6

Toxicological Effects of Ozone and Related Photochemical Oxidants

6.1 Introduction

A wide range of effects of ozone (O_3) has been demonstrated in laboratory animals (see reviews by U.S. Environmental Protection Agency [1986], Lippmann [1989, 1993], and Graham et al. [1991]). The major research findings are that environmentally relevant levels of O_3 cause lung inflammation; decreases in host defenses against infectious lung disease; acute changes in lung function, structure, and metabolism; chronic changes in lung structure and lung disease, some elements of which are irreversible; and systemic effects on target organs (e.g., liver, immune system) distant from the lung. The research also has served to expand understanding of the mechanisms of toxicity and relationships between concentration (C) and duration of exposure (time [T]). The framework for presenting the health studies of O_3 in animals begins with a discussion of respiratory tract effects and is followed by a presentation of systemic effects and interaction of O_3 with other common co-occurring pollutants. Respiratory tract effects are often interrelated; however, for purposes of presentation, effects on lung inflammation and permeability, host defenses, morphology, pulmonary function, biochemistry, and mutagenic/carcinogenic potential are discussed separately in the main text, drawing correlations where appropriate. This type of organization enables focus on specific effect categories. In the few cases where one study addresses several different categories of endpoints, cross references are made to the appropriate sections of the chapter. Each major section on a specific effect category is followed by a summary for that section. The summary and conclusions section for the entire chapter (Section 6.5) attempts to draw together findings on related endpoints and to highlight key issues, such as the relative importance of exposure concentrations and durations and the identification of potential risk factors.

A purpose of this criteria document is also to describe any key health effects of photochemical oxidants in addition to O_3. Nitrogen dioxide (NO_2) and nitric oxide are the other two primary photochemical oxidants; they have been evaluated recently in another criteria document (U.S. Environmental Protection Agency, 1993). Formaldehyde (HCHO), which is formed photochemically and can be toxic, also has been reviewed recently by the U.S. Environmental Protection Agency (EPA) (Grindstaff et al., 1991). Literature searches did not reveal any animal toxicology inhalation studies of peroxyacetyl nitrate (PAN) since the last O_3 document (U.S. Environmental Protection Agency, 1986). A myriad of other

individual photochemical oxidants are formed in ambient air (Chapter 3), but they have not been investigated by animal inhalation toxicology. The very few publications on the effects of exposures to a mixture of oxidants are summarized in Section 6.4, which discusses pollutant interactions. Therefore, other than in Section 6.4, this chapter does not address other photochemical oxidants. Even so, considering the limited literature within the aforementioned documents, the available evidence from animal toxicology studies shows that O_3 is the most potent of the oxidants for noncancer effects at environmentally relevant concentrations.

The animal toxicology database for O_3 is extremely large, making it necessary to adopt conventions for presenting succinctly the pertinent findings. Priority was placed on analysis of research published after closure of the previous O_3 criteria document (U.S. Environmental Protection Agency, 1986); however, for the purposes of broader interpretation, the older literature is very briefly summarized. Generally, only the highlights of the key recent studies and their interpretation are provided here. Confirmatory recent studies are mentioned and presented in the tables. Furthermore, studies having O_3 concentrations $\geq$1.0 ppm are highlighted with rare exception (e.g., genotoxicity studies). Genotoxicity studies at O_3 concentrations higher than 1.0 ppm were included to enable coverage of all the specific endpoints, some of which were tested only above 1.0 ppm. In most other cases, however, the 1-ppm cut point allows portrayal of the full array of the effects of O_3 that may occur from ambient air exposure and also avoids the potential for confounding mechanisms that can occur at very high, environmentally unrealistic concentrations. For example, very high levels of O_3 can cause severe pulmonary edema, resulting in types and magnitudes of pulmonary function changes that would not occur in ambient air. In summarizing the literature, changes from control are described if they were statistically significant at $p < 0.05$, rather than citing the probability values for each study. Where appropriate, critique of a statistical procedure is mentioned. A probability value is provided if it aids the understanding of trends observed in a study (e.g., $p < 0.1$).

As stated above, only literature published since the last O_3 criteria document is described in detail here. The earlier findings are summarized to facilitate cross-referencing. For example, in some cases, the older work is presented in overview in the beginning of each main section; in other cases, the overview is at the subsection level. Generally, the newer literature elucidates the influence of different exposure regimens and the mechanisms of several key effects, rather than portrays undiscovered categories of effects. The newer knowledge on molecular and biochemical interactions increases the understanding of mechanisms of effects. For example, it is unlikely that the O_3 molecule itself penetrates the lung and enters the circulation. As another example, the relationship between inflammation measured in tissue and lung lavage assists in the interpretation of lung lavage findings. Information on the immune system suggests that the cell-mediated limb may be more susceptible than the humoral limb. The ability of O_3 to decrease antibacterial host defenses has long been recognized, but only recently have viral defenses been analyzed. Much remains to be learned, but apparently antibacterial defenses are more at risk from O_3 exposure than antiviral defenses. Cellular and interstitial changes in the lungs of O_3-exposed animals were among the very early studies, with newer work adding to a detailed understanding of morphologic lesions in the pulmonary region identified through advanced morphometric procedures. Scientists just recently have begun to study the effects of O_3 on the nose and have discovered epithelial changes, identifying this tissue as a significant target site of O_3. One new body of information concerns the influence of exposure concentration, duration, and pattern. For several endpoints (e.g., increased lung

permeability), under acute exposure conditions, concentration has more impact than exposure duration. The importance of exposure duration is clearly illustrated by the newer chronic studies that show different patterns of effects (compared to acute exposures). The most sensitive indicators appear to be morphological changes (compared to pulmonary function changes) which is consistent with the concept that functional abnormalities follow morphological changes and may not become apparent until a given threshold is achieved. Studies using intermittent exposures (e.g., exposures every day versus every other week or month for equal times) indicate that interrupted exposures can produce equal or, in some cases, enhanced effects compared to uninterrupted exposures, suggesting a cumulative effect. Thus, seasonal "lows" in O_3 do not have benefit in these animal studies. Newer work also has mimicked and extended human clinical studies of repeated exposures. As with humans, the pulmonary function of rats was attenuated with several days of exposure. However, other changes (e.g., cellular) did not attenuate in the rat, illustrating the need for comprehensive evaluations. For the first time, a classical cancer bioassay has been performed with O_3. It helps put some of the earlier genotoxicity and carcinogenicity studies in perspective. This brief identification of the newer additions to the O_3 database is not meant to be a summary of effects; that is the last section of the chapter. Rather, it does show the importance of considering all the literature, not just the newer work in interpreting the effects of O_3. As mentioned, it was not feasible to repeat the 1986 O_3 criteria document herein; this makes it necessary to use both the current and the former document in evaluations.

Animal toxicological studies of O_3 are of major interest because they illustrate a fuller array of effects and exposure conditions than can be investigated in humans. Most experts accept a qualitative animal-to-human extrapolation (i.e., O_3 effects observed in several animal species can occur in humans if causative exposure concentrations, durations, and patterns also occur). However, there is less consensus on an approach to quantitative extrapolation (e.g., the exposures at which effects in animals actually occur in humans). Chapter 8, on extrapolation, provides more information on this topic.

6.2 Respiratory Tract Effects of Ozone

6.2.1 Biochemical Effects

6.2.1.1 Introduction

This section outlines studies designed to identify biochemical targets of O_3, as well as biochemical measurements of antioxidant and microsomal enzyme activities, lipids, and proteins. It should be noted that interpretation of biochemical changes resulting from whole lung measurements is complicated by the heterogeneity in cell type and function present in lung tissue and the changes in cell populations that result from O_3-induced inflammatory cell infiltration and epithelial cell and fibroblast proliferations. The ability to extrapolate from in vitro to in vivo studies and from high to low levels of O_3 is further complicated by an inability to detect biochemical changes in the whole lung when only a small proportion of the lung may be affected by O_3, especially at concentrations of O_3 less than 1 ppm. Interpretation of all biochemical measurements, therefore, needs to take into account the airway sites of O_3 interaction and concomitant changes in cell populations and numbers that take place at times other than the onset of exposure.

6.2.1.2 Cellular Targets of Ozone Interaction

In vitro experiments have indicated that O_3 has the potential to interact with a wide range of different cellular components that include polyunsaturated fatty acids (PUFAs); some protein amino acid residues (cysteine, histidine, methionine, and tryptophan); and some low-molecular-weight compounds that include glutathione (GSH), urate, vitamins C and E, and free amino acids (U.S. Environmental Protection Agency, 1986; Mustafa, 1990; Pryor, 1991, 1992). The mechanisms to explain the initial biochemical and physiological effects of O_3 exposure in vivo are therefore complex. Hypotheses have been developed on the direct action of O_3 with lung macromolecules, the reaction of secondary biochemical products that could result from the generation of free radical-precursor molecules, the release of endogenous mediators of physiological response, and the reactive oxygen intermediates and proteinases associated with the activities of inflammatory cells that subsequently infiltrate into O_3-damaged lungs (see Section 6.2.2). Based on some theoretical calculations, Pryor (1992) hypothesized that, because O_3 is so reactive, it most likely does not penetrate beyond the surface-lining fluids of the lung except in those terminal airway regions having minimal lining thickness where epithelial cells may well be relatively unprotected by either mucus or surfactant. In a review, Pryor (1991) proposed that O_3-induced cell damage more likely results from the reactions of more stable but less reactive ozonide, aldehyde, and hydroperoxide products of O_3 interaction with surface-lining fluid components than from direct interactions of O_3 with intracellular components. Although the alveolar lining fluid is relatively rich in saturated phospholipids, it does contain some lipids with unsaturated fatty acids, cholesterol, a protein A component, and small-molecular-weight compounds (e.g., GSH and uric acid) that have been shown to react with O_3 in both in vitro and in vivo studies (Effros et al., 1990; King and Clements, 1985; Shelley et al., 1984).

Polyunsaturated Fatty Acids

Hitherto, the major products of O_3-lipid interaction that account for cell membrane damage have been assumed to be lipid hydroperoxides. However, evidence for the production of hydrogen peroxide and aldehydes has been demonstrated. It has been proposed that, although Criegee ozonation (Figure 6-1) will ultimately lead to the production of ozonides in a lipophilic environment, in the aqueous environment of lung airways, the carbonyl oxide intermediate can form a hydroxyhydroperoxy compound, which on elimination of hydrogen peroxide yields another aldehyde or, in the presence of iron ions, can form an aldehyde and the very reactive hydroxyl radical (Teige et al., 1974; Pryor, 1991). Ozonation of aqueous emulsions of PUFAs, rat erythrocyte ghost membranes, and rat bronchoalveolar lavage (BAL) fluid has shown hydrogen peroxide and aldehyde generation with a much smaller proportion of ozonides and lipid hydroperoxides (Pryor et al., 1991). A mechanistic study by Santrock et al. (1992) of the ozonation of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine in unilamellar phospholipids confirmed the generation of the hydroxyperoxy compounds, which subsequently result in the generation of hydrogen peroxide and aldehydes with further oxidation to carboxylic acids (Figure 6-1). Similar studies conducted under nonaqueous conditions have demonstrated the production of secondary ozonides that, under physiological conditions, would be expected to decompose rapidly to reactive products (Lai et al., 1990). Madden et al. (1993) have demonstrated recently production of arachidonate-derived aldehydic substances and hydrogen peroxide from in vitro O_3 exposure (0.1 and 1.0 ppm for 1 h) of arachidonate in both a cell-free system and

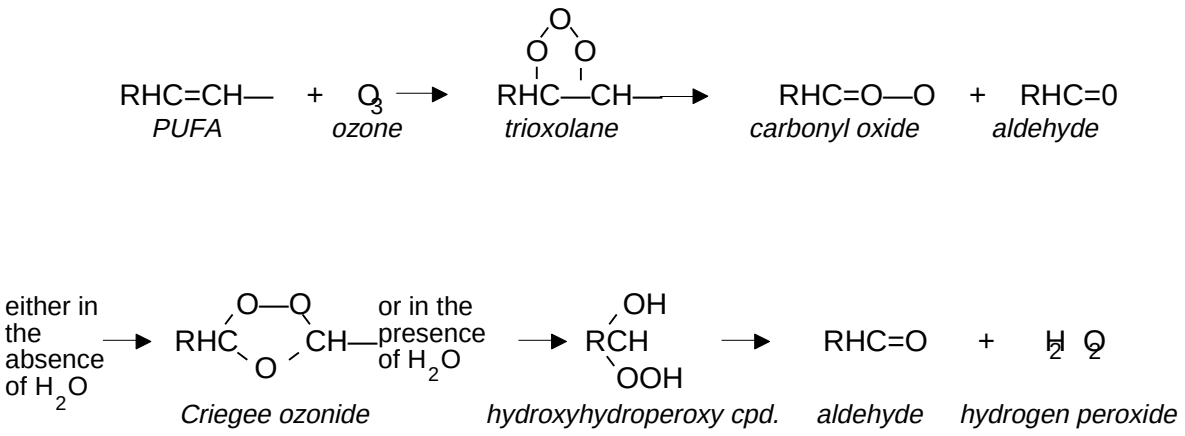

Figure 6-1. Major secondary products of ozone interaction with lung cells.

cultured human bronchial epithelial cells. Ozonides, aldehydes, hydrogen and lipid peroxides, and related reactive oxygen intermediates, together with the phospholipid from which the aldehyde has been removed, represent major products of O_3 interaction with lung cells that all have the potential to cause damage to membranes (see Figure 6-1).

Evidence that interaction of O_3 with PUFAs takes place in vivo has not been so easily obtained. Goheen et al. (1986) investigated the effects of fat-free diets on rats exposed to air or to 0.96 ppm O_3 for 0, 1, 2, and 4 weeks and concluded that O_3 does not oxidize significant levels of the PUFAs linoleate (18:2) and arachidonate (20:4). However, cleavage of lung fatty acid double bonds has been demonstrated in an in vivo study reported by Rabinowitz and Bassett (1988) that involved rat exposures for 4 h to 2 ppm O_3. These authors, by using hydrogen peroxide treatment to convert ozonides and aldehydes to carboxylic acids, were able to demonstrate O_3-induced increases in glutaric and nonanoic acids that are the ozonolysis breakdown products of lung tissue arachidonic and oleic acids, respectively. More recent studies directed towards developing suitable biomarkers and dosimeters for O_3 exposure have analyzed rat BAL lipids after a 12-h exposure to 1.3 ppm and demonstrated the appearance of the aldehydes nonanal and heptanal (Cueto et al., 1992). Pryor et al. (1992) also have been able to identify cholesterol ozonation products extracted from whole lung tissue with the same exposure of rats to 1.3 ppm O_3 for 12 h.

Evidence of the role of hydrogen peroxide in O_3-induced lung damage has been described by Warren et al. (1988), who demonstrated diminished O_3-induced increased BAL protein in rats after 1 day of exposure to 0.64 ppm O_3, when treated with the hydrogen peroxide scavenger dimethylthiourea before exposure. Hitherto, the exhalation of ethane and pentane and tissue measurements of diene-conjugates and thiobarbituric acid reactive substances (TBARS) have been used as evidence for O_3-induced free radical autoxidation of lipids (U.S. Environmental Protection Agency, 1986). However, these measurements have been found to be relatively insensitive for use in inhalation experiments under conditions of low O_3 concentrations (<0.5 ppm). Ichinose and Sagai (1989) were unable to demonstrate any changes in lung TBARS as a result of exposing rats for 2 weeks to 0.4 ppm O_3. As noted by

Pryor (1991), malondialdehyde and other thiobarbituric-acid-reacting aldehydes can be produced by Criegee ozonation of olefinic fatty acids that include arachidonate, as well as by free radical peroxidative processes. In addition, malondialdehyde, being volatile as well as highly reactive, may be lost readily from the lung or during sample preparation. However, measurements of TBARS continue to be used for in vitro experiments designed to demonstrate possible mechanisms by which such agents as taurine (Banks et al., 1991) and uric acid (Meadows and Smith, 1987; Peden et al., 1993) may protect against O_3-induced lipid damage. Rietjens et al. (1987b), by preincubating rat alveolar macrophages (AMs) with either arachidonate (20:4) or phosphatidylcholine to alter PUFA content and membrane fluidity, respectively, demonstrated that PUFA content (not membrane fluidity) determined sensitivity to O_3 damage, measured as decreases in phagocytic activity.

Evidence for free-radical-mediated autoxidation comes indirectly from the demonstration that vitamin E depletion increases O_3 toxicity, as reported previously (U.S. Environmental Protection Agency, 1986) and more recently (Elsayed, 1987; Elsayed et al., 1988). More direct evidence for free radical generation has been obtained using electron spin-trapping technology that correlated increased radical signals in isolated lung lipids from rats exposed to increasing O_3 concentrations (0 to 1.5 ppm, effect beginning at about 0.5 ppm; 2 h) under conditions of carbon dioxide (CO_2)-stimulated respiration (Kennedy et al., 1992). However, the possible contribution of activated inflammatory cell generation of reactive oxygen intermediates to these observed free radical alterations to lung lipids needs to be considered.

Antioxidants

Although vitamin E directly reacts with O_3 at the same rate as PUFAs, vitamin C appears to react more effectively (Pryor, 1991), which, together with intracellular taurine (Banks et al., 1991) and BAL uric acid (Meadows and Smith, 1987; Peden et al., 1993) found in nasal and lung-lining fluids, may act as direct scavengers of O_3. Ozone-induced increases in lung polyamine metabolism in vitamin E-deficient rats suggests their possible role as antioxidants (Elsayed, 1987). Glutathione in its reduced form (GSH) represents another potential direct O_3 scavenger. In addition to being a major intracellular antioxidant, GSH is a component of airway-lining fluids found in BAL. Ozone would have to penetrate the cellular membrane without reaction if it is to directly interact with intracellular GSH, an event considered to be unlikely (Pryor, 1991, 1992). Previously observed oxidation of glutathione and, in some cases, its loss from the lung may more likely reflect its reaction with an O_3-derived oxidant, such as a hydroperoxide or an ozonide, mediated by glutathione peroxidase (GSHPx) and glutathione-S-transferases, respectively (Rietjens et al., 1987a), resulting in the formation of glutathione disulphide or mixed disulphides with sulphydryl (SH)-containing proteins. Although ozone-induced formation of glutathione sulfonate has been reported in vitro, such irreversible oxidation of GSH has not been observed in vivo (U.S. Environmental Protection Agency, 1986; Mustafa, 1990).

Proteins

Early studies reported that nonprotein sulfydryls (NPSHs) and the activities of various cytosolic, microsomal, and mitochondrial enzymes are decreased immediately following short-term exposures to relatively high levels (2 to 4 ppm) of O_3 (U.S. Environmental Protection Agency, 1986; Mustafa, 1990). However, although these early

biochemical effects could not be demonstrated after the first day of exposure to the lower O_3 concentration of 0.8 ppm, the methods employed may not have been sensitive enough to detect coenzyme and enzyme changes in the centriacinar region (CAR), which is a primary target of O_3. However, together with surfactant lipids, the surfactant protein A also has been examined as a potential target of O_3 interaction (Oosting et al., 1991c, 1992). In vitro studies by these authors have suggested that either hydrogen peroxide- or O_3-induced oxidation of methionine and tryptophan residues account for the observed changes in physicochemical properties of canine and human surfactant protein A, measured as an impairment of self-association and a decreased ability to aggregate phospholipid vesicles and to bind mannose (Oosting et al., 1991c). Similar responses were found in vivo; surfactant isolated from rats exposed for 12 h to 0.4 ppm O_3 was less able to stimulate AM superoxide anion generation than surfactant obtained from air-exposed control rats (Oosting et al., 1992). The previously reported presence of giant lamellar bodies in O_3-exposed rat lungs following exposure to 0.3 ppm for 3 h/day for 16 days is also consistent with the hypothesis that O_3 reacts with surfactant protein A (Shimura et al., 1984) and thereby interferes with its homeostatic role in surfactant release from alveolar Type 2 cell lamellar bodies and its subsequent reuptake by Type 2 cells and AMs.

6.2.1.3 Effects of Ozone Exposure on Lung Lipid Metabolism

Arachidonate Metabolites

Ozone-induced damage to airway epithelia (Leikauf et al., 1988) and AMs (Madden et al., 1991) in vitro has been associated with the production of arachidonic acid metabolites by both cyclooxygenase and lipoxygenase pathways. These metabolites have been implicated in a variety of different physiological processes that include changes in airway permeability, infiltration of polymorphonuclear leukocytes (PMNs) and eosinophils, and airway smooth-muscle reactivity, discussed elsewhere in this chapter (Sections 6.2.2 and 6.2.5). Leikauf et al. (1993) have examined the effects of fatty acid O_3-degradation products on human airway epithelial eicosanoid metabolism and concluded that the stimulating effects were increased with product chain length, with the 3-, 6-, 9-hydroxyhydroperoxides being more potent than their corresponding aldehydes. Madden et al. (1993) concluded that aldehydic degradation products of arachidonate, but not hydrogen peroxide, increased in vitro polarization of leukocytes, and decreased peripheral blood T-cell mitogenesis and natural killer (NK) cell cytotoxicity. In vivo experiments on rabbits, guinea pigs, mice, and rats of different ages exposed to $\geq$1.0 ppm O_3 have demonstrated increases in the products of arachidonic acid metabolism (see Section 6.2.2).

Surfactant

Although alveolar surfactant lipids purified from lavage fluids have been shown to be relatively enriched in saturated lipids, a varying percentage of the lipids do contain unsaturated fatty acids depending on the species studied (King and Clements, 1985; Shelley et al., 1984). These unsaturated lipids, together with the apoprotein as possible targets of O_3 interaction, may be expected to have an altered composition as a result of O_3 inhalation. However, surfactant-enriched material isolated by BAL from rats following an 8-h exposure to 0.8 ppm O_3 retained its ability to lower surface tension in spite of an increase in protein content (Nachtman et al., 1986). In long-term exposure studies, monkeys were exposed for 8 h/day to 0.15 and 0.3 ppm O_3 for 21 and 90 days (Rao et al., 1985a,b). In contrast to

measurements of total lung lipids that demonstrate a relative decrease in PUFAs after 21 days of exposure (Rao et al., 1985a), there was a relative increase in the proportion of PUFA in the percentage of BAL unsaturated fatty acids (increases from 34% in air controls to 41, 42, and 45% in BAL lipids recovered from monkeys exposed for 21 days to 0.15 ppm, 90 days to 0.15 ppm, and 90 days to 0.3 ppm O_3, respectively) (Rao et al., 1985b). The major increases were observed in linoleate (18:2) and arachidonate (20:4). Because these PUFAs are potential targets for O_3 interaction, their increase, rather than a decrease, in BAL fluid may best be explained by changes in surfactant lipid production associated with alveolar Type 2 epithelial proliferation (Section 6.2.4). Interestingly, a relative decrease in cholesterol ester with a concomitant increase in phosphatidylcholine was observed, which supports the hypothesis that cholesterol may represent a major target of O_3 interaction (Rao et al., 1985b; Pryor et al., 1992). The observed O_3-induced changes in BAL PUFA composition were consistent with those previously reported for rats by Roehm et al. (1972), but only for BAL lipids isolated from vitamin E-depleted rats following 6 weeks of exposure to 0.5 ppm O_3. Wright et al. (1990) were unable to detect changes in BAL lipid and fatty acids recovered from normally fed rats following 0.12-, 0.25-, and 0.5-ppm O_3 exposures for 20 h/day for 18 mo. Results from these studies are summarized in Table 6-1.

