIG. 8. Apparent reaction of ozone with NO_2 (consumption) as a function of mean chamber residence time. Chamber ozone and NO_2 concentrations were allowed to equilibrate to 0.6 and 10.8 ppm, respectively. NO_2 delivery was then stopped and the chamber ozone concentration was allowed to re-equilibrate in the absence of NO_2. Reacted ozone was calculated as the difference between equilibrium ozone concentrations analyzed in the presence and absence of NO_2. Asterisks denote theoretical concentration of reacted ozone as predicted by the equation $d[O_3]/dt = -k[O_3][NO_2]$, where $k = 0.0555$ ppm$^{-1} \times$ min^{-1} (cf. Fig. 5).

other work in the literature, based upon our rationale that we wanted to expose rats to equally toxic concentrations of the two gases (Last *et al.*, 1983).

Figures 1 and 2 demonstrate that both ozone and NO_2 need to be given concurrently for synergistic effects to be seen. This is in agreement with previous reports that have also shown that sequential exposures to ozone and NO_2 lead to additive rather than synergistic toxicologic effects (Watanabe *et al.*, 1980). Concurrent exposure would be required if chemical products from the reaction of ozone and NO_2 were to be necessary for the greater than additive toxicologic response. However, the possibility of a physiologically based toxicological interaction being the basis for the observed synergy is not excluded. For example, NO_2 is known to react with constituents of the epithelial lining fluid, creating many by-products including nitric and nitrous acids (Postlethwait and Bidani, 1990). Since acid aerosols are known to potentiate the pulmonary effects of ozone (Warren and Last, 1987) and nitrogen dioxide (Last and Warren, 1987), this could also serve as a potential mechanistic basis for the observed toxicologic interaction.

From the concentration data shown in Figs. 3 and 4 it is clear that the degree of synergy is highly concentration dependent and that there is a definite threshold response. However, we should emphasize that our data suggest that this threshold is found at differing concentrations for each of the biologic endpoints we measured. For example, at 0.4 ppm of O_3 + 7.2 ppm of NO_2, numbers of epithelial cells significantly deviate from the additive model of interaction, but neither lavageable protein nor lavageable PMNs show greater than additive response at these concentrations. PMNs are not significantly increased above additive response at concentrations below 0.6 ppm of O_3 + 10.8 ppm of NO_2, whereas total protein content of lavage fluid deviates from additive interaction only at the highest concentrations tested (0.8 ppm of O_3 + 14.4 ppm of NO_2).

In a previous report (Gelzleichter *et al.*, 1992), we exposed rats to mixtures of O_3 and NO_2 at the same concentrations of oxidants used in Figs. 3 and 4, except that the daily duration of exposure was altered in such a way that the cumulative concentration $\times$ time product (ppm · hr) for both O_3 and NO_2 remained constant over a 3-day period. For example, 0.4 ppm O_3 + 7.2 ppm NO_2 for 12 hr/day should be the equivalent cumulative C $\times$ T product as is 0.8 ppm O_3 + 14.4 ppm NO_2 for 6 hr/day. In both cases the cumulative exposure was to 14.4 ppm · hr O_3 and 259.2 ppm · hr of NO_2. It is apparent that if the concentrations of ozone and NO_2 are held constant while the daily duration of exposure is increased, the threshold levels for synergistic interactions are found at lower concentrations for all of the assays performed. So not only does the threshold level for synergistic interaction vary with assay, but it also varies with the duration of exposure. Experiments in which we held NO_2 concentrations constant while varying ozone concentrations also showed the same epithelial cell > PMN > protein order of sensitivity. Figure 7 shows the degree of deviation from additive interactions when NO_2 is held constant. Note that total epithelial cells in lung lavage fluid appears to be the parameter we evaluated that exhibits synergy at the lowest concentration of ozone, and that only 0.2 ppm of O_3 is needed to induce a synergistic response. These ozone levels are often encountered in urban settings.

It has been hypothesized that chemical reaction products could be responsible for the observed synergy between O_3 and NO_2. Ozone and nitrogen dioxide are known to irreversibly react according to the following reaction:

$$O_3 + NO_2 \rightarrow O_2 + NO_3. \quad (1)$$

If it is assumed that $[NO_2] \gg [O_3]$, the reaction obeys pseudo-first-order kinetics according to the following equation (Wu *et al.*, 1973):

$$d[O_3]/dt = -k[O_3][NO_2],$$

$$\text{where } k = 0.0647 \text{ ppm}^{-1} \text{ min}^{-1}. \quad (2)$$

Thus, under our experimental conditions (2-min residence time in exposure chambers) we would expect to see substantial reaction occurring. Therefore, we measured the consumption of both O_3 and NO_2 under our experimental conditions. It is important to point out that these experiments were carried out at night in the dark. The nitrate radical is known to react in the dark via several mechanisms. Listed

below are the major dark reactions known to exist (Sverdrup *et al.*, 1987).

$$NO_3 + NO_2 \leftrightarrows N_2O_5, \text{ Keq} = 4.1 \text{ min}^{-1}; \quad (3)$$

$$N_2O_5 + H_2O \rightarrow HNO_3 \; k = 4 \times 10^{-7} \text{ ppm}^{-1} \text{ min}^{-1}; \quad (4)$$

$$NO_3 + NO \rightarrow 2NO_2 \; k = 2.8 \times 10^{4} \text{ ppm}^{-1} \text{ min}^{-1}. \quad (5)$$