Tissue Lipids

In vivo pulse labeling with carbon-14-labeled acetate was used to estimate phospholipid biosynthesis (Wright et al., 1990). Although found to be diminished at certain time points (3 and 12 mo), no consistent trend could be demonstrated that would suggest that O_3 exposures of less than 0.5 ppm alter lung surfactant homeostasis. Bassett and Rabinowitz (1985), using isolated perfused lungs taken from rats after 3 days of continuous exposure to 0.6 ppm O_3, demonstrated an enhanced incorporation of glucose carbons into both fatty acid and glycerol-glyceride moieties of total lung lipids by 180 and 95%, respectively. The relative increase in carbon incorporation into free fatty acids, phosphatidic acid, phosphatidyl inositol, and sphingosine containing lipids was consistent with the needs of a dividing cell population for increased lipids synthesis associated with alveolar epithelial proliferative repair. It should be noted that, in a separate study, under the same exposure conditions of 0.6 ppm O_3 for 3 days, rat lungs demonstrated increased glycolytic activity and generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) consistent with the energy and synthetic needs of a lung undergoing repair of O_3-induced damage (Bassett and Bowen-Kelly, 1986). Results from these studies are summarized in Table 6-1.

6.2.1.4 Effects of Ozone on Lung Antioxidant Systems

The O_3-induced increased levels of the antioxidant NPSHs, identified mainly as GSH in the lung, and the enzyme activities involved in GSH utilization, GSHPx and glutathione-*S*-transferase (GST), and for maintaining GSH in a reduced state, glutathione reductase (GR) and the NADPH-linked dehydrogenases of glucose-6-phosphate (G6PD) and 6-phosphogluconate (6PGD), typically have been attributed to concurrent morphological changes rather than to any specific biochemical response (U.S. Environmental Protection Agency, 1986). Numerous studies conducted in mice, rats, and monkeys show increases in many of these enzyme activities at exposures as low as 0.2 ppm O_3 for 1 week (rat) (U.S. Environmental Protection Agency, 1986). The earlier research also included studies of age-dependent responsiveness of rats (Tyson et al., 1982; Lunan et al., 1977; Elsayed et al.,

1982). Rats ranging in age from 5 to 90 days old were exposed to 0.8 or 0.9 ppm O_3 for 3 or 4 days or for about 20 days, depending on the experiment. Ozone altered activities of antioxidant enzymes in an age-dependent manner. Generally, prior to weaning, enzyme

Table 6-1. Effects of Ozone Exposure on Lung Lipids[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	μg/m³				
0.12 0.25 0.5	235 490 980	20 h/day, 7 days/week for 18 mo	Rat, M (F344) 28 days old	Age-related increase in BAL and tissue phospholipids generally unaffected by O_3 exposure; at 0.5 ppm, total phospholipid increased at 6 and 12 mo.	Wright et al. (1990)
0.15 0.3	353 588	8 h/day for 90 days	Monkey (Bonnet)	Fraction of total lung lipid fatty acids that were PUFAs decreased from 22 to 9% and 6% following 0.15-ppm and 0.3-ppm exposures, respectively.	Rao et al. (1985a)
0.15 0.3	353 588	8 h/day for 21 and 90 days	Monkey (Bonnet)	BAL PUFAs (linoleate [18:2] and arachidonate [20:4]) increased, with a relative decrease in cholesterol esters.	Rao et al. (1985b)
0.5 0.6	980 1,176	Continuous for 0-4 weeks	Rat, M (S-D) 50 g	No change in lung fatty acid content; no acceleration of essential fatty acid deficiency in rats on fat-free diet.	Goheen et al. (1986)
0.5 1.0 1.5 2.0	980 1,960 2,940 3,920	2 h	Rat, M (CD) 65-85 days old	Extracted lung lipid EPR signal intensity proportional to O_3 concentration following pretreatment with spin-trapping agent and CO_2 stimulation of respiration in vivo.	Kennedy et al. (1992)
0.58	1,137	Continuous for 3 days	Rat, M (Wistar) 220-250 g	Increased lipid synthesis associated with increased glucose catabolism for ATP and NADPH generation.	Bassett and Bowen-Kelly (1986)
0.6	1,176	Continuous for 3 days	Rat, M (Wistar) 220-250 g	Increased synthesis of perfused lung glyceride-glycerol and fatty acid moieties of neutral lipids and phospholipids from glucose carbons, with a greater proportion of shingosine and inositol synthesis.	Bassett and Rabinowitz (1985)
0.8	1,568	18 h	Rat (F344) 260 g	Increase in BAL protein; no alteration in surface-tension-lowering ability of BAL.	Nachtman et al. (1986)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

activities decreased, and, at older ages, they increased. The reasons for these differences are not known, but may be due to differences in (1) dose of O_3 to the lung (due to differences in exposure concentrations in huddled neonates on bedding prior to weaning or to differences in doses delivered to lung target sites), (2) basal levels of antioxidants and antioxidant enzymes, or (3) cellular sensitivity. Increased lung enzyme activities can result from either increased activity within a particular cell population or increased numbers of cells with that activity. Age, nutritional, and species differences in O_3-mediated responses must therefore be interpreted with consideration of the underlying morphological changes (Section 6.2.4). Relevant studies are summarized in Table 6-2.

An increase in lung alveolar Type 2 cells and in infiltrating inflammatory cells adequately explained the observed increases in succinate oxidase, G6PD, and 6PGD activities observed after 3 days of continuous exposure of rats to 0.75 ppm O_3 when represented on a per-milligram-deoxyribonucleic-acid (DNA) basis (Bassett et al., 1988a). These cell types are enriched in mitochondria and in NADPH-generating capacity, needed for both lipid biosynthesis and GSH maintenance. Similarly, no significant changes in these enzyme activities could be detected after 3 days of exposure to the lower O_3 concentration of 0.35 ppm, further illustrating the need to take into account the concomitant changes in cell population and number when interpreting whole-lung enzyme measurements. Increases of 150 and 108%, respectively, were observed in the per-milligram DNA activities of the ornithine carboxylase and *S*-adenosyl-methionine decarboxylase enzymes involved in polyamine synthesis, which, together with enhanced tritiated thymidine incorporation into DNA, have been considered to be more sensitive measures of biochemical changes in lungs of rats exposed continuously for 3 days to 0.45 ppm O_3 (Elsayed et al., 1990).

The potential role of superoxide dismutase (SOD) and catalase in protecting the lung against O_3 toxicity is not clear. Bassett et al. (1989), using a pretreatment with a phenyl-urea compound (*N*[2·(2-oxo-1-imidazolindinyl)ethyl]-*N*′-phenylurea; EDU) that increased rat lung SOD and catalase activities, failed to demonstrate any protection against acute lung injury from a single 3-h exposure to 2.0 ppm O_3. However, Zidenberg-Cherr et al. (1991) have demonstrated that copper (Cu)- and manganese (Mn)-deprived mice may be more susceptible to continuous O_3 exposure of 1.2 ppm for 7 days. Rahman and Massaro (1992) have demonstrated protection against edemagenic exposures to ozone (2.5 ppm for 24 h) in rats pretreated with endotoxin. Endotoxin pretreatment is associated with increases in lung tissue mitochondrial Mn-SOD activity without any concomitant increases in catalase, GSHPx, and the cytosolic Cu,Zn-SOD enzymes. Although it is difficult to conclude that mitochondrial SOD might directly protect against O_3 interactions, these results do suggest a central role of mitochondrial SOD in the protection of the cell against oxidative stress (Rahman and Massaro, 1992).

Rahman et al. (1991) also have demonstrated that lungs from O_3-exposed rats had increased activities of Cu, Zn-SOD, Mn-SOD, catalase, and GSHPx after 5 days of exposure to 0.7 ppm O_3. These increases were attributed to enhanced gene expression, indicated by higher messenger ribonucleic acid (mRNA) concentrations, rather than to the infiltration of cells enriched with these enzyme activities. Chronic exposure of rats to an urban pattern of O_3 for 12 mo did not affect total SOD activity in rats, although GSHPx and GR activities per lung were increased (Grose et al., 1989). Use of microdissection techniques following 90 days and 20 mo of rat exposures to 0, 0.5, and 1.0 ppm O_3 have shown concentration-dependent

increases in SOD, GST, and GSHPx per milligram of DNA in the distal bronchioles. In contrast, decreases in GST and GSHPx activities in major bronchi and

Table 6-2. Effects of Ozone Exposure on Lung Antioxidants[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m^3				
0.06 base, 0.25 spike	118 base, 490 spike	Base 13 h/day, 7 days/week; ramped spike 9 h/day, 5 days/week for 12 mo	Rat, M (F344)	Whole lung increase in GSHPx and GSH reductase activities. SOD activity and NPSH content not affected.	Grose et al. (1989)
0.12 0.2 0.64	235 392 1,254	Continuous for 7 days	Rat, M (S-D) 250-300 g	Pretreatment with the H_2O_2 scavenger dimethylurea decreased O_3-induced tissue DNA and protein and BAL protein, acid phosphatase, and *N*-acetyl-D-glucosaminidase. No effect of vitamin E or -carotene.	Warren et al. (1988)
0.12 0.5 1.0	235 980 1,960	6 h/day, 5 days/week for 90 days or 20 mo	Rat, M and F (F344)	Using microdissection techniques and representing data as units/mg DNA, GST, GSHPx, and SOD were increased in distal bronchioles after 90 days and 20 mo in a concentration-dependent fashion. After 90 days, SOD and GST were lower in major daughter bronchi. After 20 mo, SOD was increased in distal trachea; GSHPx was decreased in major bronchi but enhanced in minor bronchi; and GST decreased in major bronchi.	Plopper et al. (1994b)
0.35 0.75	686 1,470	Continuous for 3 days	Rat, M (Wistar) 200-250 g	0.75 ppm O_3-induced whole lung increases in GSHPx and GR not significant when corrected for increases in cell number. Increases in succinate oxidase, G6PD and 6PGD activities per mg DNA were consistent with increased Type 2 and inflammatory cell content. No increases per mg DNA at 0.35 ppm O_3.	Bassett et al. (1988a)
0.4	784	Continuous for 2 weeks	Rat, M (Wistar) 6 weeks old Guinea pig (Hartley) 6 weeks old	Small increases in whole rat lung levels of NPSH, vitamin C, GSHPx. Guinea pig GSHPx and GSH transferase activities decreased.	Ichinose and Sagai (1989)
0.41	800	12 h during day or night for 3 days or continuous for 72 h	Rat, M (Wistar) Guinea pig, M (Hartley) 9 weeks old	Rats: No effect of daytime exposure. Nighttime or continuous exposure increased activities of LDH, G6PD, GR, and GSHPx. Guinea pig: No daytime-only exposure. No effect on GR or GSHPx, G6PD increased after nighttime or continuous exposure; lactate dehydrogenase activity increased only after continuous exposure.	Van Bree et al. (1992)
0.45	882	Continuous for 2 days	Rat, M (S-D) 90 days old	Large increase in ornithine decarboxylase activity and DNA labeling reflecting polyamine metabolism and DNA synthesis and/or repair, respectively.	Elsayed et al. (1990)
0.5	980	Continuous for 5 days	Rat (Long-Evans) 10 weeks old	Ozone increased lung putrescine in both vitamin E-deficient or 1,000 IU/kg groups, but increases in spermidine content and decarboxylase activities of ornithine and *S*-adenosylmethionione only in vitamin E-deficient group.	Elsayed (1987)

Table 6-2 (cont'd). Effects of Ozone Exposure on Lung Antioxidants[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m^3				
0.5	980	Continuous for 5 days	Rat (Long-Evans) 10 weeks old	Ozone increased lung vitamin E level in supplemented rats and remained unchanged in all other tissues measured.	Elsayed et al. (1990)
0.5	980	2.25 h/day for 5 days	Rat, M (F344) 110 days old	Lung GSH initially enhanced, declining to control levels by Day 4. Lung ascorbate levels enhanced on Days 3 and 5 only.	Tepper et al. (1989)
0.64	1,254	Continuous for 7 days	Rat, M (S-D) 3-5 weeks old	The whole lung O_3-induced increase in ascorbate and GSH content unaffected by protein deficient diets.	Dubick et al. (1985)
0.64	1,254	Continuous for 7 days	Rat, M (S-D) 52 and 295 g	Whole adult lung contents of Cu,Zn-SOD and GSHPx increased by O_3 in all diet groups (ad libitum, 4-16% protein diets); GSHPx only increased in weanling rats fed 16% protein diet. Mn-SOD only increased in lungs from 4 and 16% protein-fed adult lungs.	Heng et al. (1987)
0.7	1,373	Continuous for 1-5 days	Rat, M (S-D) 45, 80, and 300 g	By 5 days, increased lung Cu,Zn-SOD, Mn-SOD, catalase, and GSHPx per DNA in all age groups. Adult lungs: Concomitant increases in mRNAs for Cu,Zn-SOD, catalase, and GSHPx without differences in mRNA stability.	Rahman et al. (1991)
0.8	1,568	8 h/day for 2 mo	Rat, M (S-D) 2 mo old	Absence of vitamin E exacerbates O_3-induced damage related to increases in whole lung levels of metabolic enzymes. No additional amelioration by diet supplementation above 50 IU vitamin E.	Elsayed et al. (1988)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

an increase in GSHPx were observed in minor bronchi after 20 mo of O_3 exposure (Plopper et al., 1994b). Rahman et al. (1991) concluded that changes in antioxidant enzyme activities in some cases could be associated with alterations in cellular pathology (see Section 6.2.4), whereas, in other cases, no correlation could be made even though the results were represented on a per-milligram-DNA basis. The observed changes in antioxidant enzyme activities appear to be site-specific and different at different airway locations. The response are concentration dependent and altered by the length of O_3 exposure (Plopper et al., 1994b).

Representing data on a per-gram, wet-lung basis, Ichinose and Sagai (1989) demonstrated increases in lung NPSH, vitamin C, and GSHPx but observed no effect on vitamin E levels, after continuous exposure of rats to 0.4 ppm O_3 for 2 weeks. In contrast, guinea pig lungs exhibited no changes in these antioxidant components when similarly exposed. However, although using the higher concentration of 0.64 ppm for 7 days of continuous O_3 exposure, Dubick et al. (1985) demonstrated that whole lung content of ascorbate and GSH was elevated, these changes were not significantly different when the data were represented on a per-100-g, wet-tissue basis. Rat BAL analysis following a 12-mo exposure to an urban pattern of O_3 demonstrated decreased vitamin E and enhanced ascorbate and protein levels (Grose et al., 1989). Because these antioxidants also have been shown to be targets of ozone interaction, any observed increases in their steady-state level suggest an increase in the ratio of production to degradation that could reflect either enhancement in cellular functions in response to continued O_3 exposure or alteration in the number of cells associated with their production.

In order to demonstrate that dietary vitamin E reduces the effects of O_3 exposure on lung biochemical parameters, comparisons between vitamin E-depleted and -supplemented diets have been used (U.S. Environmental Protection Agency, 1986) and reviewed by Pryor (1991). Elsayed et al. (1988) fed rats a test diet containing 0 or 50 International Units (IU) of vitamin E per kilogram for 2 mo prior to exposure to 0.8 ppm O_3 for 8 h/day for 7 days. Ozone exposure increased the whole-lung activities of mitochondrial, microsomal, and cytosolic enzymes. Vitamin E deficiency alone had no significant effect on these lung enzyme measurements, which were taken on a per-lung basis, but the addition of 50 IU vitamin E per kilogram to the diet prior to O_3 exposure diminished the observed O_3-induced increases in mitochondrial succinate cytochrome *c* reductase and GSHPx, microsomal NADPH cytochrome *c* reductase, and cytosolic GSHPx and SOD observed in vitamin E-deficient rats by up to 50%. Additional experiments using a relatively low range of vitamin E supplementation for short time periods demonstrated that, although absence of vitamin E in the diet exacerbates the effects of O_3 on lung injury, the magnitude of a protective effect does not increase proportionately with increased dietary vitamin E. These data support the conclusion that any supplementation beyond the normal recommended daily allowance for vitamin E may not necessarily provide humans with any additional protection against the effects of ambient O_3 exposure (Pryor, 1991). However, possible failure in these animal experiments to reach a steady-state tissue level of vitamin E may have obscured protective effects.

6.2.1.5 Effects of Ozone on Lung Protein Metabolism

Exposure of rodents to 0.45 ppm O_3 has been associated with increases in lung collagen, collagen synthesis, and prolyl hydroxylase activity associated with fibrogenesis (U.S. Environmental Protection Agency, 1986). These earlier studies showed an influence of exposure pattern on the responses. When rats were exposed to 0.8 ppm O_3 for 7 days, prolyl

hydroxylase activity continued to increase, but hydroxyproline content plateaued about Day 3 of exposure and remained elevated 28 days after exposure ceased (Hussain et al., 1976a,b). Last et al. (1984b) employed 90-day exposure regimens of rats to 0.96 ppm O_3 that included (1) a continuous 90-day exposure and (2) intermittent periods of 5 days (8 h/day) of O_3 and 9 days of air, repeated seven times with a total of 35 O_3 exposure days over a 90-day period. Both groups had equivalent increases in lung collagen content. When durations were decreased to 3 weeks, the continuous and intermittent (1 week O_3, then 2 weeks air) regimens resulted in equivalent increases in lung collagen. In nonhuman primates receiving 0.25 ppm O_3 daily or seasonally (every other month) for 18 mo, only the seasonal group had an increase in collagen (Section 6.2.4, Tyler et al., 1988). Results from studies of lung protein metabolism are summarized in Table 6-3.

More recently, Choi et al. (1994) examined the earliest time points from the onset of continuous O_3 exposure of rats to 1.0 ppm that caused alterations in extracellular matrix protein gene expression. These authors demonstrated an early increase in lung fibronectin mRNA at 2 days, which preceded an increase in Type I collagen mRNA observed at 4 days; however, increased collagen content indicated by lung hydroxyproline content was not significantly enhanced until after 7 days of exposure. Pickrell et al. (1987a) demonstrated concentration-dependent decreases in antiproteinase activities in serum and lung tissue of rats exposed to 0.5 and 1.0 ppm O_3 for 48 h. Exposure to 1.0 ppm was accompanied by a concomitant increase in inflammatory-cell-derived proteinases. A second study that examined lung collagen metabolism and proteinolysis in rat lungs exposed to 0.57 and 1.1 ppm O_3 for 19 h/day for 11 days suggested that collagen accumulation, in part, may result from decreased collagen degradation (Pickrell et al., 1987b).

Chronic exposures of monkeys to 0.61 ppm 8 h/day for 1 year demonstrated increased lung collagen content, even 6 mo postexposure (Last et al., 1984b). Further analysis also has demonstrated that the collagen isolated from these O_3-exposed lungs exhibited abnormalities, as indicated by increased levels of the difunctional cross-link dehydrodihydroxylysinonorleucine (DHLNL) and of the ratio of DHLNL to hydroxylysinonorleucine (HLNL) (Reiser et al., 1987). Although collagen content remained elevated, difunctional DHLNL and HLNL cross-link levels returned to normal by 6 mo postexposure, whereas trifunctional mature cross-links (hydroxypyridinium) remained elevated. These data suggest that structurally abnormal collagen is actively synthesized during O_3 exposure and that it becomes irreversibly deposited in the lungs.

Because O_3-induced lung effects are multifocal by nature, it is reasonable that changes in collagen content within the lung may not be easily detectable by measuring alterations in whole lung hydroxyproline at earlier time points or in those experiments that have used lower O_3 concentrations. For example, Wright et al. (1988) calculated values for the extent of lung collagen deposition using measured synthesis rates and concluded that 18 mo of exposure (20 h/day) of rats to concentrations up to 0.5 ppm O_3 did not change either synthesis or accumulation of lung collagen. On the other hand, Chang et al. (1992) demonstrated sustained thickening of rat lung extracellular matrix on long-term exposure to a simulated urban pattern of O_3 exposure (baseline of 0.06 ppm, 7 days/week, with a slow rising peak for 9 h/day, 5 days/week to 0.25 ppm) of up to 38 weeks. More recently, Last et al. (1993a, 1994) observed excess stainable collagen in the lung CAR of rats exposed to 0.5 and 1.0 ppm O_3 for 6 h/day, 5 days a week for 20 mo. Biochemical analyses demonstrated slight

but significant increases in collagen with relatively more hydroxylysine-derived cross-links in female but not male rats, when compared with age-matched,

Table 6-3. Effects of Ozone Exposure on Lung Proteins[a]

Ozone Concentration ppm	Ozone Concentration g/m^3	Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
0.12 0.25 0.5	235 490 980	20 h/day for 18 mo	Rat, M (F344) 28 days old	Age-related increases in hydroxyproline content as a measure of collagen were unaffected by O_3 exposure.	Wright et al. (1988)
0.125 0.25 0.5	245 490 980	1 year	Rat (F344) 6 weeks old	No changes in collagen content, increased turnover at 0.25 ppm after 3 or more mo of exposure.	Filipowicz and McCauley (1986b)
0.12 0.5 1.0	235 980 1,960	6 h/day 5 days/week 20 mo	Rat, M and F (F344) 4-5 weeks old	Excess stainable collagen in the CAR at 0.5 ppm. Biochemical analysis demonstrated slight but significant increases in collagen in female but not male rats exposed to 0.5 ppm with increased hydroxylysine-derived cross-links.	Last et al. (1993a, 1994)
0.25	490	8h/day 7 days/week, "daily" for 18 mo or "seasonal" O_3 odd months for 18-mo period (9 mo of O_3)	*Macaca fascicularis* 6 mo old	Increased collagen content in seasonal group only.	Tyler et al. (1988)
0.4	784	12 h	Rat, M (Wistar) 8 weeks old	Surfactant less able to stimulate AM superoxide anion generation, confirming in vitro results suggesting damage to surfactant protein A.	Oosting et al. (1992)
0.5	980	4 h/day for 2 days and 6 weeks	Sheep, F 23-41 kg	At 2 days, increased sulfated glycoproteins secretion; at 6 weeks, diminished tracheal mucosal gland hyperplasia secretion.	Phipps et al. (1986)
0.5 1.0 1.5	980 1,960 2,940	Continuous for 48 h	Rat, F (F344) 12-14 weeks old	Concentration-dependent decrease in antiproteinase activity at 0.5 and 1.0 ppm. Increases in acid proteinase activity 1.0 and 1.5 ppm correlated with increased inflammatory cell content.	Pickrell et al. (1987a)
0.57 1.1	1,117 2,156	19 h/day for 11 days	Rat, F (F344) 120-180 g	After 11 days of 1.1 ppm O_3, inflammatory cell infiltrate and Type 2 cell and fibroblast proliferation, increased cathepsin D and AM elastase activity, decreased rate of intracellular collagen degradation, and increased extracellular matrix collagen turnover (indicated by enhanced BAL hydroxyproline). These changes preceded increased collagen content observed 50 days PE.	Pickrell et al. (1987b)
0.61	1,196	8 h/day for 1 year	Monkey (Cynomolgus) 6-7 mo old	Increased lung collagen content associated with elevated abnormal cross-links that were irreversibly deposited.	Reiser et al. (1987)

Table 6-3 (cont'd). Effects of Ozone Exposure on Lung Proteins[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m^3				
0.8	1,568	Continuous for 3 days	Rat (S-D) 24-365 days old	Increased lung dry weight, protein, and collagen synthesis greatest after 60 days of age.	
0.8	1,568	6 h/night for up to 90 days	Rat, M (S-D) 10-12 weeks old	Increased lung content of collagen. No change in hydroxypyridinium or elastin.	Last et al. (1993b)
1.0	1,960	Continuous for 14 days	Rat, M (Wistar) 200-250 g	Lung mRNAs for c-myc proto-oncogene and fibronectin enhanced on Day 2 and Type I collagen mRNA not increased until Day 4, preceding increases in collagen hydroxyproline observed on Day 7.	Choi et al. (1994)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

air-exposed control animals. It should be noted that no excess of mRNA for Type I procollagen was observed by in situ hybridization in lungs of rats exposed to 1.0 ppm for 20 mo, although increases after a 2-mo exposure under similar conditions did indicate some increase expression of this mRNA in alveolar interstitial cells (Last et al., 1993a).

Ozone exposure also affects airway secretion of mucous glycoproteins. After 2 days of exposure of sheep to 0.5 ppm O_3, with subsequent evaluation of tracheal sulfated glycoprotein and ion fluxes in vitro, there was an increase in basal secretion that was associated with a moderate hypertrophy of lower tracheal submucosal glands (Phipps et al., 1986). Although 7 days of exposure resulted in hypertrophy of upper and lower tracheal submucosal glands, glycoprotein secretion was reduced, but chloride secretion was increased, which can be explained by a relative decrease in gland mucous content.

6.2.1.6 Effects of Ozone Exposure on Lung Xenobiotic Metabolism

Previous studies have demonstrated that exposure to 0.75 to 1.0 ppm O_3 for a few hours diminishes microsomal cytochrome P-450 content and decreases the activities of benzo[*a*]pyrene hydroxylase and benzphetamine *N*-demethylase of lungs isolated from several different experimental animal species (U.S. Environmental Protection Agency, 1986). Because bronchiolar Clara cells and alveolar Type 2 cells are considered to be relatively enriched with microsomal cytochrome P-450 enzyme systems, it is reasonable that damage and subsequent proliferative repair of these cell types would be expected to change the lung's capacity to conduct xenobiotic metabolism. In a series of rat studies, Takahashi et al. (1985) and Takahashi and Miura (1985, 1987, 1989, 1990) have demonstrated that, although intermittent exposure of 0.4 ppm O_3 for 7 h/day for 14 days did not affect microsomal metabolism, increasing the concentration to 0.8 ppm (Takahashi et al., 1985) or exposing the rats continually to 0.2 and 0.4 ppm for 14 days (Takahashi and Miura, 1985) increased cytochrome P-450 content and the activities of cytochrome P-450 reductase, benzo[*a*]pyrene hydroxylase, and 7-ethoxycoumarin *O*-deethylase (see Table 6-4). These increased microsomal activities were sustained in rats exposed continuously for up to 12 weeks to 0.1 to 0.4 ppm O_3, with a greater response being observed in the activity of benzphetamine *N*-demethylase, suggesting preferential increase in the associated P-450 cytochrome isozyme (Takahashi and Miura, 1987). Ozone-induced increases in cytochrome P-450 also have been shown not to result in concomitant increases in microsomal xenobiotic metabolism (Rietjens et al., 1988). Rat lung microsomal benzo[*a*]pyrene oxidation and benzphetamine demethylation were found to be enhanced after a 6-mo continuous exposure to 0.5 ppm O_3 (Filipowicz and McCauley, 1986a). More recent studies have explored O_3-induced changes in cytochrome P-450 isozyme patterns and correlated changes in lung xenobiotic metabolism with Clara cell enlargement and increased numbers during a 14-day exposure of rats to 0.4 ppm O_3 (Takahashi and Miura, 1990; Suzuki et al., 1992). These authors also demonstrated, by immuno-electron microscopy, the presence of cytochrome P-450b (IIB1) in the Clara cell endoplasmic reticulum.

Changes in the extent and pattern of formation of benzo[*a*]pyrene products were investigated by Bassett et al. (1988c) in lungs from rats undergoing epithelial proliferative repair resulting from 3 days of continuous exposure to 0.6 ppm O_3. Although metabolism to all benzo[*a*]pyrene metabolites was enhanced 4.7-fold, the relative proportion of metabolism involving quinone formation was enhanced from 10 to 25%. The toxicity of other inhaled pollutants that undergo lung xenobiotic metabolism may therefore be dependent not only on

Table 6-4. Effects of Ozone Exposure on Lung Xenobiotic Metabolism[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m^3				
0.1 0.2 0.4	196 392 784	Continuous for 4-12 weeks	Rat, M (Wistar) 19-22 weeks old	Concentration-dependent increases in NADPH-cytochrome P-450 reductase activity and cytochrome P-450 content during 4-12 weeks exposure to 0.2 and 0.4 ppm O_3, reaching a maximum at 12 weeks with concomitant increases in benzo[*a*]pyrene hydroxylase and 7-ethoxycoumarin *O*-deethylase activities. NADH-cytochrome b_5 reductase activity unaffected. Four weeks at 0.1 and 0.2 ppm demonstrated a preferential increase in benzphetamine *N*-demethylase activity, with no alterations in coumarin hydroxylase activity.	Takahashi and Miura (1987)
0.2	392	Continuous for 2 weeks	Rat, M (Wistar) 19-22 weeks old	Increases in cytochrome P-450 isozymes ascribed to constitutive types rather than induction of other types.	Takahashi and Miura (1990)
0.2 0.4	392 784	Continuous for 7 and 14 days	Rat, M (Wistar) 22-24 weeks old	By 14 days, NADPH-cytochrome P-450 reductase activity and cytochrome P-450 content enhanced with concomitant increases in benzo[*a*]pyrene hydroxylase and 7-ethoxycoumarin *O*-deethylase activities by Day 7; no change in NADH-cytochrome b_5.	Takahashi and Miura (1985)
0.4 0.8	784 1,568	7 h/day for 14 days	Rat, M (Wistar)	No effect at 0.4 ppm. 0.8 ppm increased NADPH-cytochrome P-450 reductase activity and cytochrome P-450 content, with concomitant increases on Day 7 in benzo[*a*]pyrene hydroxylase and 7-ethoxycoumarin *O*-deethylase activities that further increased by Day 14.	Takahashi et al. (1985)
0.4	784	Continuous for 6 h, 1-14 days	Rat, M (Wistar) 5 weeks old	By 24 h, Clara cell number decreased, but by 14 days had increased. Increase in cytochrome P-450b (IIB1) on Days 7 and 14.	Suzuki et al. (1992)
0.5	980	Continuous for 1 year	Rat, M (F344)	Microsomal benzo[*a*]pyrene oxidation and benzphetamine demethylase activities enhanced after 6 mo and 1 year of exposure.	Filipowicz and McCauley (1986a)
0.6	1,176	Continuous for 3 days	Rat, M (Wistar) 200-220 g	In isolated perfused lung, increase in overall benzo[*a*]pyrene metabolism but with a greater proportion being metabolized to quinones.	Bassett et al. (1988c)
0.8	1,600	Continuous for 7 days	Rat, M (Wistar) 8 weeks old	Cytochrome P-450, cytochrome b_5, and NADPH-cytochrome P-450 reductase enhanced per lung and per gram lung but not per milligram microsomal protein. No concomitant increases in all cytochrome P-450-dependent reactions, suggesting alterations in isozyme patterns.	Rietjens et al. (1988)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

O_3-induced changes in airway protective barrier function and clearance mechanisms, but also on O_3-induced changes in epithelial cell activation and detoxification reactions.

6.2.1.7 Summary

In vitro studies have provided an indication of a wide range of initial biochemical targets of ozone interaction that include lipid PUFAs, SH-containing proteins, and small-molecular-weight electron donors such as GSH and vitamins E and C. Demonstration that these interactions occur in vivo and are responsible for subsequent cytotoxicity has been more difficult to characterize and mainly has required the use of relatively high (>1 ppm) concentrations of O_3. However, because of the high reactivity of ozone and the relatively high abundance of PUFAs in both cell membranes and epithelial lining fluids, PUFAs are considered to be the most likely initial target of interaction of O_3 with the lung. Current understanding of this interaction is that, in the relatively aqueous environment of the lung airways, hydroxyhydroperoxy intermediates are formed that break down to form aldehydes and hydrogen peroxide. Alternatively, it has been proposed that, in more hydrophobic environments (e.g., within a cell membrane), O_3 interaction with PUFAs yields ozonides and their free radical products. Ozonides, aldehydes, hydrogen peroxide, and other lipid break-down products and oxygen intermediates are therefore considered to be secondary products of the initial O_3 interaction with PUFAs that would account for the observed alterations of cell lipids, SH-containing enzyme proteins, and antioxidants associated with O_3-induced cell damage. As a result of these observations, it has been hypothesized that O_3 most likely does not reach far beyond the surface lining fluids of the upper and lower airways, which are rich in mucopolysaccharides and surfactant lipids, respectively. However, at points where coverage is either discontinuous or thin, epithelial cell components might be expected to directly interact with inhaled O_3.

A wide array of lung biochemical measurements have been made at different times from the onset of O_3-exposure. These measurements have included lung lipids, antioxidants, and enzyme and structural proteins that, in some cases, can be attributed to particular cell populations. However, many of these biochemical determinations cannot be interpreted fully without consideration of the changes in cell population that occur as a result of O_3 exposure. In addition, the sensitivity of some of these measurements has been limited by the relatively small percentage of the whole lung affected by O_3 exposure. The more recent biochemical determinations being made on airway samples isolated by regional microdissection should help overcome some of these limitations.

In vivo experiments have demonstrated cleavage of total lung lipid PUFA double bonds, with arachidonate being a major target of O_3 interaction, the breakdown of cholesterol, and the production of aldehydes and hydrogen peroxide (results that are consistent with ozonation of cell membrane and epithelial lining lipids). The protein A component of the alveolar surfactant system also has been identified as a possible primary target of O_3 interaction. Changes observed in lung lipid biosynthesis during the first few days from the onset of O_3 exposure can be accounted for by concomitant alveolar epithelial proliferative repair. However, lavage-recovered lipids from monkeys following O_3 exposures of 0.12 ppm for 90 days have demonstrated a relative increase in PUFAs and decrease in cholesterol-esters, suggesting some long-term alteration in surfactant lipid composition. However, age-related changes in lavage-recovered lipids and total lung lipid biosynthesis have been shown to be relatively unaffected in rats exposed to 0.5 ppm O_3 for periods of up to 18 mo.

Many studies have utilized whole-lung measurements of antioxidant enzyme changes as indicators of biochemical responses to O_3 exposure. The increased levels of the cytosolic enzymes G6PD, 6PGD, GR, and GSHPx and mitochondrial succinate dehydrogenase observed during the first week from the onset of exposure to O_3 levels of 0.5 to 1.0 ppm are most likely a result of the epithelial proliferation and infiltration of inflammatory cells taking place during this period. Failure to observe similar biochemical changes at lower O_3 concentrations most likely reflects an inability to detect focal changes of altered pathology when using whole-lung tissue samples. Longer-term exposure of rats to an urban pattern of O_3 with daily peaks of 0.25 ppm has demonstrated increases in tissue GSHPx and GR but not SOD. These enzyme changes could reflect changes in either cellular antioxidant capacity in response to chronic O_3 exposure or the steady-state cell population.

Although no long-term changes in collagen content have been observed in rats exposed to <0.5 ppm O_3 for 18 mo, extracellular matrix thickening has been observed in rats exposed to an urban pattern of O_3 with daily peaks of 0.25 ppm for 38 weeks. Exposure of female but not male rats for 20 mo to concentrations of 0.5 and 1.0 ppm O_3 for 6 h/day has demonstrated increased centriacinar stainable collagen and collagen and difunctional cross-links. Similar results were obtained in lungs from monkeys exposed to 0.61 ppm O_3 for 1 year, providing a sensitive indicator that long-term O_3 exposure does cause some fibrogenic alterations to the lung extracellular matrix.

Ozone-induced changes in the extent and pattern of lung microsomal metabolism of xenobiotics have provided consistent results, which may, in part, reflect changes in the numbers and function of bronchiolar epithelial Clara cell and alveolar epithelial Type 2 cells at different durations of O_3 exposure. These cell types are relatively enriched with cytochrome P-450-dependent enzyme systems. Changes in both lung activation and detoxification reactions represent important effects when considering whether or not low-level O_3 exposures alter the ability of the lung to deal adequately with the co-exposure to inhaled xenobiotics found in urban air.

6.2.2 Lung Inflammation and Permeability Changes

6.2.2.1 Introduction

The barrier functions of the airway epithelia have been investigated by isotope tracer techniques for detecting mucosal permeability and by analysis of the BAL for total protein and albumin concentrations. Under normal conditions, the airway epithelia restrict the penetration of exogenous particles and macromolecules from airway lumen into airway interstitium and blood. The integrity of the zonula occludens (tight junctions) is regarded as a major factor in providing barrier properties to the airway epithelia so that only a small amount of intratracheally introduced tracers finds its way across the airway epithelia into the blood. However, disruption of the epithelial barrier creates a leak across the airway mucosa, resulting in increased permeability of serum proteins into the air spaces and of intraluminal exogenous tracers into the blood. Therefore, permeability is generally detected by either the tracer transport from airway spaces to blood or measurement of total protein and albumin in the BAL. Both of these measures are, therefore, taken into account in discussing permeability changes in this section. Although BAL protein measurement offers a good marker for detecting permeability changes, it is important to note that the proteins in the BAL can result from tissue injury and secretory activity, in addition to leakage of the serum proteins across the airway mucosa (Hatch et al., 1989; Hatch, 1992).

Inflammatory cells in the lung constitute an important component of the pulmonary defense system. In their unstimulated state, the inflammatory cells present no danger to other cells or tissues, but, on activation, they are capable of generating proteolytic enzymes such as elastase and reactive oxygen species such as superoxide, hydrogen peroxide (H_2O_2), and the hydroxyl radical. These oxidants can cause substantial injury to cell membranes and intracellular components by their effects on membrane lipids and proteins (biochemical effects of O_3 were described in Section 6.1). Ozone exposure also can cause the epithelial or activated inflammatory cells to liberate arachidonic acid, which is free to enter enzymatic lipoxygenase or cyclooxygenase pathways that lead to the production of leukotrienes (LTs) and prostaglandins (PGs), respectively. Although some of the studies indicate a lack of change in the production and release of cellular mediators following O_3 exposure, other studies demonstrate an elevation in the levels of arachidonic acid and its metabolites in the bronchial washings of rats, as well as humans (see Chapter 7) exposed to O_3 under controlled conditions. The changes in the lung levels of arachidonic acid metabolites generally were observed in animals exposed to O_3 concentrations higher than 0.5 ppm. These cellular mediators can cause a wide range of pathophysiological changes. For example, LTB_4 can cause PMN aggregation and degranulation in vitro and margination of circulating PMNs to capillary endothelium in vivo, whereas LTC_4 and LTD_4 can cause contraction of vascular smooth muscle, PGE_1 has bronchodilator activity, and LTD_4 and $PGF_{2\alpha}$ are regarded as bronchoconstrictors. Because of the toxic potential of the products released by PMNs, AMs, mast cells, and other inflammatory cells, it has been suggested that the recruitment of these cells into the pulmonary interstitium is associated with lung injury and associated edema. An inflammatory response in the lung and an elevation of transmucosal permeability are observed after O_3 exposure, but the interdependence of these two events is a topic of debate. Although AMs are involved in cellular changes during the course of inflammation, AMs are discussed only in terms of their primary function in the section on host defense (Section 6.2.3.4).

The previous O_3 criteria document (U.S. Environmental Protection Agency, 1986) discussed studies available at that time on the inflammatory and permeability effects of O_3. These studies recognized the increased thickness of the alveolar septa, presumably due to increased cellularity after acute exposure to O_3 and excess collagen after chronic exposure to O_3. The inflammatory cell response was reported in rats and monkeys receiving single or repeated exposures to O_3 concentrations ranging from 0.2 to 0.8 ppm (Castleman et al., 1980; Brummer et al., 1977; Moore and Schwartz, 1981; Crapo et al., 1984). Exposures to O_3 also resulted in increased mucosal permeability, as detected by the nonspecific diffusion of phenol red from the lung into circulation (Williams et al., 1980) or the appearance of serum proteins in the air spaces. Increased BAL levels of total protein, albumin, and immunoglobulin (Ig) G were detected in rats, dogs, and guinea pigs exposed acutely to O_3 concentrations ranging from 0.1 to 2.5 ppm (Alpert et al., 1971; Reasor et al., 1979; Hu et al., 1982). For example, Hu et al. (1982) found that a 72-h exposure of guinea pigs to 0.26 ppm O_3 increased BAL protein immediately after exposure and that, when the exposure duration was decreased to 3 h, protein increased 10 to 15 h postexposure (not immediately after exposure ceased).

6.2.2.2 Permeability Changes

A number of studies have demonstrated an increase in airway mucosal permeability following inhalation exposure to O_3 concentrations of 1.0 ppm (Table 6-5).

Table 6-5. Lung Inflammation and Permeability Changes Associated with Ozone Exposure[a]

Ozone Concentration ppm	Ozone Concentration μg/m	Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
0.1 1.2	196 2,352	2 h/day for 1, 2, 6, and 13 days	Rabbit, M (NZW) 2-4 mo old	Increase in AM number at 7 days following single exposure to 0.1 ppm and increase in number of AMs and PMNs on 1-day after cessation of 6 or 13 days of exposure. Increase in number of PMNs at 24 h after single exposure to 1.2 ppm.	Driscoll et al. (1987)
0.1 0.3 1.0	196 588 1,960	2 h	Rabbit, M (NZW) 15-16 weeks old	Increase in levels of PGE_2 and $PGF_{2\alpha}$ in BAL immediately after exposure to 1.0 ppm O_3 only. No significant effects were observed on the levels of 6-keto-$PGF_{1\alpha}$, TXB_2 or LTB_4	Schlesinger et al. (1990)
0.1 0.3 1.2	196 588 2,352	2 h In vitro and in vivo	Rabbit, M (NZW) 2-4 mo old	In vitro: Increase in PGE_2 after 0.3 ppm and increase in $PGF_{2\alpha}$ after 1.2 ppm by AMs. In vivo: Increase in the release of PGE_2 and $PGF_{2\alpha}$ by AMs after 1.2 ppm, but no effect of 0.1 ppm.	Driscoll et al. (1988)
0.1 0.3 1.0	196 588 1,960	2 h In vitro	Rat (S-D) 12-18 weeks old	Increased production of arachidonic acid metabolites by AMs at 1.0 ppm only.	Madden et al. (1991)
0.1 to 10	196 to 19,600	2 h In vitro	Cow	Increased production of PGE_2 and $PGF_{2\alpha}$ by tracheal epithelial cells after exposure to 0.1 and 0.3 ppm. Increased production of other arachidonic acid metabolites at > 1.0 ppm.	Leikauf et al. (1988)
0.1 0.2 0.4 0.8	196 392 784 1,568	2, 4, and 8 h	Rat (F344) 90 days old Guinea pig (Hartley) 60 days old	C × T exposure design; BAL 25 h after exposure started. PMNs measured in rats only; no C and T interaction; effect dependent on C. Exponential and polynomial response surface model used. Similar protein responses at low C × T products; generally, the influence of T increased as C increased. Exponential model explained 86% of the data.	Highfill et al. (1992)
0.1 0.3 1.2	196 588 2,352	2 h In vitro	Rabbit, M (NZW) 2-4 mo old	Exposure of AMs to ≥0.3 ppm O_3 resulted in increased secretion of factors capable of stimulating migration of inflammatory cells.	Driscoll and Schlesinger (1988)
0.1 0.2 1.2 4.0	196 392 2,352 7,840	Continuous for 1 to 12 weeks	Rat, M (Wistar) 16 weeks old for 1 week exposure; 21 weeks old for longer exposures	Number of AMs in BAL increased after exposure for 11 weeks to 0.2 ppm. Infiltration of PMNs did not occur.	Mochitate et al. (1992)
0.12 0.3	235 588	24 h 48 h and 72 h	Mice, M (C57BL/6J[B6]); (C3H/HeJ[C3]) 6-8 weeks old	BAL immediately PE. Comparable increases in BAL protein, AMs, PMNs, and lymphocytes in the two strains after exposure to 0.12 ppm, but greater number of inflammatory cells and protein concentration in B6 than in C3 mice after exposure to 0.3 ppm.	Kleeberger et al. (1993a)

Table 6-5 (cont'd). Lung Inflammation and Permeability Changes Associated with Ozone Exposure[a]

Ozone Concentration ppm	Ozone Concentration µg/m	Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
0.12 0.8 1.5	235 1,568 2,940	6 h	Rat, F (F344/N) 12-18 weeks old	Increased number of PMNs in nasal lavage, but not in BAL at 18 h after 0.12 ppm; increased number of PMNs in BAL, but not in nose after 1.5 ppm; number of PMNs decreased with time in nose, with a concomitant increase in BAL PMNs after 0.8 ppm.	Hotchkiss et al. (1989a)
0.12 0.8 1.5	235 1,568 2,940	6 h	Rat, M (F344/N) 12-18 weeks old	AMs and PMNs increased in number in BAL at various times PE at ≥0.8 ppm.	Hotchkiss et al. (1989b)
0.12 to 0.96	255 to 1,882	6 h, 24 h, or 2 days	Rat, M (S-D) 250-300 g	Total protein in BAL increased after exposure to ≥0.4 ppm for 6 h and ≥0.12 ppm for 1 or 2 days. Transport of radiolabeled albumin from blood to the airways increased after 6- or 24-h exposure to ≥0.4 ppm and after 2 days exposure to 0.2 ppm.	Guth et al. (1986)
0.2 0.5 1.0 2.0	392 980 1,960 3,920	4 h	Mouse (Swiss Albino) 19-25 g Guinea pig (Hartley) 314-522 g Rat (S-D) 280-350 g Rabbit (NZW) 1.7-2.5 kg Hamster (Golden Syrian) 94-107 g	Species differences in responsiveness. At 18-20 h PE, total protein in BAL increased in guinea pigs exposed to 0.2 ppm, whereas mice, hamsters, and rats responded to ≥1.0 ppm, and rabbits responded only to 2.0 ppm.	Hatch et al. (1986)
0.2 0.4 0.6 0.8	392 784 1,178 1,568	6, 8, 12, and 24 h/day for 3 days	Rat (S-D) 10-12 weeks old	C and T matched such that all $C \times T = 14.4$ ppm · h. BAL immediately after exposure ceased. Increase in PMNs equivalent in all O_3 groups. Increase in protein equivalent for 6-, 8-, and 12-h exposure groups, all of which are greater than protein in 24-h groups. Equivalent results for BAL PMNs.	Gelzleichter et al. (1992b)
0.2 0.4 0.6 0.8	392 784 1,176 1,568	7 h/day for 1, 2, or 4 days	Rat, M (PVG) 12-16 weeks old	BAL approximately 17 h PE. The proportion of AMs in the BAL decreased, with a concomitant increase in the proportion of PMNs after 1 or 2 days exposure to ≥0.6 ppm O_3. No significant effect on total number of lavageable cells or on the ability of neutrophils to injure epithelial cells.	Donaldson et al. (1991, 1993)
0.25 peak over a bkg of 0.06	490 118	13 h bkg, rose to peak and returned to bkg over 9 h	Rat, M (F344) 60 days old	Interstitial AMs increased in number in proximal alveolar region and TBs at one week of exposure, but the effects had subsided by 3 weeks of exposure.	Chang et al. (1992)