If we assume these to be the major gas phase reactions, then mathematical modelling using classical kinetics theory would predict that >95% of the reaction products will be in the form of N_2O_5, with the remainder consisting almost entirely of HNO_3. This modelling is in good agreement with published physical data under similar conditions (Sverdrup *et al.*, 1987). However, it must be pointed out that there are a large variety of minor reactions of NO_2 and nitrate radical known to exist, and some of these different reaction pathways become significant if other assumptions are made. In strong sunlight, photochemical reactions compete with these "dark" reactions. In urban settings where photochemical oxidants are being produced, these "light" reactions predominate and no appreciable amount of nitrogen pentoxide accumulates. It remains to be determined how these photochemical reactions might alter the observed synergistic toxicologic effects between O_3 and NO_2. However, as the ambient atmosphere is moved to indoor settings or at night, the dark reactions again become important. As the highest concentrations of NO_2 are usually found indoors, this is a potential setting where appreciable amounts of nitrogen pentoxide as well as other reaction products of $O_3 + NO_2$ could accumulate. However, the high chemical reactivity of O_3 tends to prevent indoor concentrations of ozone from being as high as those outdoors. Our examination of the kinetic curves shows that we first begin to see synergy occurring when the amount of ozone consumed equals the amount of steady-state ozone. At the highest concentrations, where dramatic synergistic responses were recorded, there is twice as much reacted ozone as there is steady-state ozone. Therefore, for these specific assays we do see a relationship between chemical reactivity and synergistic effects. The experimentally determined concentrations of products formed are in excellent agreement with the theoretical amounts predicted by Equations 1 and 2. Therefore, Equation 2 can accurately predict chemical reactivity if steady-state levels of O_3 and NO_2, as well as chamber residence durations (turnover rates), are known.

In experiments designed to hold the ratio of the concentrations of reacted ozone to unreacted ozone constant, we also observed that the degree of synergy was ozone concentration dependent. However, threshold levels were found at lower ozone concentrations than were observed in Figs. 3 and 4. In fact, 0.2 ppm O_3 was adequate to elicit a synergistic increase in the number of epithelial cells in the total lung lavage fluid. This concentration of ozone may actually be encountered in ambient urban air at peak hourly concentrations.

In a preliminary experiment, we attempted to further test the hypothesis that N_2O_5 (or a reaction product for which N_2O_5 is a surrogate) was the newly formed chemical species responsible for the observed synergistic interaction between NO_2 and O_3. We performed the experiment illustrated in Fig. 8, where concentrations of NO_2 and O_3 were held constant, but residence time in the chamber (flow rate) was varied. We observed a positive trend in lavageable protein that correlated with increased gas residence time in the chambers; however, no clear trend in any of the cellular measurements was observed. Since we could not directly measure N_2O_5 in the chambers, we did not pursue these experiments further, as the possibility of further chemical reactions occurring under these conditions could not be directly evaluated.

In summary, the toxicologic interaction between ozone and NO_2 is highly concentration dependent. This interaction clearly demonstrates a threshold level below which detectable synergistic effects as detected by the assays used herein are not seen. Furthermore, threshold levels are found at concentrations very much higher than would be encountered in environmental or occupational settings. However, the concentrations of O_3 and NO_2 where such thresholds are found are highly dependent on the dose rate of exposure, with shorter durations (higher dose rates) leading to lower threshold levels for synergy. We should emphasize that the concentration at which these thresholds are observed are highly assay specific as well. We might speculate that there could be a biological basis for the observed assay-specific thresholds. That is, the least sensitive assays were based upon endpoints that are, at least theoretically, reversible. For example, protein content of lavage fluid presumably measures net accumulation of edema fluid, which in turn represents an equilibrium between fluid leakage across the epithelium and its resorption. Accumulation of PMNs also represents an equilibrium between airway influx and outward migration of these motile inflammatory cells. However, our most sensitive assay, that of epithelial cell accumulation in the lavage fluid, is theoretically irreversible over the time scales examined as it measures the sloughing of necrotic cells into the airway lumen. Such cells are dead and therefore have no motility except as they are broken down and/or phagocytosed into alveolar macrophages for their clearance, or are cleared slowly by the mucociliary escalator.

Although synergistic toxicologic responses were not seen except under circumstances where substantial chemical reaction between ozone and NO_2 was observed, we cannot as yet directly correlate increased toxicologic response with any specific reaction product formed in the chambers, even though we can predict the responses observed with reasonable accuracy using N_2O_5 as a surrogate for the toxic species.

ACKNOWLEDGMENTS

This work was supported in part by a contract from the Center for Indoor Air Research (90-20) and by grants from the U.S. National Institutes of

Health, RR-00169 and ES-00628. Thomas Gelzleichter was a predoctoral trainee of the NIEHS, supported by ES-07059. We thank Brian Tarkington and Tim Duvall for design and operation of the exposure facilities used in these studies and Susan Hawk for her skilled technical assistance.

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