Table 6-5 (cont'd). Lung Inflammation and Permeability Changes Associated with Ozone Exposure[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m				
0.3 2.0	588 3,920	24, 48, or 72 h for 0.30 ppm and 3 h for 2.0 ppm	Mice (C57BL/ 6J[B6]); (C3H/ HeJ[C3]) DBA/2J (D2), hybrids, and recombinant inbred strains (R1): BXD and BXH 6-8 weeks old	Inflammatory response was greater in B6 than in C3 or D2 mice. F1 progeny was categorized as resistant; F2 generation segregated into 45:16 for resistant vs. susceptible phenotypes. Among BXD R1 strains, 4 of 10 responded discordantly to the two exposures (0.3 and 2.0 ppm). Among BXD R1, 4 of 16 were discordant.	Kleeberger et al. (1990, 1993b)
0.35 0.5 1.0	686 980 1,960	2.25 h/day for 5 days	Rat, M (F344) 3-4 mo old	Persistent increase in BAL protein and progressive inflammation at 0.5 ppm.	Tepper et al. (1989)
0.35 0.5 0.65 0.8	686 980 1,274 1,568	2, 4, and 7 h	Rat, M (F344) 13 weeks old	C × T exposure design. All exposures included 45 min of CO_2 for 1 h to increase ventilation. BAL after pulmonary function tests completed. The quadratic model explained 92% of the variance. The models suggest that C may have a more dominant influence than T.	Tepper et al. (1994, in draft)
0.38 0.76 1.28 2.04 0.13 0.26 0.38	750 1,500 2,500 4,000 250 500 750	1, 2, 4, and 8 h daytime 4, 8, and 12 h nighttime	Rat (Wistar) 7 weeks old	C × T exposure design. BAL protein measured at various times PE. Daytime exposures: At 0.76 ppm, maximal increase 22 h after exposure started; after 4 and 8 h of exposure, protein still elevated at 54 h from start of exposure. Nighttime exposures: Temporal increase and decrease of protein more gradual, with maximal response at 36 h after exposure started. Protein still elevated 72 h after start of 8- or 12-h exposure to 0.26 or 0.38 ppm. Smallest tested C × T effect was with 0.13 ppm × 4 h. Both: Multivariate regression analysis. Polynomial function shows that T has progressive influence as C increases.	Rombout et al. (1989)
0.4 0.6	784 1,176	8 h/days for 90 days	Monkey, M (Bonnet) 5.2-8 years old	Inflammatory response in RBs at 0.64 ppm.	Moffatt et al. (1987)
0.4	800	12 h during day or night	Rat, M (Wistar) Guinea pig, M (Hartley) 9 weeks old	Nighttime exposure of rats resulted in greater increase in BAL protein, albumin, and PMNs than the daytime exposure. A similar difference was not observed in guinea pigs.	Van Bree et al. (1992)

Table 6-5 (cont'd). Lung Inflammation and Permeability Changes Associated with Ozone Exposure[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
ppm	g/m				
0.5	980	2 h	Dog, M (Mongrel) 15 ± 0.9 kg, Baboon, M 25-40 kg	No effect on levels of 6-keto PGF_{1}, PGE_2, TXA_2, TXB_2, or PGF_{2} in BAL.	Fouke et al. (1990, 1991)
0.5	980	Continuous exposure for 1-14 days	Mouse, F (Swiss) 20-25 g	PGE and total protein levels in BAL increased after the exposure, peaked at 3 days, then declined with time, but remained higher than the controls at 7 days; protein still increased at 14 days. Total cells in BAL decreased on Days 1 to 3 after exposure.	Canning et al. (1991)
0.5 1.0	980 1,960	4 h	Guinea pig, M (Hartley) 300-400 g	Depleting lungs of ascorbic acid enhanced effects of 0.5 but not 1.0 ppm on BAL protein. Depletion of lung nonprotein sulfhydryl had no effect.	Slade et al. (1989)
0.75	1,410	Continuous exposure for 3 days	Rat, M (Wistar) 200-250 g	Increased number of PMNs and AMs and elevated levels of albumin in the BAL. At 4 days PE, no PMNs were detected, but AM numbers and albumin levels were elevated.	Bassett et al. (1988a)
0.8	1,568	2 h	Rat, M (S-D) 300 g	Transient increase in tracheal and bronchoalveolar permeability, as revealed by tracer transport from airways to blood and tracer localization in intercellular spaces.	Bhalla et al. (1986) Bhalla and Crocker (1986)
0.6 0.8	1,176 1,568	2 h exposures during rest or exercise	Rat, M (S-D) 47-52 days old	Airway permeability increased after exposure of resting animals; trends of greater and more persistent effects in exercising group.	Bhalla et al. (1987)
0.8	1,568	2 h	Rat, M (S-D) 50-60 days old	Increased transport of radiolabeled tracers from blood to the air spaces following exposure.	Bhalla and Crocker (1987)
0.8	1,568	3 h	Rat, M (S-D) 250-300 g	The number of PMNs in lung parenchyma increased immediately after exposure, peaked at 8 h PE, and returned to baseline by 16 h PE. Total protein and albumin levels in BAL increased immediately after exposure, peaked at 8 h PE, and then declined with time, but the albumin levels were higher than the controls at 24 h PE.	Bhalla and Young (1992)
0.8	1,568	3 h	Rat, M (S-D) 250-300 g	Time-related changes in tracheal permeability, detected by tracer transport, and PMN influx in tracheal wall following exposure. Increase in permeability prior to increase in PMNs.	Young and Bhalla (1992)
0.8	1,568	2 h	Rat, M (F344) 11-12 weeks old	Increased DTPA transport across the tracheal mucosa and elevated levels of protein and albumin in BAL. Effects attenuated in leukopenic rats or rats pretreated with indomethacin or FPL 55712.	Bhalla et al. (1992)

Table 6-5 (cont'd). Lung Inflammation and Permeability Changes Associated with Ozone Exposure[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
ppm	g/m				
0.8	1,568	2 h	Rat, M (F344) 250-275 g	PMNs isolated from blood of O_3-exposed rats displayed deformation of shape, indicative of motility and greater cell adhesion than the PMNs from air-exposed rats.	Bhalla et al. (1993)
0.8	1,568	3 h	Mouse, F (CD-1) 5 and 9 weeks old	Increase in PGE_2 in BAL in 5-week-old mice only; effect blunted by indomethacin pretreatment.	Gilmour et al. (1993b)
0.96	1,882	8 h	Monkey, M (Rhesus) 2-8.5 years old	Number of labeled PMNs into lung tissue and BAL increased immediately after exposure, peaked at 12 h PE, and returned to baseline by 24 h PE. Total labeled and unlabeled PMNs in BAL remained elevated at 24 h, but returned to control levels by 72 h PE. Total protein in BAL was elevated only at 24 h PE.	Hyde et al. (1992)
1.0	1,960	4 to 24 h	Rat (S-D) 63-70 days old	Total protein and PMNs in BAL and PMNs in the CAR of the lung increased with exposure duration, but the number of AMs in BAL decreased. Treatment with anti-rat-PMN serum resulted in elimination of PMNs in BAL, but it did not affect the O_3-induced increase in BAL protein.	Pino et al. (1992a,b)
1.0	1,960	5 min O_3 delivered to a localized area of lung via a Teflon catheter fitted to bronchoscope	Dog, M (Mongrel) 21.2 ± 0.5 kg	Number of PMNs in the subepithelial tissue increased at 1-3 h PE. Number of BAL PMNs increased at 24, but not at 1-3 h PE.	Kleeberger et al. (1989)
1.0	1,960	6 h	Rat, F (S-D) 8-9 and 13-17 weeks old	BAL 16 h PE. Enhanced responsiveness to O_3-induced inflammation and elevated protein levels in BAL developed during pregnancy, was maintained during lactation, and disappeared following lactation.	Gunnison et al. (1992b)
1.0	1,960	2, 4, or 6 h	Rat, M (S-D) 13, 18 days and 8 and 16 weeks old	BAL immediately after exposure. PGE_2 concentrations in BAL greatest in 13-day-old rats after 2 h of exposure, but in older rats the response was seen after 6 h of exposure. In 13-day-old rats, 50% of leukocytes in BAL were dead after 6 h of exposure; no such effect on 16-week-old adults. No age dependence for BAL protein increase or PMN increase.	Gunnison et al. (1992a)
1.0	1,960	2 h	Rat (S-D) 18 days or 14 weeks old Rabbit (NZW) 6, 11, 16, or 30 weeks old	BAL immediately after exposure. In youngest animals, greater amounts of PGE_2 and PGF_{2}. In youngest rabbits, 6-keto PGF_{1} and TXB_2 increased. No effect on LTB_4. No age dependent effects on BAL protein or cell number.	Gunnison et al. (1990)

Table 6-5 (cont'd). Lung Inflammation and Permeability Changes Associated with Ozone Exposure[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
ppm	g/m				
1.0	1,960	1 h	Guinea pig, M (Hartley) 300-400 g	Appearance of horseradish peroxidase in plasma, following its intratracheal administration, was accelerated at 2 and 8 h PE, but not at 24 h PE.	Miller et al. (1986)
1.0	1,960	1 h	Guinea pig, M (Hartley) 250-300 g	The concentrations of PGE_1, 6-keto PGF_{1}, and TXB_2 in BAL increased at various times following exposure.	Miller et al. (1987)
1.0	1,960	3 h isolated perfused lung	Rat (S-D) 350 ± 42 g	No effect on BAL protein.	Joad et al. (1993)
1.8	3,528	2 or 4 h	Rat, M (Wistar) 200-250 g	A decrease in number of AMs in BAL immediately after exposure. PMNs and albumin content of BAL increased at 1 day PE. Increased albumin levels, but not PMNs, persisted on Day 3 PE.	Bassett et al. (1988b)
2.0	3,920	4 h	Guinea pig, M (Hartley) 300-350 g	Interstitial PMNs increased in number immediately after exposure, but declined by 24 h PE. BAL PMNs were maximal by 3-6 h and remained elevated by 3 days PE.	Schultheis and Bassett (1991)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

In rats exposed for 2 h to 0.8 ppm O_3, labeled tracers, such as diethylenetriaminepentaacetate (DTPA) and bovine serum albumin, introduced into the airway lumen were transferred to blood to a greater extent than in the air-exposed rats (Bhalla et al., 1986; Bhalla and Crocker, 1986; Crocker and Bhalla, 1986). The rapidly rising concentration of the tracers in the blood during the initial period of instillation of the tracers into the airways reflected both the accumulation, due to slow instillation over a 5-min period, of the tracers in the airway lumen and subsequent transfer across the respiratory epithelium. The changes in permeability observed in this study were transient in nature, returning to the baseline value by 24 h postexposure in the trachea and by 48 h in the distal airways. Reversible increases in airway epithelial permeability also were observed in guinea pigs acutely exposed to 1 ppm O_3 (Miller et al., 1986). The rate of appearance of intratracheally administered horseradish peroxidase increased in blood at 2 and 8 h after O_3 exposure, as compared to rats at 24 h postexposure to O_3 and air-exposed controls. When rats were exercised at a level that increased the minute ventilation ($\dot{V}_E$) twofold, the effect of 0.8 ppm O_3 was not only greater than in rats exposed at rest, but the increased permeability persisted longer (Bhalla et al., 1987).

Guth et al. (1986) analyzed the permeability effects of O_3 by injecting radiolabeled albumin into the blood and measuring it in the BAL, as well as by measuring the total protein concentration in the BAL. This study revealed a concentration-dependent increase in permeability following a 6-h exposure of rats to 0.4 ppm or following 1 or 2 days of exposure to 0.12 ppm O_3. For example, after a 2-day exposure to 0.12 ppm, there was a 71% increase in BAL protein. Tracer transport also was increased in rats exposed for 2 h to 0.8 or 2.0 ppm O_3 (Crocker and Bhalla, 1986; Bhalla and Crocker, 1987).

The relative influence of concentration and duration of O_3 exposure was evaluated by three laboratories using BAL protein as an indicator of effects. In the first study, Rombout et al. (1989), exposed rats for 1, 2, 4, or 8 h to 0.38, 0.76, 1.28, or 2.04 ppm O_3 during the daytime (16 C × T products). A similar nighttime exposure study was conducted using 0.13 to 0.38 ppm O_3 and 4, 8, or 12 h of exposure (nine C × T products). The smallest C × T product causing an increase in protein was 0.52 ppm · h (0.13 ppm × 4 h). A multivariate regression analysis accounted for 88.6% of the variance in the daytime data and 73.2% in the nighttime data. Animals exposed during the night were more responsive. A quadratic polynomial function showed that the influence of T increased with increasing C and that the influence of T was still important at the lowest O_3 concentration tested (0.13 ppm). The second study employed rats and guinea pigs, each having 12 C × T products (0.1, 0.2, 0.4, and 0.8 ppm O_3; 2, 4, and 8 h) (Highfill et al., 1992). Using additional modeling approaches, they obtained similar results to those of Rombout et al. (1989) . For example, the exponential response surface model explained 86% of the variance in the data and showed that the influence of T increased as C increased. However, at low C × T products, similar BAL protein increases were observed. Further modeling of these data (Highfill and Costa, 1995) again showed that C and T had interdependent influences. Tepper et al. (1994) performed a similar C × T study with 12 C × T products (0.35 to 0.8 ppm O_3, 2 to 7 h). However, rats were exposed to 8% CO_2 for 45 min of each hour to increase ventilation, and BAL was conducted on lungs that had been measured for pulmonary function. The response surface predicted by the modeling again indicated that the influence of T increased as C increased. Histopathological observations in the rats support the findings that C had more influence than T. Tepper et al. (1994) compared their analysis of BAL protein to that of Highfill et al. (1992) and found very good agreement, even though there were experimental differences. However,

in Tepper et al. (1994), there were larger constants for C terms, indicating that C had a greater influence than in the Highfill et al. (1992) study, probably because Tepper and co-workers increased ventilation (and hence O_3 dose) by using concurrent CO_2 exposures.

Gelzleichter et al. (1992b) exposed rats to a single O_3 C × T (14.4 ppm · h) composed of 16 products (0.2 to 0.8 ppm O_3, 6 to 24 h/day for 3 days). They found that the 24 h/day exposure groups had significantly fewer responses than the other groups, which were all equivalent. Thus, in this study, C and T had equivalent influences on the response, except when T was 24 h/day. This study was well conducted, but had some basic differences from the Rombout et al. (1989) and Highfill et al. (1992) studies in that the longer exposure durations (i.e., 24 h/day) involved a mixture of daytime and nighttime exposure that likely altered the dose-rate of O_3. Also, Gelzleichter et al. (1992b) used one C × T product, whereas the other studies used several C × T products.

6.2.2.3 Concomitant Changes in Permeability and Inflammatory Cell Populations in the Lung

Polymorphonuclear leukocyte infiltration in the lung following O_3 exposure has been investigated in a number of studies (Table 6-1), either by analyzing the cellular content of the BAL or by counting PMNs in lung sections. Bassett et al. (1988a) found an increase in the number of inflammatory cells in the BAL of rats continuously exposed for 3 days to 0.75 ppm O_3. The inflammatory response was accompanied by elevated levels of albumin and lactate dehydrogenase, suggesting increased permeability and cellular injury. Comparable changes were also observed in rats acutely exposed to a higher O_3 concentration (Bassett et al., 1988b). In another study, a random count of PMNs in the lung sections at 4-h intervals, following a 3-h exposure of rats to 0.8 ppm O_3, revealed a gradual increase in the number of PMNs, with a peak at 8 h postexposure and a return to the baseline value by 16 h postexposure (Bhalla and Young, 1992). The total protein and albumin concentrations in the BAL also increased after the exposure, peaking at 8 h postexposure. Although the protein concentrations returned to baseline by 16 h postexposure, the albumin levels remained above the controls after 24 h. Alveolar changes, consisting of thickened septa, parenchymal cellularity, and increased numbers of free cells, began to increase between 12 and 16 h postexposure and were still increasing at 24 h postexposure.

In trachea of rats exposed for 3 h to 0.8 ppm O_3, a peak of PMN infiltration at 12 h postexposure was preceded by a decline in the number of PMNs in pulmonary capillaries, suggesting exit of PMNs from the blood vessels and their migration across the endothelial cells into the tracheal wall (Young and Bhalla, 1992). Although a significant change in the tracheal population of PMNs did not occur until 12 h after the end of exposure, tracheal permeability, as detected by DTPA transport, increased immediately following O_3 exposure. The results of this study suggest that the initial changes in tracheal permeability may be independent of an inflammatory response, but the recruited PMNs may serve to sustain the increased permeability and to amplify O_3 effects at later stages. This conclusion was based on the observed shift of PMNs from the vascular compartment into the tracheal wall and a concurrent peak of increased permeability. In comparable studies, Pino et al. (1992a) exposed rats to 1.0 ppm O_3 for periods ranging from 4 to 24 h. Total protein and the number of PMNs in the BAL increased with time, with the maximum increase at the end of 24 h of continuous exposure. The number of AMs was lower in the exposed animals than in the controls. By morphometry, the peak PMN response in the terminal bronchioles (TBs) and alveolar ducts

(ADs) occurred at 4 h after an 8-h exposure. In dogs, local exposure of peripheral airways to 1 ppm O_3 for a short period (5 min) produced a recognizable inflammatory response (Kleeberger et al., 1989). An increase in the number of PMNs was detected in the subepithelial tissue within 3 h after 5 min of exposure of the dogs, but the response had subsided 24 h later. In BAL, on the other hand, an increase in the number of PMNs was not observed at 3 h postexposure; the number of PMNs increased at 24 h.

Hotchkiss et al. (1989a,b) have investigated the effects on AMs and PMNs of a 6-h O_3 exposure of rats to 0.12, 0.8, or 1.5 ppm O_3 and compared the inflammatory responses by nasal lavage and BAL, as well as by morphometry in the nose and the CAR of the lung, a site at which abnormal cellular changes generally occur following O_3 exposure. Animals were examined 3, 18, 42, or 66 h after exposure ceased. From lavage data, 0.12 ppm O_3 had no effect. At 0.8 ppm, there was an increase in the number of nasal PMNs lavaged immediately after exposure, which tapered off (no significant change at 42 h postexposure). In contrast, BAL PMNs increased later, beginning at 18 h postexposure and peaking at 42 h postexposure. From morphometric data, 0.12 ppm O_3 caused an increase in nasal PMNs 66 h postexposure. At 0.8 ppm, nasal PMNs increased to their greatest extent immediately after exposure and still were increased at later time periods. However, PMNs in the lung increased only at 18 and 66 h postexposure. The interpretation of these results was based on the presence of potential competing mechanisms in the nose and lungs. Therefore, the attenuation of the nasal effects are matched by simultaneous enhancement of the inflammatory response in the lung. Whether such a balance between nasal and alveolar PMNs represents a specialization restricted to rats or is a more general phenomenon remains to be investigated. A similar balance was not observed in humans exposed to O_3 (see Chapter 7). Subtle differences in species, O_3 concentrations, and exposure durations, however, need to be considered when making interspecies comparisons.

Hyde et al. (1992) investigated the inflammatory response in monkeys exposed to 0.96 ppm O_3 for 8 h. Polymorphonuclear leukocytes were isolated from peripheral blood, labeled with indium-111-labeled tropolonate and infused into the cephalic vein of monkeys 4 h before necropsy. Labeled PMNs in the lung tissue and the BAL peaked at 12 h and returned to control values by 24 h postexposure. The total number of labeled and unlabeled PMNs in the BAL, however, remained elevated at 24 h postexposure, but returned to baseline by 72 h. Furthermore, the PMN peak at 24 h postexposure coincided with the maximum increase in BAL protein at this time point. These studies suggest a strong correlation between BAL protein concentration, epithelial necrosis, and inflammatory cells (especially eosinophils) in bronchi, but not in the trachea or bronchioles. This observation may represent a species-specific response. In rats, the inflammatory response in the terminal airways involved an increase in the number of migratory cells, including PMNs, but not eosinophils (Pino et al., 1992a). The available literature suggests that the precise time point at which the maximum change in the number of inflammatory cells occurs is variable and may be dependent on several factors, including animal species, concentration, duration of exposure, and mode of analysis (i.e., BAL versus morphometry of lung parenchyma). Because the PMNs sampled by BAL represent only a small fraction of the cells shown to be present in the air spaces by morphometry (Downey et al., 1993), the inflammatory response detected by analyzing BAL may or may not match the response obtained by microscopic analysis of tissue sections. Even when the PMN response detected by the BAL analysis accurately reflects the tissue PMNs (Hotchkiss et al., 1989a), the times at which the PMN response peaks do not necessarily coincide when the analyses are made by the two procedures. Therefore, the mode of analysis

(BAL versus morphometry) and the time at which this analysis is made need to be taken into account when analyzing the inflammatory response. This recommendation is consistent with the conclusion of Schultheis and Bassett (1991) that BAL does not necessarily reflect cellular changes in the lung interstitium.

Another approach to studying the inflammatory impact of O_3 and its effects on airway permeability is based on exposure of rats to drugs that destroy leukocytes or block the activity of chemical mediators released by these cells. To determine whether the PMNs play a role in O_3-induced increased permeability, Pino et al. (1992b) studied O_3 effects in PMN-depleted rats. Although ip injection of anti-PMN serum resulted in a nearly complete depletion of PMNs in rats, it did not affect the increase in BAL protein following an 8-h exposure to 1.0 ppm O_3. In comparable studies, rats were rendered leukopenic by ip injection of cyclophosphamide (Bhalla et al., 1992). A 2-h exposure of untreated rats to 0.8 ppm O_3 caused a significant increase in the tracheal mucosal permeability, as measured by enhanced trachea-to-blood transport of ^{99m}Tc-radiolabeled DTPA immediately postexposure and accumulation of protein and albumin in BAL at 12 h postexposure. Pretreatment with cyclophosphamide, a potent immunosuppressive agent, which can be toxic to the lung, did not change baseline values but did eliminate the O_3 response. The reasons for the discrepancy between these results and the results of the anti-PMN serum treatment study of Pino et al. (1992b) are not entirely clear, but it is likely that the O_3 effects are dependent, in part at least, on an interaction between different inflammatory cell types. Therefore, it is not unreasonable to assume that, in the absence of PMNs, their role is taken up by another cell type. The attenuation of O_3 effects also was observed in rats pretreated with indomethacin, an inhibitor of cyclooxygenase products, and FPL55712, which blocks LTD_4 activity by preventing its binding to the receptors (Bhalla et al., 1992). Based on these results, it was proposed that, although O_3 is capable of producing direct injury to cells, inflammatory cells and their products may contribute to the injury process (Bhalla et al., 1992). This conclusion is supported by the recent studies of Joad et al. (1993). In the isolated perfused rat lung, PMNs (but not O_3) increased BAL protein concentration. However, PMNs acted synergistically with O_3 in the induction of epithelial injury in the bronchioles. The recent demonstration of the effects of O_3 (0.8 ppm, 2 h) on some of the cellular activities of vascular PMNs (Bhalla et al., 1993) further suggests potential mechanisms involved in the stimulation of PMNs and the induction of inflammatory response. Polymorphonuclear leukocytes isolated from the blood of rats exposed to O_3 displayed shape changes, indicative of cell motility, and greater adhesion to epithelial cells in culture than did the PMNs from rats exposed to purified air.

6.2.2.4 Sensitive Populations

In addition to investigating the inflammatory response and permeability changes in healthy adult animals, studies in recent years have analyzed the effects of O_3 on lung inflammation and airway permeability in different animal species, in potentially susceptible subpopulations, and under special conditions (Table 6-1). Hatch et al. (1986) performed an interspecies comparison to determine their relative responsiveness to O_3. Although the baseline BAL protein concentration of all the species was nearly the same, there were noticeable differences in changes in BAL protein concentration among different species following their exposure to O_3. Significant changes were observed in guinea pigs exposed to 0.2 ppm O_3. Mice, hamsters, and rats responded at O_3 concentrations of 1 ppm and above, but rabbits responded only to 2 ppm O_3. In the case of rats, no differences were observed in the

sensitivity between males and females. When Slade et al. (1989) depleted guinea pigs of lung ascorbic acid, they were more susceptible to an O_3-induced increase in BAL protein when the 4-h exposure was to 0.5 but not 1.0 ppm. Depletion of lung nonprotein sulfhydryls did not enhance susceptibility. Although Hu et al. (1982) report no elevation in BAL protein in O_3-exposed, vitamin C-deficient guinea pigs, when their data were statistically reanalyzed by Slade et al. (1989), vitamin C deficiency enhanced the effects of 0.5 but not 1.0 ppm O_3.

Kleeberger et al. (1990) found interstrain differences in inbred mice with regards to inflammation and permeability changes following high-concentration (2-ppm) O_3 exposure for 3 h that led the investigators to propose that the PMN response to O_3 may be controlled by a single autosomal recessive gene at a chromosomal location designated as "Inf" (inflammation) locus. In the follow-up studies, Kleeberger et al. (1993a) exposed the "susceptible" (C57BL/6J) and the "resistant" (C3H/HeJ) strains of mice to lower concentrations of O_3. Although changes in inflammatory response and BAL protein were observed after exposure for 24 to 72 h to 0.12 as well as 0.3 ppm O_3, the elevation in response in the susceptible strain over that in the resistant strain was observed only at 0.3 ppm O_3. Further studies with recombinant inbred strains of mice suggested that genes at different loci may be responsible for responses to 24-h (Inf locus) and 48-h (Inf-2 locus) O_3 exposures (Kleeberger et al., 1993b).

Gunnison et al. (1992a) exposed rats aged 13 and 18 days and 8 and 16 weeks old to 1 ppm O_3 for 2, 4, or 6 h. In the experiments to be discussed here, BAL was performed immediately after exposure. Ozone exposure resulted in an increase in protein in the BAL and a decrease in the number of leukocytes, but this decrease was not specific for a certain age group. A weak relationship was observed between age and the number of lavageable PMNs; a slightly greater influx of PMNs was observed in the younger rats. A strong inverse relationship was, however, observed between age and leukocyte viability. Approximately 50% of the total leukocytes recovered in the BAL from 13-day-old rats exposed for 6 h were dead, as compared to about 10% dead in the 16-week-old rats. Furthermore, 13-day-old animals were more responsive to a 2-h O_3 exposure than the other age groups of rats in terms of PGE_2 levels in BAL; PGE levels were enhanced more in older animals with the longer exposure durations. The authors attribute the increase in PGE_2 to an increased release of arachidonic acid, rather than an effect on metabolism or formation on PGE_2. Gunnison et al. (1990) also have shown that levels of several eicosanoids in rabbits show a similar pattern of age-responsiveness.

Factors such as physical activity and pregnancy, in addition to age, can modify the airway sensitivity of rats to O_3. Van Bree et al. (1992) have reported circadian variation in response to O_3. In rats exposed to 0.4 ppm O_3 for 12 h, about 70% more PMNs were recovered in the BAL after nighttime exposure than after daytime exposure. This increase was attributed to greater physical activity and increased ventilation in the nocturnal animals. In guinea pigs, a similar difference between daytime and nighttime exposures was not observed; instead, the variations appeared to be related to random physical activity. The nighttime exposures also caused a greater increase in BAL protein and albumin in rats but not in guinea pigs. Gunnison et al. (1992b) have found that pregnant rats are more responsive to O_3 (1 ppm for 6 h) than virgin females, as measured by an enhanced inflammatory response and as detected by the analysis of protein, PMNs, leukocytes, and enzyme activities in BAL at 18 h postexposure. When O_3 exposure occurred on Day 17 of pregnancy or Days 3, 13, and 20 of lactation, the magnitude of the increase in BAL protein and number of PMNs was greater than the magnitude of increase in virgin rats. No such increased responsiveness was observed in

rats at Day 10 to 12 of pregnancy or 14 days after lactation ceased. Enzyme changes followed a similar pattern.

6.2.2.5 Repeated Exposures

The magnitudes of some of the effects of O_3 observed after an acute exposure were smaller following repeated exposure. This phenomenon has been referred to as tolerance, adaptation, or attenuation. Most of the older literature is on tolerance, which classically is defined as the phenomenon in which a previous exposure to a nonlethal concentration of O_3 provides protection against an otherwise lethal level. These studies and others at high O_3 levels are discussed in the last O_3 criteria document (U.S. Environmental Protection Agency, 1986).

Tepper et al. (1989) observed attenuation of pulmonary function changes in rats exposed for 2.25 h/day for 5 days, but a corresponding attenuation of lung inflammation did not occur. Histologic examination of the lung sections revealed substantially more inflammatory cells in alveoli after 5 days of exposure to 0.5 ppm O_3 than after a single exposure to the same O_3 concentration. Increased protein concentration in the BAL observed after a single exposure also persisted after 5 days of repeated exposures. The morphologic studies of Moffatt et al. (1987) identified an inflammatory response in the respiratory bronchioles (RBs) of bonnet monkeys exposed for 8 h/day for 90 days to 0.64 ppm O_3. Significantly greater numbers of AMs, mast cells, and PMNs reflected persistence of inflammation following repeated exposures. Chang et al. (1992) exposed rats to an ambient pattern of O_3. In this morphometric study, the responses (epithelial inflammation in the proximal alveolar region and the TBs, interstitial edema, and infiltration of AMs) to 1 week of O_3 exposure had subsided after 3 and 13 weeks of exposure. Donaldson et al. (1993) did not find a change in the total number of cells in BAL of rats exposed for 7 h/day for 4 days to O_3 concentrations ranging from 0.2 to 0.8 ppm. The number of PMNs, however, increased after exposure to 0.6 and 0.8 ppm O_3. This increase was greatest after the first day of exposure, but it was resolved by Day 4. In the studies of Mochitate et al. (1992), the number of BAL AMs of rats exposed to 0.2 ppm O_3 for 11 weeks was about 60% greater than in the air-exposed controls. The preferential increase in the number of small AMs was not dependent on an enhancement of DNA synthesis. It was concluded that AMs adapt to long-term exposures as a result of recruitment of immature AMs from an influx of monocytes. No increase in the number of PMNs was observed in the BAL of exposed rats.

When analyzing the PMN data from different studies like the ones discussed above, it is important to make a distinction between the PMN response in the lung interstitium versus that observed in the BAL. It is possible that, although the inflammatory response may persist after repeated exposures, the PMNs do not necessarily continue to migrate from pulmonary interstitium into the air spaces. As a result, the inflammatory response is detected in the histological sections of the lung but not in the BAL.

6.2.2.6 Mediators of Inflammation and Permeability

Although the presence of PMNs in the lung in large numbers is regarded as evidence of a morphological response to O_3, the release of chemical mediators by inflammatory cells indicates a state of activation and represents the functional modification as a consequence of O_3 exposure. Mediators with biological and chemotactic properties have been shown to be released as a result of stimulation or injury of AMs, epithelial cells, and PMNs. Arachidonic

acid metabolites play an important role in a variety of processes, including inflammatory response and permeability changes. Driscoll and Schlesinger (1988) found that, although AMs isolated from rabbit lungs continually released chemoattractant factors for monocytes and PMNs, an in vitro exposure of AMs to 0.3 and 1.3 ppm O_3 resulted in the increased secretion of factors that stimulated the migration of PMNs. Driscoll et al. (1988) also found increased eicosanoid biosynthesis following O_3 exposure. In the latter studies, elevated levels of PGE_2 and $PGF_{2\alpha}$ were detected in the supernatant following in vitro exposure of rabbit AMs to 0.3 and 1.2 ppm O_3 for 2 h. In a parallel in vivo study, an effect was seen only at the higher O_3 concentration. In vitro exposure in a roller-bottle system of rat AMs to 1 ppm (but not 0.1 ppm) O_3 causes stimulation of both cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism, as shown by substantial increases in the levels of 6-keto-$PGF_{1\alpha}$, thromboxane B_2 (TXB_2), PGE_2, LTB_4, LTD_4, and 15-hydroxy-eicosatetraenoic acid (15-HETE) in the supernatant of the AM culture (Madden et al., 1991). The authors attribute these effects to both an increase in the availability of arachidonic acid and a stimulation of cycloxygenase and lipoxygenase activities. Another in vitro study also demonstrated effects on arachidonic acid metabolism (Leikauf et al., 1988) . Interpretation of these in vitro studies is difficult. When Gunnison et al. (1990) compared the effect of in vitro and in vivo exposures of AM to O_3 on eicosanoid metabolism of AMs in culture, a disparity was found. Cultured AMs from O_3-exposed rabbits had a decrease in the elaboration of $PGF_{2\alpha}$; in vitro exposure caused an increase.

Changes in the levels of eicosanoids also have been observed in vivo studies. Schlesinger et al. (1990) found elevation of PGE_2 and $PGF_{1\alpha}$ in BAL of rabbits immediately following a 2-h exposure to 1 ppm O_3. Age may play a role. Five-week-old (but not 9-week-old) mice had increased levels of PGE_2 in BAL (Gilmour et al., 1993b). Lower O_3 concentrations did not affect the levels of BAL eicosanoids. Hyde et al. (1992) found an increase in BAL concentrations of $PGF_{2\alpha}$, PGD_2, and PGE_2 following an 8-h exposure of monkeys to 0.96 ppm O_3. Prostaglandin concentrations in the BAL, detected using an antibody that did not distinguish PGE_1 from PGE_2, also increased with time following a continuous exposure of mice to 0.5 ppm O_3 (Canning et al., 1991). The peak levels of PGE at 3 days were followed by a decline with time, but the levels remained higher than the controls after 14 days of exposure. The time course of changes in the PGE levels was matched by the time sequence of changes in BAL protein following exposure to 0.5 ppm O_3. Plasma concentrations of 6-keto-$PGF_{1\alpha}$ and PGE_1 also were elevated in guinea pigs exposed for 1 h to 1 ppm O_3 (Miller et al., 1987). Kleeberger et al. (1989) delivered 1 ppm O_3 for 5 min to a lobar bronchus of dogs using a wedged bronchoscope. An analysis of the lavage fluid collected at 1 min postexposure revealed significant increases in the concentrations of PGD_2 and histamine. Although, in this study, the concentration of TXB_2 did not change after O_3 exposure, significant increases in concentrations of TXB_2 in the plasma and BAL were observed following acute exposure of guinea pigs to 1 ppm O_3 (Miller et al., 1987) and humans to lower levels of O_3 (see Chapter 7). In addition, increased plasma concentrations of $PGF_{1\alpha}$ and PGE_1 were observed in the guinea pigs exposed to O_3. Fouke et al. (1990, 1991) were unable to detect changes in the BAL concentrations of 6-keto-$PGF_{1\alpha}$, PGE_2, TXB_2, and $PGF_{2\alpha}$ in baboons and mongrel dogs exposed to 0.5 ppm O_3 for 2 h. The reasons for the lack of this response in the baboon are not entirely clear, but the lower O_3 concentration used in this study, as compared to the exposure concentrations in dogs and guinea pigs, and species differences offer possible explanations for the discrepancy. Prostaglandin E_1 and E_2 have been shown to

influence the inflammatory processes in the lung. Intrabronchial or iv administration of PGE_2 was accompanied by increased accumulation of the inflammatory cells in the lung and elevation of BAL protein (Downey et al., 1988). A possible mechanism involved in the proinflammatory effects of the PGEs included arteriolar vasodilation without venodilation, resulting in increased transfer of proteins and cells from blood into the lung by hydrostatic pressure.

6.2.2.7 Summary

The airway epithelial lining serves as an efficient barrier against penetration of exogenous particles and macromolecules into the lung tissue and circulation and against entry of endogenous fluids, cells, and mediators into the air spaces. Disruption of this barrier following O_3 exposure represents a state of compromised epithelial defenses, leading to increased transepithelial permeability. Inflammatory cells represent another important component of pulmonary defenses. The recruitment of these cells into the lung following O_3 exposure could result in the release of mediators capable of damaging other cells in the lung.

Toxicological studies from several laboratories demonstrate alterations in epithelial permeability and inflammatory responses in animals exposed to O_3 concentrations of 1.0 ppm and below. In these studies, an inflammatory response, as detected by an increase in the number of PMNs in the BAL or in lung parenchyma, was accompanied by either an increased tracer transport across the airway mucosa or an elevation in the levels of total protein or albumin in the BAL. These changes were observed in animals exposed to O_3 concentrations as low as 0.1 ppm in rabbits (2 h/day for 6 days of exposure [Driscoll et al., 1987]; 0.12 ppm in mice (24 h-exposure [Kleeberger et al., 1993a]) and rats (6-h exposure [Hotchkiss et al., 1989a] and 24-h exposure [Guth et al., 1986]); and 0.2 ppm in guinea pigs (4-h exposure [Hatch et al., 1986]). Although monkeys also exhibit inflammatory responses, concentrations this low have not been tested in this species. The magnitude of response and the time at which it peaked appeared to vary with O_3 concentration, exposure duration, and the mode of analysis. Investigations of C $\times$ T relationships for BAL protein in both rats and guinea pigs showed that T had increasing influence as C increased (Rombout et al., 1989; Highfill et al., 1992; Highfill and Costa, 1995; Tepper et al., 1994). However, at low C $\times$ T products, similar increases were observed (Highfill et al., 1992). The responsiveness to O_3 also depended on the animal species tested (Hatch et al., 1986) and increased under certain conditions, such as physical activity (Van Bree et al., 1992) and pregnancy and lactation (Gunnison et al., 1992b).

To determine the impact of inflammatory cells on O_3-induced airway permeability, rats were exposed to drugs that either destroyed the inflammatory cells or blocked the activity of their products. Treatment of rats with anti-PMN serum resulted in the depletion of PMNs but did not affect the increase in BAL protein produced by O_3 exposure (Pino et al., 1992b). Depletion of all the leukocytes by cyclophosphamide or treatment of rats with PG and LT antagonists resulted in an attenuation of the O_3 effects on permeability (Bhalla et al., 1992).

Inflammatory cells, when activated, are capable of releasing mediators with pathophysiologic and a variety of modulating activities. An increase in the release of arachidonic acid metabolites following O_3 exposure has been shown after both in vitro (Driscoll and Schlesinger, 1988; Driscoll et al., 1988; Madden et al., 1991; Leikauf et al., 1988) and in vivo exposures (Schlesinger et al., 1990; Miller et al., 1987; Canning et al., 1991).

Some of the effects seen after an acute exposure to O_3 are modified on repeated exposure. The responses following repeated exposures included persistence or an increase in the number of PMNs or AMs on exposure of rats to 0.5 ppm (2.25 h/day) for 5 days (Tepper et al., 1989) or exposure of monkeys to 0.64 ppm for 90 days (Moffatt et al., 1987). Other studies report a reduced inflammatory response following repeated exposures (Chang et al., 1992; Donaldson et al., 1993). The section on morphometry (Section 6.2.4) has an extended discussion of microscopically evaluated inflammatory responses.

In brief, these studies show that acute exposures to O_3, at concentrations of 0.12 ppm and above, are capable of producing inflammatory and permeability changes in laboratory animals. It is clear that an assessment of the effects of O_3 and interpretation of the results requires that several factors be taken into consideration; these include O_3 concentration, duration of exposure, exposure conditions (e.g., repeated versus continuous exposure, daytime versus nighttime exposure, rest versus exercise during exposure), animal species, method of evaluation, sensitive populations, and time of analysis postexposure.

6.2.3 Effects on Host Defense Mechanisms

6.2.3.1 Introduction

The mammalian respiratory tract has a number of closely integrated defense mechanisms that, when functioning normally, provide protection from the adverse effects of a wide variety of inhaled particles and microbes (Green et al., 1977; Kelley, 1990; Schlesinger, 1989; Sibille and Reynolds, 1990). For simplicity, these interrelated defenses can be divided into two major parts: (1) nonspecific (transport and phagocytosis) and (2) specific (immunologic) defense mechanisms. A variety of sensitive and reliable methods have been used to assess the effects of O_3 on these components of the lung's defense system to provide a better understanding of the health effects associated with the inhalation of this pollutant.

The previous *Air Quality Criteria for Ozone and Other Photochemical Oxidants* (U.S. Environmental Protection Agency, 1986) provided a review and evaluation of the scientific literature published up to 1986 regarding the effects of O_3 on host defenses. This section briefly summarizes the existing database through 1986; describes the data generated since 1986; and, where appropriate, provides interpretations of the data. This section also discusses the various components of host defenses, such as the mucociliary escalator, the phagocytic and regulatory role of the AMs, the immune system, and integrated mechanisms that are studied by investigating the host's response to experimental pulmonary infections.

6.2.3.2 Mucociliary Clearance

This nonspecific defense mechanism removes particles deposited on the mucous layer of the conducting airways by ciliary action. Ciliary movement directs particles trapped on the overlying mucous layer toward the pharynx, where it is swallowed or expectorated. The effectiveness of the mucociliary transport system can be measured by the rate of transport of deposited particles, the frequency of ciliary beating, and the structural integrity of the cells that line the conducting airways. Impaired mucociliary clearance can result in an unwanted accumulation of cellular secretions, increased infections, chronic bronchitis, and complications associated with chronic obstructive pulmonary disease.

Studies cited in the previous criteria document (U.S. Environmental Protection Agency, 1986) provided evidence on the effect of O_3 on the morphologic integrity of the mucociliary escalator and its ability to transport deposited particles from the respiratory tract.

For example, a number of studies with various animal species reported morphologic damage to the cells of the tracheobronchial tree from exposures to O_3 (see Section 6.2.4). The cilia had become noticeably shorter or were completely absent. Based on such morphologic observations, related effects such as ciliostasis, increased mucous secretion, and a slowing of mucociliary transport rates might be expected. Functional studies on mucociliary transport of deposited particles from the respiratory tract have, in general, observed a delay in particle clearance in early time periods following acute exposure. For example, a 4-h exposure of rats to 0.8 ppm O_3 slowed early clearance of inhaled latex spheres (Phalen et al., 1980).

Since the publication of the previous criteria document (U.S. Environmental Protection Agency, 1986), several studies have been performed on the effects of acute O_3 exposure on the mucociliary transport apparatus (Table 6-6). Retarded mucociliary particle clearance was observed following a 2-h exposure of rabbits to 0.6 ppm O_3; extended exposures (up to 14 days) caused no effects (Schlesinger and Driscoll, 1987). Acute exposure of adult sheep for 4 h/day for 2 days to 0.5 ppm O_3 increased basal secretion of glycoproteins in sheep trachea, whereas a longer exposure (4 h/day, 5 days/week for 6 weeks) to 0.5 ppm O_3 reduced tracheal glycoprotein secretions (Phipps et al., 1986). In a similar manner, continuous exposure of ferrets to 1.0 ppm O_3 for 3 days increased tracheal gland secretion of glycoproteins, which remained elevated following 7 days of exposure (McBride et al., 1991). Because the integrity of the periciliary space is vital for efficient mucociliary action, O_3-induced hyper- or hyposecretion by the mucous glands along the conducting airways can alter the effectiveness of the mucociliary escalator.

Mariassy et al. (1990) exposed sheep during the first week of life to 1.0 ppm O_3 for 4 h/day for 5 days and observed retardation of normal morphologic development of the tracheal epithelium and a decrease in the tracheal mucous velocity. In a similar manner, exposure of sheep during the first week of life for 4 h/day for 5 days to 1.0 ppm O_3 decreased epithelial mucosa density and retarded the developmental decrease of tracheal mucous cells and their carbohydrate composition (Mariassy et al., 1989). Finally, exposure of adult sheep for either 2 h or for 5 h/day for 4 days to 1.0 ppm O_3 decreased tracheal mucous velocity (Allegra et al., 1991).

6.2.3.3 Alveolobronchiolar Transport Mechanism

In addition to the transporting of particles deposited on the mucous surface layer of the conducting airways, particles deposited in the deep lung may be removed either up the respiratory tract or through interstitial pathways to the lymphatic system (Green, 1973). The pivotal mechanism of alveolobronchiolar transport involves the movement of AMs with phagocytized particles to the bottom of the mucociliary escalator. Failure of the AMs to phagocytize and sequester the deposited particles from the vulnerable respiratory membrane can lead to particle entry into the interstitial spaces. Once lodged in the interstitium, particle removal is more difficult and, depending on the toxic or infectious nature of the particle, its interstitial location may allow the particle to set up a focus for pathologic processes. Although Phalen et al. (1980) and Kenoyer et al. (1981) observed decreases in early (tracheobronchial) clearance after acute O_3 exposure of rats; late (alveolar) clearance was accelerated.

Table 6-6. Effects of Ozone on Host Defense Mechanisms: Physical Clearance[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m				
0.06 base, spike rising to 0.25	118 base, spike rising to 490	13 h/day, 7 days/week base; ramped spike 9 h/day, 5 days/week; 6 weeks	Rat, M (F344) 35 days old	Increased retention of asbestos fibers in the lung parenchyma.	Pinkerton et al. (1989)
0.1 0.6 1.2	196 1,176 2,352	2 h/day for 1 or 13 days	Rabbit, M (NZW) 2.5-3.0 kg	Acceleration of early alveolar particle clearance at 0.1 and 0.6 ppm for 13 days. After single exposure, increased clearance at 0.1 ppm and decrease at 1.2 ppm.	Driscoll et al. (1986)
0.1 0.6 1.2	196 1,176 2,352	2 h/day for 1 or 13 days	Rabbit, M (NZW) 2.5-3.0 kg	Acceleration of early alveolar particle clearance at 0.1 and 0.6 ppm for 13 days. After single exposure, increased clearance at 0.1 ppm and decrease at 1.2 ppm	Driscoll et al. (1986)
0.1 0.25 0.6	196 490 1,176	2 h/day for 14 days	Rabbit, M (NZW) 2.5-2.7 kg	Retarded mucociliary particle clearance at 0.6 ppm only following a single 2-h exposure; no effect of 14-day exposure.	Schlesinger and Driscoll (1987)
0.5	980	4 h/day, 5 days/week for 6 weeks	Sheep, F 23-41 kg	Increase of tracheal glycoprotein secretion following acute exposure (4 h/day for 2 days) with a decrease following longer term exposure.	Phipps et al. (1986)
1.0	1,960	24 h/day for 7 days	Ferret	Increased secretion of glycoconjugates by tracheal glands.	McBride et al. (1991)
1.0	1,960	4 h/day for 5 days	Sheep 1st week of life	Retardation of normal morphologic development of the tracheal epithelium. Decreased tracheal mucous velocity. Decreased tracheal mucosa epithelial density. Retardation of developmental decrease of tracheal mucous cells and their carbohydrate composition.	Mariassy et al. (1989, 1990)
1.0	1,960	2 h and 5 h/day for 4 days	Sheep 26-41 kg	Decreased tracheal mucous velocity.	Allegra et al. (1991)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

Exposure of rabbits for 2 h/day for 13 days to 0.1 and 0.6 ppm O_3 resulted in acceleration of early alveolar clearance of polystyrene latex particles (Driscoll et al., 1986). After a single exposure to 0.1 ppm, the greatest acceleration occurred over the period shortly after exposure ceased (1 to 4 days), although the effect was still observed at Day 14 postexposure. A single exposure to 0.6 ppm caused no effect, whereas a higher concentration (1.2 ppm) retarded clearance. To investigate the effects of longer term O_3 exposure on alveolobronchiolar clearance, rats were exposed to an urban pattern of O_3 (continuous 0.06 ppm, 7 days/week with a slow rise to a peak of 0.25 ppm and subsequent decrease to 0.06 ppm over a 9-h period for 5 days/week) for 6 weeks and were exposed 3 days later to chrysotile asbestos, which can cause pulmonary fibrosis and neoplasia (Pinkerton et al., 1989). Ozone did not affect the deposition of asbestos at the first AD bifurcation, the site of maximal asbestos and O_3 deposition. However, 30 days later, the lungs of the O_3-exposed animals had twice the number and mass of asbestos fibers as the air-exposed rats.

6.2.3.4 Alveolar Macrophages

Within the gaseous exchange region of the lung, the first line of defense against microorganisms and nonviable particles that reach the alveolar surface is the AM. This resident phagocyte is responsible for a variety of activities, including the detoxification and removal of inhaled particles, maintenance of pulmonary sterility, and interaction with lymphocytes for immunologic protection. Under normal conditions, AMs seek out particles deposited on the alveolar surface and ingest them, thereby sequestering the particles from the vulnerable respiratory membrane. If the particle is insoluble, the AMs serve as a repository for the transport of the particle from the alveolus to the bottom of the mucociliary escalator located at the far distal portion of the conducting airways. Degradable particles are detoxified by powerful lysosomal enzymes, whereas microorganisms are killed by biochemical mechanisms, such as superoxide anion radicals and lysosomal enzymes. To adequately fulfill their defense function, the AMs must maintain active mobility, a high degree of phagocytic activity, and an optimally functioning biochemical and enzyme system.

As discussed in the previous criteria document (U.S. Environmental Protection Agency, 1986), short periods of O_3 exposure can cause a reduction in the number of free AMs available for pulmonary defense, and these AMs are more fragile, less phagocytic, and have decreased lysosomal enzyme activities. The lowest O_3 concentration showing AM effects in this early work was 0.25 ppm; a 3-h exposure of rabbits decreased lysosomal enzyme activities (Hurst et al., 1970).

Since the publication of the previous criteria document, the studies performed have been, in general, confirmatory of previous observations (Table 6-7). Morphologic observations showed that continuous exposure for 7 days to 0.13, 0.25, 0.5, and 0.77 ppm O_3 resulted in concentration-related increases in the number of rat AMs at 5 days postexposure, as well as increased AM size and morphologic changes consisting of surface microvilli and bleb formation (Dormans et al., 1990). Other morphological studies discussed in Section 6.2.4 also show increased numbers of AMs. A 2-h exposure to 0.1 ppm O_3 did not affect the AM number in the BAL when the analyses were made immediately postexposure, but, 7 days later, the total number of AMs increased by about 70% (Driscoll et al., 1987). On repeated exposure for 6 or 13 days, the number of AMs increased on the day after exposure. A single exposure to a higher concentration (1.2 ppm O_3) did not affect the number of AMs when assessed immediately after exposure. It is assumed that, although

Table 6-7. Effects of Ozone on Host Defense Mechanisms: Macrophage Alterations[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m				
0.05 0.1 0.2 0.4	98 196 392 784	16 h	Rat, M (Wistar) 210 ± 10 (SD)g	Increased adherence to nylon fibers at 0.05 and 0.1 ppm, but not at 0.2 and 0.4 ppm.	Veninga and Evelyn (1986)
0.1 0.2	196 392	Continuous for 11 weeks	Rat, M (Wistar) 16-21 weeks old	Increases in enzyme activity.	Mochitate et al. (1992)
0.1 1.2	196 2,352	2 h/day for 1, 2, 6, and 13 days	Rabbit, M (NZW) 2-4 mo old	Single exposure: Decreased phagocytosis at 0.1 and 1.2 ppm immediately and 1 day after exposure; recovery by 7 days after exposure in the 0.1-ppm group. Multiple exposure to 0.1 ppm only: decreased phagocytosis 1 day after 2 and 6 days of exposure.	Driscoll et al. (1987)
0.1 1.2	196 2,352	2 h	Rabbit (NZW)	Increased release of PGE_2 and PGF_{2} at 1.2 ppm; no effect at 0.1 ppm.	Driscoll et al. (1988)
0.11 to 3.6	216 to 7,056	3 h	Mouse, M (Swiss) Rat, F (S-D)	Concentration-dependent decrease in superoxide anion radical production; mouse more sensitive. No effect on murine AM phagocytosis at 0.42, 0.95, 1.0, and 1.2 ppm.	Ryer-Powder et al. (1988) Amoruso et al. (1989)
0.12 0.8 1.5	235 1,568 2,940	6 h	Rat, M (F344) 12-18 weeks old	At 42 and 66 h PE, concentration-dependent increase in mitotic index beginning at 0.8 ppm; increased size at 1.5 ppm at 18 and 42 h PE.	Hotchkiss et al. (1989b)
0.12 0.27 0.8	235 529 1,568	6 h	Rat, F (F344) 12-14 weeks old	Increase in mitotic index and chromosome damage at 0.27 and 0.8 ppm, no effect at 0.12 ppm.	Rithidech et al. (1990)
0.12 0.25 0.5	235 490 980	20 h/day for 1, 2, 3, 7, and 14 days	Rat, M (F433)	Increase in AM DNA synthesis at 2 and 3 days at 0.25 ppm O_3 and at 1, 2, and 3 days at 0.50 ppm O_3.	Wright et al. (1987)
0.13 to 0.77	250 to 1,500	Continuous for 7 days	Rat, M (Wistar) 8 weeks old	Concentration-related effects on number, size, and surface morphology.	Dormans et al. (1990)
0.13 0.26 0.51 0.77	250 500 1,000 1,500	Continuous for 7 days	Rat, M (Wistar) 8 weeks old	Decreased phagocytic ingestion (at all concentrations) and intracellular killing (at 0.26 ppm) of *Listeria monocytogenes*.	Van Loveren et al. (1988)
0.2	392	Continuous for 14 days	Rat, M (Wistar) 10 weeks old	Increases in enzyme activity at 3 days. Increased number of AMs by Day 3.	Mochitate and Miura (1989)

Table 6-7 (cont'd). Effects of Ozone on Host Defense Mechanisms: Macrophage Alterations[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m				
0.4	784	3, 6, or 12 h; 12 h/day for 1, 3, or 7 days	Mouse, M (NIH) 23-28 g Rat, M (Wistar) 180-200 g	Decreased Fc-receptor mediated phagocytosis of AMs from mice at Days 1 and 7. Decreased phagocytosis at 6 h, increased phagocytosis of AMs from rats on Day 1. Decrease in superoxide anion production at some exposures.	Oosting et al. (1991a)
0.4	784	6 or 12 h, or 12 h/day for 3 or 7 days	Mouse, M (NIH) 25-30 g Rat, M (Wistar) 200-250 g	Increased ATP levels in mouse AMs only after 7 days of exposure.	Oosting et al. (1991b)
0.4 0.8	784 1,568	3 h	Mouse, F (C3H/HeJ C57Bl/6) 30 days old	Decreased phagocytosis of *Streptococcus zooepidemicus* (in vivo assay) and latex beads (in vitro assay). C57B1 mice more susceptible than C3H at 0.4 ppm only for bacteria phagocytosis, reverse susceptibility for latex beads.	Gilmour et al. (1993a)
0.4 0.8	784 1,568	3 h	Mouse, F (CD-1) 5 and 9 weeks old	Decreased in vivo phagocytosis of *Streptococcus zooepidemicus* independent of age. Decrease in number of phagocytic cells and number of bacteria/AM.	Gilmour et al. (1993b)
0.4 0.8	784 1,568	3 h	Mouse, F (C3H/HeJ C57Bl/6) 30 days old Rat, F (F344)	Decreased phagocytosis of latex beads.	Gilmour and Selgrade (1993)
0.5	980	24 h/day for 14 days	Mouse, F (Swiss) 20-25 g	Decreased Fc-receptor mediated phagocytosis of AMs at Days 1-14, with trend towards maximal decrease on Day 3. Effect correlated with increases in PGE. Decreased phagocytosis of peritoneal AMs.	Canning et al. (1991)
0.5	980	24 h/day for 14 days	Mouse, F (Swiss) 20-25 g	Decreased Fc-receptor mediated phagocytosis on Days 1, 3, and 7, but not at Day 14.	Gilmour et al. (1991)
0.64	1,254	23 h/day for 27 days	Rat, M (S-D) 130-150 g	Decreased lysozyme enzyme content during chronic *Pseudomonas aeruginosa* bacterial infection.	Sherwood et al. (1986)
1.0	1,960	2 h/day for 3 days	Rabbit, M (NZW) 14 weeks old	Decreased cytotoxicity vs. xenogeneic tumor cells. No effect on TNF- and H_2O_2 production. Depression of superoxide anion production immediately after exposure, with an increase at 24 h.	Zelikoff et al. (1991)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

the lower O_3 concentrations are stimulatory for AMs activity, the higher concentrations are inhibitory because of their ability to produce substantial cellular injury. The number of AMs in BAL is reduced after a single exposure to 0.8 or 1.8 ppm O_3 (Bassett et al., 1988b; Bhalla and Young, 1992; Donaldson et al., 1993). Hotchkiss et al. (1989b) also observed a slight decrease in the number of AMs in the BAL immediately after a 6-h exposure to 0.8 and 1.5 ppm O_3, but the number of AMs increased at 42 and 66 h after the exposure. The results of the studies on AMs in general indicate that, following O_3 exposure, there is a short-term reduction in the number of lavageable AMs, but these cells increase in number over several days following exposure.

Exposure of rats to 0.12, 0.8, and 1.5 ppm O_3 for 6 h also resulted in a concentration-dependent increase in mitotic index at 0.8 ppm at 92 h after postexposure; AM size was only increased at the highest concentration (18 and 48 h postexposure) (Hotchkiss et al., 1989b). In a similar study, exposure of rats for 6 h to 0.12, 0.27, and 0.8 ppm O_3 resulted in AM chromosome damage at the two higher concentrations 28 h after exposure (Rithidech et al., 1990).

Several studies have investigated the effect of O_3 exposure on AM phagocytosis. Exposure of C3H/HeJ and C57Bl/6 mice for 3 h to 0.4 and 0.8 ppm O_3 decreased AM phagocytosis of *Streptococcus zooepidemicus* and latex beads (Gilmour et al., 1993a). In a similar study, a 3-h exposure of 5- and 9-week-old CD-1 mice decreased AM phagocytosis of *S. zooepidemicus*, but there was no effect of age (Gilmour et al., 1993b). Decreased phagocytic ingestion of *Listeria monocytogenes* also was observed following continuous exposure of rats to 0.13, 0.26, 0.51, and 0.77 ppm for 7 days; only the two lower concentrations inhibited intracellular killing (Van Loveren et al., 1988). Although lower O_3 concentrations were not tested, rats exposed to 1.02 ppm O_3 were unable to clear *Listeria* from their lungs. Exposure of rabbits to 0.1 ppm O_3 for 2 h/day resulted in decreased AM phagocytosis of latex microspheres after 2 or 6 (but not 13) days of exposure (Driscoll et al., 1987). In the same study, a single exposure to 0.1 or 1.2 ppm decreased AM phagocytosis immediately after exposure; recovery occurred by 7 days postexposure in the 0.1-ppm group but not in the 1.2-ppm group. That repeated exposures to O_3 results in an initial suppression of AM phagocytosis, which is followed by recovery of phagocytic potential while exposure continues (Driscoll et al., 1987), was confirmed by the studies of Gilmour et al. (1991) and Canning et al. (1991). Using identical exposure systems, it was observed that continuous exposure of mice to 0.5 ppm O_3 decreased AM Fc-receptor-mediated phagocytosis of sheep erythrocytes on Days 1, 3, 5, 7, and 8 of exposure, with return to control phagocytic levels by Day 14. This temporal trend paralleled the pattern of O_3-induced reduction of lung bactericidal activity against *Staphylococcus aureus*.

Interspecies comparisons of AM phagocytic potential were made by Gilmour and Selgrade (1993), who exposed C3H/HeJ and C57Bl/6 mice and Fischer 344 rats to 0.4 and 0.8 ppm O_3 for 3 h. Alveolar macrophage phagocytosis of latex beads was suppressed in all animals immediately after 0.4 ppm O_3 exposure, with the percent suppression greater in both strains of mice as compared to similarly treated rats. No differences in phagocytic suppression were observed between 0.4- and 0.8-ppm-O_3-exposed rats or the C57Bl/6 mice, but phagocytosis by AMs from 0.8-ppm-O_3-exposed C3H/HeJ mice was more suppressed as compared to the 0.4-ppm-O_3-exposed group. In a similar comparative study, Oosting et al. (1991a) exposed mice and rats to 0.4 ppm O_3 for single (3, 6, and 12 h) and repeated (12 h/day for 7 days) regimens. A decrease was observed in rat and mouse AM Fc-receptor mediated

phagocytosis following the single O_3 exposure protocol. With the repeated O_3 exposure protocol, rat AM phagocytosis was increased a day after exposure with no significant changes on Days 3 and 7. In contrast, phagocytosis by mouse AMs was suppressed at Day 1 of exposure and still did not recover at Day 7. In the same study, when mice were allowed to recover for 4 days following 3 days of O_3 exposure, phagocytosis by AMs was increased. These interspecies comparisons on the effect of O_3 exposure on AM phagocytic potential indicate that mice may be more susceptible than rats.

A species comparison of superoxide anion radical production between mouse AMs and rat AMs following a single 3-h exposure to O_3 concentrations ranging from 0.11 to 3.6 ppm showed the O_3 concentration that inhibits superoxide anion radical production by 50% to be 0.41 ppm for mouse AMs and 3.0 ppm for rat AMs (Ryer-Powder et al., 1988). Oosting et al. (1991a) also found a species difference in superoxide anion production using a more varied exposure-duration protocol; mice appeared to be more responsive than rats. This oxygen radical is important in antibacterial activity, and both sets of authors suggest that O_3-induced impairment of pulmonary antibacterial defenses may be related to decreases in superoxide anion radical production. Decreased lysozyme enzyme levels in rat AMs also were observed during chronic *Pseudomonas aeruginosa* bacterial infection following exposure to 0.64 ppm O_3 for 23 h/day for 27 days (Sherwood et al., 1986).

Exposure of rats for 16 h to 0.05, 0.1, 0.2, and 0.4 ppm O_3 increased AM adherence to nylon fibers at 0.05 and 0.1 ppm, but had no effect at 0.2 and 0.4 ppm (Veninga and Evelyn, 1986). Increased metabolic activity of AMs retrieved from rats following continuous O_3 exposure for 14 weeks to 0.1 and 0.2 ppm was observed by Mochitate et al. (1992). In a similar study, long-term O_3 exposure of rats (continuous 0.2 ppm for 11 weeks) continued to increase AM metabolic activity (Mochitate and Miura, 1989). Exposure of mice and rats for 14 h/day for 7 days to 0.4 ppm O_3 also increased adenosine triphosphate (ATP) levels in the mouse AMs, but had no effect on ATP levels in rat AMs (Oosting et al., 1991b).

In addition to their phagocytic function and particle removal, AMs also play several other roles in host defense that include (1) a regulatory role through their release of mediators (soluble substances secreted by the AMs that produce biologic effects on other cells) such as tumor necrosis factor, interleukin-1, and PGs; (2) activities associated with tumor surveillance; and (3) accessory cell function in antigen presentation to lymphocytes in the initiation of the immune response. Investigating the effect of a single 2-h exposure of rabbits to 0.1 and 1.2 ppm O_3, Driscoll et al. (1988) observed an increased release of PGE_2 and $PGF_{2\alpha}$ by AMs following exposure to 1.2 but not 0.1 ppm. Prostaglandin E_2 can depress AM and natural killer cell cytotoxicity to tumor cells. Perhaps this is a mechanism involved in the depression of AM-mediated cytotoxicity toward xenogeneic tumor cells following exposure of rabbits for 2 h/day for 3 days to 1.0 ppm O_3 (Zelikoff et al., 1991). No studies were found on the effects of O_3 on antigen presentation.

6.2.3.5 Immunology

In addition to the above nonspecific defense mechanisms, the respiratory system also has specific immunologic mechanisms that can be initiated by inhaled antigens. There are two types of immune mechanisms: antibody (humoral)-mediated and cell-mediated.
In general, humoral mechanisms neutralize viruses and microbial toxins, enhance the ingestion of bacteria by phagocytes, and play an important role in defense of the lung against fungal and parasitic infections. Cell-mediated mechanisms enhance the microbiocidal capacity of AMs in

defense to intracellular bacteria such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, whereas another arm of the cellular immune response generates a class of lymphocytes that are cytotoxic for virus-infected cells. Both the humoral and cell-mediated responses protect the respiratory tract against infectious agents and operate in three major temporal waves: (1) natural killer cells (nonspecific lymphocytes that can destroy bacteria, viruses, and tumor cells), (2) cytotoxic T lymphocytes (lymphocytes that lyse specifically recognized targets), and (3) antigen-specific antibodies.

Little information was available in the previous criteria document (U.S. Environmental Protection Agency, 1986) on the effects of O_3 on immunologic defenses. However, the data base indicated an immunotoxic effect of O_3 exposure, especially on T-cell populations. For example, Aranyi et al. (1983) found that a 90-day (5 h/day, 5 days/week) exposure of mice to 0.1 ppm O_3 suppressed blastogenesis of splenic lymphocytes to T-cell, but not B-cell mitogens; the ability of these cells to produce antibodies was not affected either. As can be seen from Table 6-8, this database has greatly expanded and also has been recently reviewed (Jakab et al., 1995). Many of the studies include both the pulmonary and systemic immune system, which, to a degree, are compartmentalized; both systems are discussed here.

Studies on the effect of O_3 exposure on the immune system can be divided into three broad categories. These are (1) measurement of lymphoid organ weights and cellular composition, (2) determination of the functional capacity of lymphocytes in the absence of antigenic stimulation, and (3) measurement of the immune response following antigenic stimulation.

Dziedzic and White (1986a) observed that exposure of mice to 0.3, 0.5, and 0.7 ppm O_3 for 20 h/day for 28 days resulted in a concentration-dependent initial depletion of cells in the mediastinal lymph nodes (MLNs) (Days 1 and 2); this was followed by a T-cell hyperplasia peaking about Days 3 and 4. There was an enhanced blastogenic response to the T-cell mitogen concanavalin A (ConA) at 0.7 ppm O_3 (only level tested). There was no effect of O_3 on cell division morphology of B cells. In a similar study, exposure of mice to 0.7 ppm O_3 for 20 h/day for 28 days resulted in an initial thymic atrophy, with return to normal thymus weights by Day 14 of exposure (Dziedzic and White, 1986b). Exposure of rats to 0.5 ppm O_3 for 20 h/day for 14 days also increased bronchus-associated lymph node and MLN cell proliferation at 2 and 3 days of exposure, but not at 1, 7, and 14 days of exposure (Dziedzic et al., 1990). Bleavins and Dziedzic (1990) observed that exposure of BALB/c mice to 0.7 ppm O_3 for 20 h/day for 14 days resulted in decreased spleen and thymus weights at Day 4, with recovery at Day 14. The absolute number of thymocytes decreased following exposure of mice to 0.7 ppm O_3 for 24 h/day for 7 days (Li and Richters, 1991a) and to 0.3 ppm O_3 for 24 h/day for 3 weeks (Li and Richters, 1991b). Although the latter exposure protocol (0.3 ppm for 24 h/day for 3 weeks) decreased the absolute number of thymocytes, an increase in the percentage of thymocytes was observed in the absence of any changes in splenic T cells (Li and Richters, 1991b). Continuous exposure of rats to □0.26 ppm O_3 for 7 days increased MLN T:B lymphocyte ratios immediately and 5 days postexposure (Van Loveren et al., 1988). Bleavins and Dziedzic (1990) observed an increased infiltration of Thy-1.2^+ lymphocytes and IgM^+ cells into the O_3-induced pulmonary lesion following exposure of mice to 0.7 ppm O_3 for 20 h/day for 14 days. The lesion was defined by quantitative histomorphometric analysis as any lung area with inflammatory cell infiltration, cellular proliferation, consolidation, or edema. Dziedzic and White (1987a) further investigated these T-cell effects by exposing normal and athymic nude

Table 6-8. Effects of Ozone on Host Defense Mechanisms: Immunology[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m				
0.06 base, spike rising to 0.25	118 base, spike rising to 490	13 h/day, 7 days/week base; ramped spike 9 h/day, 5 days/week	Rat, M (F344) 60 days old	No effect on splenic NK cell activity or splenic lymphocyte blastogenic response to T-cell mitogens (PHA and ConA) or B-cell mitogen (*S. salmonella* glycoprotein).	Selgrade et al. (1990)
0.1 0.5 1.0	196 980 1,960	23.5 h/day for 10 days	Rat, M (F344) 8-12 weeks old	Decreased lung NK cell activity at 1.0 ppm on Days 1, 5, and 7, with recovery at Day 10; decreased NK cell activity at 0.5 ppm on Day 1 (only day tested).	Burleson et al. (1989)
0.13 0.26 0.51 0.77 1.02	250 500 1,000 1,500 2,000	Continuous for 7 days	Rat, M (Wistar) 8 weeks old	At 0.26 ppm, increased T:B cell ratios in MLN. Decreased T:B cell ratios in MLN and delayed-type hypersensitivity response following *Listeria monocytogenes* immunization.	Van Loveren et al. (1988)
0.2 0.4 0.82	400 800 1,600	24 h/day for 7 days	Rat, M (Wistar) NS	Decreased lung NK cell activity at 0.82 ppm; increased lung NK cell activity at 0.2 and 0.4 ppm.	Van Loveren et al. (1990)
0.3	588	24 h/day for 3 weeks	Mouse, F (BALB/c) 3 weeks old	Increase in percentage of thymocytes with lower absolute numbers. No changes in splenic T-lymphocytes.	Li and Richters (1991b)
0.3 0.5 0.7	588 980 1,372	20 h/day for 28 days	Mouse, F (CD-1)	Concentration- dependent MLN cell depletions (Days 1 and 2) followed by T-cell hyperplasia (Days 4, 7, 14, and 28). Enhanced blastogenic response to T-cell mitogen ConA at 4 and 7 days at 0.7 ppm (other levels not tested).	Dziedzic and White (1986a)
0.5	980	24 h/day for 14 days	Mouse, F (Swiss)	Decreased antiviral serum antibody following influenza virus infection and decreased T-cells in lung tissue.	Jakab and Hmieleski (1988)
0.5	980	20 h/day for 14 days	Rat, M (F344) 11 weeks old	BALT and MLN cell proliferation at 3 days, but not at Days 1, 2, 7, and 14.	Dziedzic et al. (1990)
0.7	1,372	20 h/day for 28 days	Mouse, F (CD-1)	MLN hyperplasia. Thymic atrophy through Day 7 with return to control level at Day 14.	Dziedzic and White (1986b)
0.7	1,372	24 h/day for 7 days	Mouse, F (BALB/c) 5-12 weeks old	Decrease in the absolute number of thymocytes.	Li and Richters (1991a)
0.7	1,372	20 h/day for 7 and 14 days	Mouse, F (Athymic and euthymic)	Compared to euthymic (nu/+) mice, athymic nude (nu/nu) mice have increased lung inflammation and lesion volume, but no MLN hyperplasia.	Dziedzic and White (1987a)

Table 6-8 (cont'd). Effects of Ozone on Host Defense Mechanisms: Immunology[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	μg/m				
0.7	1,372	20 h/day for 14 days	Mouse, F (BALB/c) 10-12 weeks old	Decreased spleen and thymus weight at Day 4 with recovery at Day 14. Increased infiltration of Thy-1.2$^+$ lymphocytes and IgM$^+$ cells into the O_3-induced pulmonary lesion.	Bleavins and Dziedzic (1990)
0.8	1,568	23 h/day for 14 days	Mouse, F ($B6C3F_1$) 6-8 weeks old	Decreased blastogenesis of MLN and splenic lymphocytes to PHA mitogen on Day 1; no effect on Days 3, 7, and 14. Decreased splenic NK cell activity on Days 1 and 3; no effect on Days 7 and 14. Decreased pulmonary IgG and IgA response to ovalbumin immunization.	Gilmour and Jakab (1991)
0.8	1,568	24 h/day for 14 days	Mouse, M (BALB/c) 2-3 mo old	Suppression of delayed-type hypersensitivity response to SRBCs on Day 7; no effect on Days 1, 3, and 14. Thymic atrophy on Day 7 with decreased Thy-1.2$^+$ cells in thymus.	Fujimaki et al. (1987)
0.8	1,568	24 h/day for 4 weeks	Mouse, M (BALB/c) 7 weeks old	Suppression of serum IgG following ovalbumin immunization.	Ozawa (1986)
1.0	1,960	24 h/day for 4 weeks	Mouse, M (C57B/6 × DBA/2 F1) 11-14 weeks old	Decreased ability of spleen cells to generate a primary antibody response to SRBCs in vitro.	Wright et al. (1989)
1.0	1,960	8 h/day for 7 days	Rat, M (Long-Evans) 7-8 weeks old	No effect on lung and splenic ADCC activity. Enhanced blastogenesis of splenic lymphocytes to PHA, ConA, and lipopolysaccharide mitogens at 1.0 ppm.	Eskew et al. (1986)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

mice to 0.7 ppm O_3 for 7 or 14 days (20 h/day). After 7 days of exposure, the athymic nude mice did not have the MLN hyperplasia seen in the euthymic mice. However, the athymic nude mice had a greater inflammatory response and an increase in lung lesion volumes compared to the euthymic mice. Thus, it appears that T cells have some involvement in protecting the lungs from the morphological effects of O_3.

The above longitudinal studies on the effects of O_3 exposure on lymphoid organ cell numbers provide information on cellular traffic and cell numbers but provide few insights into the functional capacity of the lymphocytes. A number of studies have investigated the effect of O_3 exposure on the blastogenic response of lymphocytes to nonspecific mitogens. These assays measure nonspecific clonal expansion of the lymphocyte population, a critical step during the amplification of the immune response. Exposure of mice to 0.7 ppm O_3 for 20 h/day for 28 days enhanced the MLN cell blastogenic response to the T-cell mitogen ConA at 4 and 7 days of exposure, with return to control levels by Day 14 (Dziedzic and White, 1986a). In a similar manner, Gilmour and Jakab (1991) observed that continuous exposure of mice to 0.8 ppm O_3 decreased the MLN and splenic lymphocyte blastogenic response to the T-cell mitogen phytohemagglutinin (PHA) on Day 1 of exposure, with the effect abrogated after prolonged exposure. An enhanced blastogenic response of splenic lymphocytes to PHA and Con A and a B-cell mitogen (*Escherichia coli* lipopolysaccharide) was observed following exposure of rats to 1.0 ppm O_3 for 8 h/day for 7 days (Eskew et al., 1986).

Natural killer cell activity also has been studied. One such study in rats observed a decreased lung NK cell activity following 1, 5, and 7 days of exposure to 1.0 ppm for 23.5 h/day, with recovery by Day 10 (Burleson et al., 1989). In a similar experiment, Van Loveren et al. (1990) observed decreased lung NK cell activity following 7 days of continuous exposure of rats to 0.82 ppm O_3; however, exposure to 0.2 and 0.4 ppm enhanced lung NK cell activity. Exposure of mice for 23 h/day for 14 days to 0.8 ppm O_3 also decreased splenic NK cell activity on Days 1 and 3, with a return to control values on Days 7 and 14 (Gilmour and Jakab, 1991). Finally, Selgrade et al. (1990) used an experimental protocol designed to mimic diurnal urban O_3 exposure patterns. Rats were exposed to a background level of 0.06 ppm for 13 h (7 days/week), followed by a broad exposure spike (5 days/week) rising from 0.06 to 0.25 ppm and returning to 0.06 ppm over 9 h, and then followed by 2-h downtime. After 1, 3, 13, 52, or 78 weeks of exposure, spleen cells were assessed for NK cell activity and responses to T-cell mitogens (PHA and ConA) and a B-cell mitogen (*Salmonella typhimurium* glycoprotein). Ozone exposure had no effect on NK cell activity, nor were there any O_3-related changes in mitogen responses in splenic or blood leukocytes. There were also no effects of a single 3-h exposure to 1.0 ppm O_3 on spleen cell responses to the mitogens immediately after exposure or at 24, 48, and 72 h thereafter.

Several studies also have investigated the effect of O_3 exposure on the immune response following antigenic stimulation. Fujimaki (1989) observed that exposure of mice to 0.8 ppm O_3 for 24 h/day for 56 days suppressed the primary splenic antibody response to sheep red blood cells (SRBCs; T-cell-dependent antigen) but not to DNP-Ficoll (T-cell-independent antigen). In a similar study, exposure of mice to 0.8 ppm O_3 for 24 h/day for 14 days suppressed the delayed type hypersensitivity response to SRBCs on Day 7, but not on Days 1, 3, and 14 (Fujimaki et al., 1987). Suppression of serum IgG levels on ovalbumin immunization was observed following exposure of mice to 0.8 ppm O_3 for 24 h/day for 4 weeks (Ozawa, 1986). Decreased pulmonary IgG and IgA responses on ovalbumin immunization were also observed in mice during a 2-week O_3 exposure for 23 h/day to

0.8 ppm (Gilmour and Jakab, 1991). Exposure of mice to 0.5 ppm O_3 continuously for 14 days during the course of influenza virus infection also decreased the serum hemagglutinin antiviral antibody response (Jakab and Hmieleski, 1988).

Van Loveren et al. (1988) investigated antigen-specific responses following pulmonary *Listeria* infection and observed no significant changes in the delayed-type hypersensitivity response when O_3 exposure (continuous, 0.77 ppm) was for 7 days prior to infection. However, if the O_3 exposure took place when an infection with *Listeria* was also present (from Days 0 to 7 or from Days 7 to 14), the delayed-type hypersensitivity response was significantly decreased. In a similar manner, no significant changes were observed in the splenic lymphoproliferative response to *Listeria* antigen when the 0.77-ppm O_3 exposure preceded the infection, whereas the response was suppressed when the 0.77-ppm O_3 exposure occurred immediately after infection or from Days 7 to 14 after infection. In the same series of experiments, Van Loveren et al. (1988) observed that continuous exposure to 0.26 ppm O_3 impaired the increase in T:B lymphocyte ratios that occurred in response to the *Listeria* infection.

6.2.3.6 Interaction with Infectious Agents

Because respiratory infections remain one of the most common public health problems, it is important to determine whether or not exposure to air pollutants reduce susceptibility to infectious agents. Measurement of the competence of the host's antimicrobial mechanisms can best be tested by challenging air-pollutant-exposed animals and the clean-air-exposed control animals to an aerosol of viable organisms. If the test substance, such as O_3, decreases the efficiency of the host's integrated protective mechanisms (i.e., physical clearance via the mucociliary escalator, microbicidal activity of the AMs, and associated humoral and cellular immunologic events), the microorganisms are less efficiently killed in the lungs, or the organisms may even multiply, resulting in the demise of the host. The defensive function of the lung is remarkably similar across animals species, and available human data suggests that qualitative findings obtained on functional resistance mechanisms using appropriate animal models may be extrapolated to humans (Green, 1984).

The studies detailed in the previous criteria document (U.S. Environmental Protection Agency, 1986) primarily used the mouse "infectivity model" (Gardner, 1982). Briefly, animals are randomly selected to be exposed to either clean air or O_3. After exposure, the animals from both groups are combined and exposed to an aerosol of microorganisms. The vast majority of these studies have been conducted with streptococcus species. At the termination of the infectious exposure period, the animals are housed in clean air and the mortality rate in the two groups is determined during a 15-day holding period. In this system, the concentrations of O_3 used do not cause any mortality. The mortality in the control group (clean air plus exposure to the microorganism) ranges from approximately 10 to 20% and reflects the natural resistance of the host to the infectious agents. The difference in mortality between O_3-exposure groups and the controls is concentration-related (Gardner, 1982). These studies showed that, depending on the O_3 exposure protocol, a 3-h exposure to concentrations as low as 0.08 ppm O_3 can enhance the increased mortality of CD-1 mice from streptococcus infection (Coffin et al., 1967; Coffin and Gardner, 1972; Miller et al., 1978). However, although a prolonged intermittent exposure (103 days) to 0.1 ppm O_3 increased mortality in this model system, the magnitude of the effect was not substantially greater than that after acute exposure (Aranyi et al., 1983).

Another approach to assess the effect of air pollutants on host defenses is to quantitate rates of pulmonary bacterial inactivation following aerosol infection with microorganisms. In this system, the animals are exposed either to clean air or to the air pollutant and then are exposed to an aerosol of microorganisms in a manner similar to the method used for the infectivity model. However, instead of assessing enhancement of mortality, viable bacteria are quantitated in lung homogenates at various times after inhalation of the microorganisms (Goldstein et al., 1971a,b). In air-exposed control animals, there is a rapid inactivation of the inhaled microorganisms that have been deposited in the respiratory tract. However, O_3 exposure alters the ability of the microbicidal mechanisms of the lungs to function normally and bacterial inactivation proceeds at a slower rate, indicating impairment of host defenses. For example, Goldstein et al. (1971b) showed that a 4-h exposure of mice to 0.6 ppm O_3 after infection with *S. aureus* decreased lung bactericidal activity. Studies appearing in the literature since publication of the previous criteria document (U.S. Environmental Protection Agency, 1986) are described below (also see Table 6-9).

Gilmour et al. (1993a) observed that exposure of C3H/HeJ and C57Bl/6 mice for 3 h to 0.4 and 0.8 ppm O_3 resulted in decreased intrapulmonary killing of *S. zooepidemicus* in both strains of mice. Although both strains were affected, the C3H/HeJ mice appeared to be more susceptible because bactericidal activity was decreased sooner and mortality was enhanced more. Gilmour et al. (1993b) expanded these studies to CD-1 mice of different ages (5 and 9 weeks old) exposed for 3 h to 0.4 and 0.8 ppm O_3. The higher concentration decreased intrapulmonary killing 4 h after infection with *S. zooepidemicus*; there was no effect of age. However, the 5-week-old mice were more susceptible to the infection because mortalities were 9, 41, and 61% in the air, 0.4-ppm, and 0.8-ppm exposure groups, respectively; whereas only 4, 15, and 28% of the older animals died with analogous exposure. Pretreatment of the mice with indomethacin reduced the O_3-induced enhancement of PGE_2 levels in BAL as well as the enhanced mortality in the 5-week-old mice, suggesting an involvement of arachidonic acid metabolites in antibacterial defenses.

Gilmour and Selgrade (1993) studied the interspecies response to experimental *S. zooepidemicus* infection of rats and C3H/HeJ and C57Bl/5 mice following a 3-h exposure to 0.4 and 0.8 ppm O_3. Exposure of rats to O_3 suppressed intrapulmonary bacterial killing, with no differences observed between the 0.4- and 0.8-ppm O_3 exposure groups. Exposure of C57Bl/6 mice to 0.4 ppm O_3 also resulted in a suppression of bactericidal activity, and exposure to 0.8 ppm O_3 led to bacterial proliferation in the lungs, resulting in 60% mortality at Day 4. Exposure of C3H/HeJ mice to both 0.4 and 0.8 ppm O_3 resulted in bacterial proliferation with, respectively, 60 and 80% mortality at Day 4 after exposure. Increased mortality from *S. zooepidemicus* infection following 24 h/day exposure for 5 days/week for 3 weeks also was observed following 0.3 and 0.5 but not 0.1 ppm O_3 exposure (Graham et al., 1987).

To investigate the effect of longer exposures and challenges with bacteria, Gilmour et al. (1991) exposed mice continuously to 0.5 ppm O_3 for 14 days. At 1, 3, 7, and 14 days, intrapulmonary killing was assessed by inhalation challenge with *S. aureus* and *Proteus mirabilis*. Ozone exposure impaired the intrapulmonary killing of *S. aureus* at 1 and 3 days. However, with prolonged exposure, the bactericidal capacity of the lungs returned to normal. In contrast to *S. aureus*, when *P. mirabilis* was the challenge organism, O_3 exposure had no suppressive effect on pulmonary bactericidal activity. The authors attribute this difference to the defense mechanisms involved. Alveolar macrophages are

Table 6-9. Effects of Ozone on Host Defense Mechanisms: Interactions with Infectious Agents[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	μg/m				
0.1 0.3 0.5	196 588 980	24 h/day, 5 days/week for 3 weeks	Mouse, F (CD-1) 4-6 weeks old	Increased mortality from *Streptococcus zooepidemicus* infection at 0.3 and 0.5 ppm.	Graham et al. (1987)
0.13 0.26 0.51 0.77 1.02	250 500 1,000 1,500 2,000	Continuous for 7 days	Rat, M (Wistar) 8 weeks old	Decreased bactericidal activity vs. *Listeria monocytogenes* at 0.77 and 1.02 ppm. Increased mortality at 1.02 ppm.	Van Loveren et al. (1988)
0.25 0.5 1.0	490 980 1,960	3 h/day for 5 days	Mouse, F (CD-1) 3-4 weeks old	Increased mortality from influenza virus infection and increased pulmonary virus titers at 1.0 ppm when infection followed 2 days of exposure, but not at other time points. Histopathologic and pulmonary function changes more severe with this regimen. At ≥0.5 ppm, increased lung wet weight when virus given after 2 days of O_3 exposure.	Selgrade et al. (1988)
0.4 0.8	784 1,568	3 h	Mouse, F (C3H/HeJ C57Bl/6) 30 days old	Decreased bactericidal activity vs. *Streptococcus zooepidemicus*. Increased mortality in both strains, with greater mortality in the C3H/HeJ strain.	Gilmour et al. (1993a)
0.4 0.8	784 1,568	3 h	Mouse, F (CD-1) 5 and 9 weeks old	Decreased bactericidal activity vs. *Streptococcus zooepidemicus*. Increased mortality in both age groups, with greater mortality in the 5-week-old mice.	Gilmour et al. (1993b)
0.4 0.8	784 1,568	3 h	Mouse, F (C3H/HeJ C57Bl/6) Rat (F344) 30 days old	Decreased intrapulmonary killing of *Streptococcus zooepidemicus*.	Gilmour and Selgrade (1993)
0.5	980	24 h/day for 14 days	Mouse, F (Swiss) 20-23 g	Decreased bactericidal activity vs. *Staphylococcus aureus* on Days 1 and 3; no effect on Days 7 and 14. Enhanced bactericidal activity vs. *Proteus mirabilis* on Days 3, 7, and 14; no effect on Day 1.	Gilmour et al. (1991)
0.5	980	24 h/day for 15 days	Mouse, F (Swiss) 20-23 g	No effect on pulmonary virus titers during influenza virus infection. O_3 decreased lung morphological injury due to virus (Day 9).	Jakab and Hmieleski (1988)
0.5	980	24 h/day for 120 days	Mouse, F (Swiss) 20-23 g	Ozone decreased acute lung influenzal injury, but increased pulmonary fibrosis during the course of and period after influenza virus infection.	Jakab and Bassett (1990)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

active against the gram-positive *S. aureus*; AMs and PMNs defend against the gram-negative *P. mirabilis*. The effects of O_3 on bactericidal activity against *S. aureus* paralleled the effects on AM phagocytosis (early decrease, then no change). With *P. mirabilis*, there was more than a 1,000-fold increase in PMNs in the lung that was not altered by O_3, enabling bactericidal activity to occur. In a similar manner, exposure of rats for 24 h/day for 7 days to 0.13, 0.26, 0.51, 0.77, and 1.02 ppm O_3 decreased pulmonary bactericidal activity against *Listeria* at 0.77 and 1.02 ppm, with increased mortality at 1.02 ppm (Van Loveren et al., 1988). These effects were associated with increased pathologic lesions, characterized by multifocal infiltrates of histiocyctic and lymphoid cells, found in the lungs and liver of O_3-exposed and *Listeria* infected animals as compared to *Listeria* infection alone.

Fewer studies of viral infectivity have been conducted. Exposure for 15 days to 0.5 ppm O_3 during the course of murine influenza virus infection had no effect on pulmonary virus titers (Jakab and Hmieleski, 1988). A 5-day exposure for 3 h/day to 1.0 ppm O_3 with influenza virus infection on the second day of exposure had no effect on pulmonary virus titers, but did show increased mortality, increased lung wet weight, and more severe nonsuppurative pneumonitis and epithelial metaplasia and hyperplasia, with changes in lung function consistent with that effect (Selgrade et al., 1988). Lung wet weight also was increased when the mice were infected after the second day of exposure to 0.5 but not 0.25 ppm. When infection occurred on other days during the 5-day O_3 exposure, no such effects were found.

Typically, influenza virus infection causes pneumonitis characterized by severe acute lung damage that eventually resolves to persistent alveolitis and changes in the parenchyma (focal interstitial pneumonia and collagen deposition). Jakab and Bassett (1990) investigated the effect of long-term O_3 exposure (24 h/day for 120 days to 0.5 ppm) on mice administered influenza virus immediately before O_3 exposure started. The authors observed an increase in pulmonary fibrosis with the virus infection, as compared to O_3 exposure alone. During the course of the viral infection, O_3 exposure had no effect on pulmonary virus titers and reduced the virus-induced acute lung injury. However, from Day 30 after infection, increased numbers of AMs, lymphocytes, and PMNs were recovered from animals exposed to virus plus O_3, as compared to virus infection alone or O_3 exposure alone. This increased alveolitis correlated with increases in morphometrically determined lung damage and lung hydroxyproline content, a biochemical marker indicative of pulmonary fibrosis. Ozone exposure administered 10 days after viral infection enhanced lung hydroxyproline content at Day 30, as compared to either virus infection or O_3 exposure alone. Thus, O_3 enhanced postinfluenzal aveolitis and parenchymal changes. From these data, the authors speculated that the mechanism for the postinfluenza lung damage may be related to O_3 impairing the repair process of the viral-induced acute lung injury.

In the studies reported to date, it is clear that the temporal relationships between O_3 exposure and influenzal infection are important. This is not surprising because there are several waves of different antiviral defense mechanisms that might be affected differently by O_3. However, they have not been studied adequately for susceptibility to O_3. Apparently, O_3 does not alter defenses responsible for clearing virus from the lungs, as evidenced by the lack of effect of O_3 on viral titers (Selgrade et al., 1988; Jakab and Hmieleski, 1988). The interaction between virus and O_3 on histological changes in lung tissue can be damaging (Selgrade et al., 1988; Jakab and Bassett, 1990) or beneficial (Jakab and Bassett, 1990), possibly depending on the time of observation relative to the stage of the infectious process.

The exact reasons are not known, but perhaps the induction of interferon production by the virus plays a role. In noninfectious studies, Dziedzic and White (1987b) observed that interferon induction mitigates O_3-induced lung lesions, defined as areas with inflammatory cell infiltration, cellular proliferation, consolidation, or edema, and that anti-interferon treatment exacerbates those lesions.

6.2.3.7 Summary

Exposure to O_3 can result in alterations of all the defense mechanisms of the respiratory tract, including mucociliary and alveolobronchiolar clearance, functional and biochemical activities of AMs, immunologic competence, and susceptibility to respiratory infections. Structural (see Section 6.2.4), functional, and biochemical alterations in the mucociliary escalator occur after O_3 exposure. Mucociliary clearance is slowed in rabbits after a single 2-h exposure to 0.6 ppm, but repeated (up to 14-day) exposures have no such impact (Schlesinger and Driscoll, 1987). Secretions of mucous components are affected by repeated exposure (Phipps et al., 1986; McBride et al., 1991). When lambs were exposed (1.0 ppm O_3, 4 h/day, 5 days) shortly after birth, tracheal mucous components did not develop normally (Mariassy et al., 1989, 1990). In contrast, alveolar clearance of rabbits after acute O_3 exposure (0.1 ppm, 2 h/day, 1 to 4 days) is accelerated (Driscoll et al., 1986). In the same study, a 14-day exposure caused no effects, and a higher concentration (1.2 ppm) slowed alveolar clearance. A similar pattern of slowed tracheobronchial clearance and accelerated alveolar clearance occurs in rats (Phalen et al., 1980; Kenoyer et al., 1981). A subchronic (6-week) exposure of rats to an urban pattern of O_3 increased the retention of asbestos fibers (Pinkerton et al., 1989).

Although AMs have numerous functions, one primary role is to clear the lung of infections and noninfectious particles. Phagocytosis of bacteria, inert particles, and antibody-coated red blood cells (RBCs) is inhibited by acute exposure to O_3. The lowest effective concentration tested was 0.1 ppm O_3 (2 h) in rabbits (Driscoll et al., 1987). If exposures are repeated for several days, phagocytosis returns to control levels (Driscoll et al., 1987; Gilmour et al., 1991; Canning et al., 1991). The ability of AMs to produce superoxide anion radicals (important to bactericidal activity) is inhibited by acute exposure to O_3, especially in mice as compared to rats (Ryer-Powder et al., 1988; Oosting et al., 1991a). The effect is clearly evident after exposure for 3 h to 0.4 ppm, as observed by dysfunction in AM phagocytosis and enhanced susceptibility to experimental respiratory infection (Gilmour et al., 1993a, 1993b). Thus, the evidence indicates that the AM-dependent alveolobronchiolar transport mechanisms are impaired, as are their phagocytic and microbicidal activities, leading to decreased resistance to respiratory infections.

The experimental database also shows that the effects of O_3 on the immune system are complex. These effects are not yet fully evaluated, and the reported effects on immune parameters are dependent on the exposure regimen and the observation period. It appears that the T-cell-dependent functions of the immune system are more affected than B-cell-dependent functions (U.S. Environmental Protection Agency, 1986; Fujimaki, 1989). Generally, there is an early immunosuppressive effect that, with continued O_3 exposure, results in either return to normal responses or immunoenhancement. For example, in mice exposed for 28 days (20 h/day) to 0.3 to 0.7 ppm O_3, there was an early (Days 1 and 2) depletion of cells in the MLN, followed by MLN T-cell hyperplasia and increased blastogenic response to a T-cell mitogen (Dziedzic and White, 1986a). Several investigations have found an initial (Days 1 to 4)

decrease in blastogenic response to T-cell mitogens in the MLN and spleen of mice exposed for a few weeks to 0.7 or 0.8 ppm O_3 that returned to control levels by the end of the exposure (Dziedzic and White, 1986a; Gilmour and Jakab, 1991). There also are changes in cell populations in lymphatic tissues. For example, T:B-cell ratios in the MLN increase when rats are exposed for 7 days to 0.26 ppm O_3 (Van Loveren et al., 1988). Natural killer cells in the lung are affected under some circumstances. Van Loveren et al. (1990) showed that a 1-week exposure to 0.2 or 0.4 ppm O_3 increased NK cell activity, but a higher concentration (0.82 ppm) decreased it. Ozone also alters response to antigenic stimulation. For example, antibody responses to a T-cell-dependent antigen were suppressed after a 56-day exposure of mice to 0.8 ppm O_3, and a 14-day exposure to 0.5 ppm O_3 decreased the antiviral antibody response following influenza virus infection (Jakab and Hmieleski, 1988). The temporal relationship between O_3 exposure and antigenic stimulation is important. When O_3 exposure preceded *Listeria* infection, there were no effects on delayed-type hypersensitivity or splenic lymphoproliferative responses; when O_3 exposure occurred during or after *Listeria* infection was initiated, these immune responses were suppressed (Van Loveren et al., 1988). With experimental viral infections, O_3 exposure decreases the T-lymphocyte responses and the antiviral antibody response (Jakab and Hmieleski, 1988); the latter impairment may pave the way for lowered resistance to reinfection.

The significance of O_3-induced suppression of immune parameters in relation to risk of infectious disease is an example of a generic problem that remains to be clarified. For example, correlative studies between immune parameters and acquired immune deficiency syndrome have provided clear insights on the magnitude of the immune dysfunction and the progression of the disease. However, there is a paucity of such correlative studies on alterations of immune parameters and function resistance mechanisms or disease endpoints. Because the major functions of the immune apparatus are to protect against infectious agents and conduct tumor surveillance, suppression of immune parameters is considered a signal of increased susceptibility to infections and acquisition of tumors. One of the few studies that has addressed this issue (Selgrade, 1995) illustrates how the temporal relationships between exposure to a compound and exposure to infectious agents or tumor cells will have an impact on the risk associated with immune suppression in experimental animal models. The types of immune responses affected by a chemical and their importance to defense against any particular infectious agent, the recovery time of the immune response, the length of exposure, and the time required for mobilization of alternative defenses are among the factors that can impact the risk of enhanced infectious disease that might be associated with an immunosuppressive event.

In addition to a suppressive effect on pulmonary immunity, O_3 exposure also can affect systemic immunity. Although these depressive effects on the systemic compartment occur at approximately twice the O_3 exposure concentrations observed for pulmonary immunosuppression (with the exception of a study by Aranyi et al. [1983] at 0.1 ppm O_3), the observations are important because they show that the effects of O_3 exposure on host resistance are not limited to the lung alone, but may increase susceptibility to systemic infections as well as pulmonary infections. However, Selgrade et al. (1990) found no effects on selected systemic immune functions in rats exposed for up to 78 weeks to an urban pattern of O_3.

Numerous studies have confirmed that acute or short-term exposure to O_3 decreases lung bactericidal activity and increases susceptibility to respiratory bacterial infections. The lowest exposure showing such effects was 0.08 ppm (3 h) in the mouse streptococcal model (Coffin et al., 1967; Coffin and Gardner; 1972; Miller et al., 1978). Further research has

indicated that changes in antibacterial defenses are dependent not only on exposure regimens, but also on species and strain of animal, species of bacteria, and age of animal (e.g., young mice are more susceptible) (Gilmour et al., 1991, 1993a,b; Gilmour and Selgrade, 1993). Furthermore, increasing the duration of an exposure to 0.1 ppm O_3 from a few hours to 3 weeks either causes no effect or does not enhance the streptococcal-induced mortality observed after acute exposure (Graham et al., 1987; Aranyi et al., 1983). In general, the effect of O_3 exposure on antibacterial host defenses appears to be concentration- and time-dependent. Acute exposures result in an impairment of host defenses, whereas the defense parameters become reestablished with more prolonged exposures.

Effects of O_3 on the course of viral infections are more complex and highly dependent on the temporal relationship between O_3 exposure and viral infection. For example, Selgrade et al. (1988) found increases in mortality and lung wet weight in mice infected with influenza only after the second day of O_3 exposure (1 ppm, 3h/day). Jakab and Bassett (1990) found no detrimental effect of a 120-day exposure to 0.5 ppm O_3 on acute lung injury from influenza virus administered immediately before O_3 exposure started. However, O_3 enhanced postinfluenzal alveolitis and lung parenchymal changes. Because O_3 did not affect lung influenza viral titers in any of these studies, it is unlikely that O_3 has an impact on antiviral clearance mechanisms.

Ozone-induced susceptibility to experimental respiratory infections has been correlated with the immunotoxic effects of O_3 by the observation that O_3 exposure increases the severity of *Listeria* infection while concurrently suppressing the antigen-specific immune responses (Van Loveren et al., 1988).

6.2.4 Morphological Effects

6.2.4.1 Introduction

All mammalian species studied react to inhaled concentrations of <1.0 ppm O_3 in a generally similar manner, with species variation in morphological responses depending on the distribution of sensitive cells and the type of junction between gas conducting and exchange areas of the lung (U.S. Environmental Protection Agency, 1986). The cells most damaged by O_3 are the ciliated epithelial cells in airways and Type 1 cells in gas-exchange areas. Both of these cell types have very large surface areas (relative to volume) exposed to inhaled gases. The many factors that influence the distribution of inhaled O_3 within the respiratory system (see Chapter 8) result in some of the largest effective doses to the epithelial cells lining the nose and to epithelial cells located at the junction of the conducting and exchange areas, the CAR, in lungs. The 1986 criteria document did not contain studies of morphological effects of O_3 on the nose. There are species differences both in the basic structure of the CAR and in the epithelial cells that line the CAR (Tyler, 1983; Plopper, 1983). The CAR of humans, other primates, dogs, cats, and a few other domesticated species consists of the last conducting airway, the TB, several generations of RBs, alveoli that open directly into RB lumens, and ADs that branch from RBs (Figure 6-2). In lungs from many other mammals, including those most commonly used for inhalation toxicology (i.e., rats, mice, guinea pigs, and rabbits), RBs are poorly developed or absent and the CAR consists of TBs that open directly into ADs.

Epithelial degenerative changes in TBs and alveoli occur early, 2 to 4 h, in an O_3 exposure (Stephens et al., 1974a; Castleman et al., 1980). Depending on the dose to

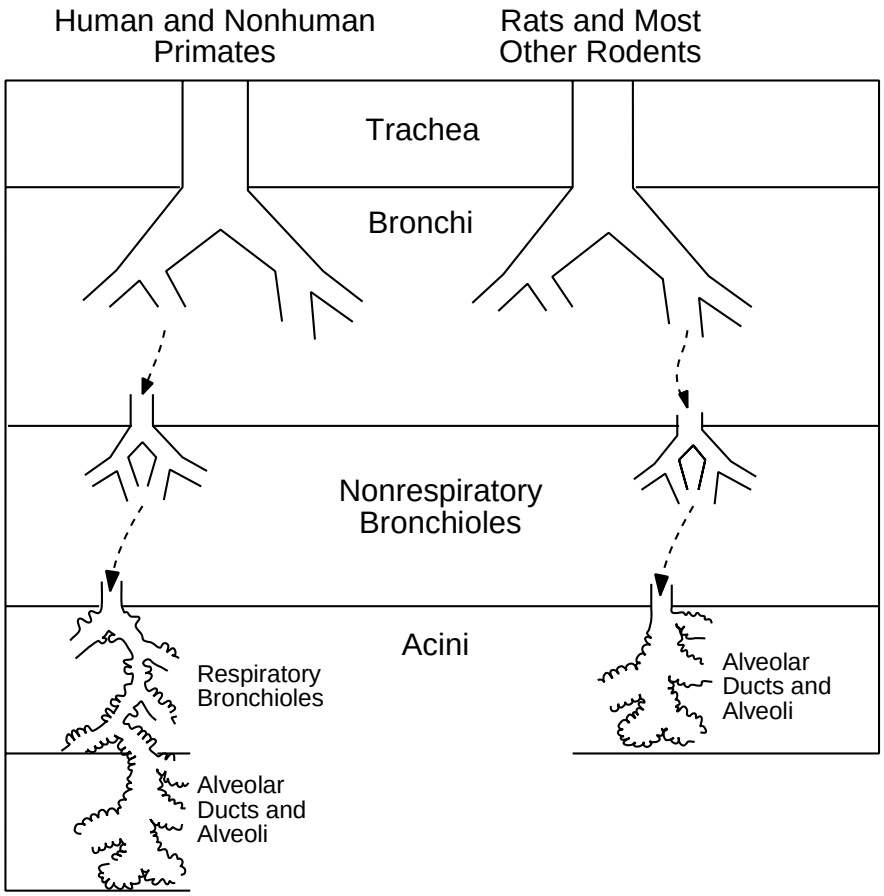

Figure 6-2. Schematic representation of intrapulmonary conducting airways and acini from animals with respiratory bronchioles (RBs) (human and nonhuman primates) and without RBs (rats and most rodents). For simplicity, several generations of bronchi and nonrespiratory bronchioles are not depicted.

Source: Redrawn from Weibel (1963) using information from Tyler and Julian (1991).

individual ciliated cells, they may lose cilia; undergo degenerative changes; or become necrotic and be sloughed into the lumen, leaving bare basement membrane until other cells replace them (Stephens et al., 1974a; Castleman et al., 1980). In the TB of the CAR, sloughed ciliated cells are replaced by nonciliated bronchiolar cells, which may become hyperplastic following longer exposures. Although these changes in TB epithelial cells can be studied readily by light microscopy (LM) or transmission electron microscopy (TEM), the surface views provided by scanning electron microscopy (SEM) provide a more comprehensive understanding of the three-dimensional aspects of CAR changes (Schwartz et al., 1976; Castleman et al., 1980).

Changes in mucus-secreting cells of conducting airways were considered minor in the 1986 criteria document (U.S. Environmental Protection Agency, 1986). Schwartz et al. (1976) did not find changes in mucous cells suggesting damage to cell organelles in rats

exposed to 0.2, 0.5, or 0.8 ppm O_3 for 7 days continuously or 8 h/day intermittently. Mellick et al. (1977) reported similar negative findings in mucous cells of monkeys following exposure to 0.5 or 0.8 ppm 8 h/day for 7 days. Wilson et al. (1984) reported more prominent small-mucous-granule (SMG) cells in the tracheas from monkeys exposed to 0.64 ppm O_3 continuously for 3 or 7 days. They speculated that these SMG cells may be related to repair processes.

Following O_3 exposure, Type 1 cells lining alveoli in the CAR, especially those opening into either RBs or ADs, may undergo vacuolization, fragmentation, or necrosis and be sloughed from the surface, leaving bare basement membrane (Stephens et al., 1974a). Although these degenerative changes, called lesions for purposes of this document, can be seen by careful LM of thin plastic sections, they are more reliably identified and the amount of damage can be estimated morphometrically using TEM (Barry et al., 1983; Crapo et al., 1984; Fujinaka et al., 1985). In alveoli, the bare basement membrane that follows O_3 exposure is recovered by Type 2 alveolar epithelial cells. Some Type 2 cells differentiate into Type 1 cells (Evans et al., 1975), but the epithelium remains thickened (Barry et al., 1983; Crapo et al., 1984).

Epithelial replacement in both TBs and alveoli can be followed and the amount can be estimated using radiolabeled thymidine and autoradiography (Evans et al., 1976a,b). Although bare basement membrane in alveoli is usually recovered by multiplication of Type 2 cells, in chronic exposures, bronchiolar cells, especially nonciliated bronchiolar cells, may cover part of the basement membrane formerly occupied by Type 1 or 2 cells. This process, termed bronchiolization (Nettesheim and Szakal, 1972), results in remodeling of CAR airways with the formation of new RBs. New RBs are identified readily by SEM or by LM in lungs from species in which RBs are normally absent or poorly developed (Boorman et al., 1980; Moore and Schwartz, 1981). In animals whose lungs normally have well- developed RBs, the extent of remodeling can be estimated using LM morphometry (Fujinaka et al., 1985).

The above epithelial changes are accompanied by an inflammatory response in the CAR characterized by increased numbers of PMNs in early stages, by increased numbers of AMs in lumens and in tissue at later stages, by hyperemia and interstitial edema, and by a fibrinous exudate (Stephens et al., 1974a; Schwartz et al., 1976; Boorman et al., 1980; Castleman et al., 1980; Fujinaka et al., 1985). As exposure continues, alveolar septa in the CAR thicken due to increased matrix, basement membrane, collagen, and fibroblasts and other cells, as well as by thickened alveolar epithelium (Boorman et al., 1980; Barry et al., 1983; Crapo et al., 1984; Fujinaka et al., 1985).

6 weeks to 0.25 ppm O_3. They reported CAR alveoli had more Type 1 and 2 epithelial cells and more AMs. The Type 1 cells were smaller in volume, covered less surface, and were thicker. They were aware of the results of Stephens et al. (1978) and speculated that the changes they described may have occurred primarily during the last 3 weeks of exposure. Boatman et al. (1983) did not find effects of O_3 on lung growth following pneumonectomy.

Several studies included both an exposure and a postexposure period during which the animals breathed air without O_3. Plopper et al. (1978) reported that CAR epithelial cells returned to normal appearance 6 days after a 72-h exposure to O_3. Incomplete resolution was reported 7 days after a 50-h O_3 exposure of monkeys (Castleman et al., 1980), 10 days after a 20-day O_3 exposure of mice (Ibrahim et al., 1980), and 62 days after a 180-day O_3 exposure of rats (Moore and Schwartz, 1981).

The previous criteria document (U.S. Environmental Protection Agency, 1986) comprehensively evaluated several citations reporting emphysema following O_3 exposure using current definitions of human emphysema (Snider et al., 1985). The morphological changes described in those earlier publications did not meet the current criteria for emphysema of the type seen in human lungs.

6.2.4.2 Sites Affected

The sites in the respiratory system that are affected will be discussed in anatomical sequence, beginning with the upper respiratory tract and proceeding downward. The upper or extrathoracic conducting airways (also referred to as the nasopharyngeal region) include the nasal cavity, pharynx, and larynx. Lower conducting airways begin with the trachea and include the bronchi and nonterminal bronchioles (also referred to as the tracheobronchial region). A summary of available information on these sites in the respiratory system is in Table 6-10, Effects of Ozone on Conducting Airways. Summaries of the information available concerning effects on the CAR, the gas exchange region (also referred to as the pulmonary region), and other pulmonary structures are divided into effects of short-term (<2-week) exposures in Table 6-11 and effects of long-term exposures in Table 6-12.

Nasal Cavity and Nasopharynx

The nasal cavity "conditions" inhaled air and in that process "scrubs" some reactive pollutants from the inhaled air, thereby reducing the concentration to which other portions of the respiratory system are exposed (Yokoyama and Frank, 1972; Miller et al., 1979). Although this scrubbing process is protective of other portions of the respiratory system, it results in a large dose of pollutant to the cells and tissues that line the nasal cavity.

There is a large range of variation in the structure of the nasal cavity among the animals used for inhalation toxicology and between those animals and humans (Schreider and Raabe, 1981). Schreider and Raabe (1981) found a striking similarity between the nasopharyngeal cavities of monkey and humans. They proposed that, with appropriate scaling, the monkey could serve as a model for aerosol and gas deposition in the nasopharyngeal region of humans. Thus, the studies on monkeys by Harkema et al. (1987) provide useful information for extrapolation to humans, as well as information concerning cellular responses in monkeys.

Table 6-10. Effects of Ozone on Conducting Airways[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	μg/m				
0.05 0.1 0.5 1.0 2.0 4.0 6.0 8.0	98 196 980 1,960 3,920 7,840 11,760 15,680	30 min (In vitro monolayer cultures)	Rabbit (NZW)	Phase contrast LM and trypan blue exclusion of tracheal epithelium. No consistent changes at 0.05 or 0.1 ppm, but 0.5 to 2.0 ppm resulted in cytoplasmic vacuolization without decreased viability. More evidence of cell damage, including gaps in previously confluent cultures and loss of cell viability at 4.0 and 8.0 ppm. Lipid peroxidation also was studied in these cultures.	Alpert et al. (1990)
0.12 0.8 1.5	235 1,568 2,940	6 h	Rat, F (F344/N) 280-400 g	LM pathology and LM morphometry of PMNs in lung and nasal epithelia. Also nasal and bronchoalveolar lavage. See Section 6.2.2. Nasal epithelium: No necrosis, loss or attenuation of cilia, or hyperplasia at any exposure. Increases in pavemented PMNs at most concentrations and times. Lung: No lesions due to 0.12 ppm. CAR lesions not obvious at 0.8 or 1.5 ppm immediately or 3 h PE, but a progressive increase in CAR lesions at all other times. CAR TBs and proximal alveolar septa thickened and increased inflammatory cells. Increased CAR tissue PMNs in 0.8- and 1.5-ppm groups at 18 and 66 h PE and 1.5-ppm group at exposure end.	Hotchkiss et al. (1989a)
0.12 0.8	235 1,568	6 h/day for 7 days	Rat, F (F344/N) 12-14 weeks old	LM pathology and morphometry of LM histochemistry of nasal epithelia. No LM pathology at 0.12 ppm, but 0.8 ppm caused hyperplasia of transitional nonciliated epithelium. In 0.8-ppm group, shortened cilia in respiratory epithelium of nasopharynx, but not of nasal septum. Increased intraepithelial mucosubstances in all areas at 7 days PE to 0.8 ppm.	Harkema et al. (1989)
0.12 0.25 0.5	235 490 980	20 h/day, 7 days/week for 2 years (4, 12, 26, 52, 78, and 104 weeks)	Rat, M (F344, CrlBR) 42 days old	Smaller body weights after 7 weeks exposure to 0.5 ppm. LM histopathology of nasal epithelia. Nose: At ≥0.25 ppm, mucous cell and respiratory epithelial hyperplasia. No lesions in mainstem or large bronchi. CAR: Described in Table 6-12.	Smiler et al. (1988)
0.12 0.27 0.8	235 529 1,568	6 h/day for 3 or 7 days	Rat, F (F344/N) 8-12 weeks old	LM pathology and LM BrdU for DNA synthesis by nasal epithelia. No LM pathology in squamous epithelium or in ciliated respiratory epithelium. Hyperplasia of nonciliated cuboidal/transitional epithelium at 0.8 ppm. BrdU uptake (DNA synthesis) increased at end of 3- and 7-day exposure to 0.8 ppm. BrdU uptake decreased in squamous epithelium only after 7 days exposure to 0.8 ppm and 7 days PE.	Johnson et al. (1990)
0.12 0.5 1.0	235 980 1,960	6 h/day, 5 days/week for 20 mo	Rat, M (F344) 6-8 weeks old	Nasal changes limited to nasal transitional nonciliated epithelium at 0.5 and 1.0 but not 0.12 ppm. LM histochemistry of intraepithelial mucosubstances in the nose and bronchi. Epithelial cell hyperplasia; mild to moderate inflammatory cell influx into the mucosa; and increased mucosubstances. Mucous flow rates decreased.	Harkema et al. (1994)

Table 6-10 (cont'd). Effects of Ozone on Conducting Airways[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
ppm	μg/m				
0.12 0.5 1.0	235 980 1,960	6 h/day, 5 days/week for 20 mo	Rat, M (F344) 6-8 weeks old	LM morphometry and histochemistry of "short-" and "long-path" conducting airways and CAR. *Trachea*: No changes in epithelial thickness, cell populations or stored glycoconjugate, but a dose-dependent loss of stored glycoconjugate was found. *Bronchi*: No changes in epithelial thickness or cell populations. Rats exposed to 1.0 ppm had increased stored gylcoconjugates in cranial (short-path) and caudal (long-path) bronchi, but not in central (short-path) bronchi. *Bronchioles*: TB of rats exposed to 1.0 ppm had thicker epithelium with increased V_v of nonciliated bronchiolar cells. V_v also increased in caudal (long-path) TB of 0.5-ppm exposed group. Mass ($\mu m^3/\mu m^2$) (V_s) of nonciliated cells increased in caudal (long-path) TBs of all exposed rats, but not in short-path TBs. *CAR*: Increased V_s of bronchiolar epithelium in former ADs in cranial and caudal CARs of rats exposed to 1.0 ppm and in cranial CAR of rats exposed to 0.5 ppm.	Plopper et al. (1994a)
0.12 0.24 0.48	235 470 940	3, 6, 12, or 24 h	Rat, F (F344/N) 11-13 weeks old	LM pathology and semiautomatic image analysis system for DNA synthesis (BrdU uptake) of epithelium in nasal maxilloturbinates. C × T design. No effects at 0.12 or 1.44 ppm·h. For a given C × T, increased DNA synthesis equal at different Cs and Ts. Nonlinear increase in DNA synthesis as C × T increased.	Henderson et al. (1993)
0.15 0.3	294 588	8 h/day for 6 days to 0.15 ppm or for 90 days to 0.15 or 0.3 ppm	*Macaca radiata*, M, F 2-6 years old, 2.3-9.7 kg	LM, SEM, and TEM morphometry of nasal epithelia. Respiratory epithelium: Ciliated cell necrosis, shortened cilia, and increased small mucous granule cells at all exposures, even at 0.15 ppm for 6 days. Transitional epithelium: Decreased nonciliated cells without granules, increased nonciliated cells with granules, and increased small mucous granule cells. Increased intraepithelial leukocytes only at 6 days in both types of epithelium.	Harkema et al. (1987)
0.15 0.25	294 490	8 h/day for 6 days to 0.15 ppm or for 90 days to 0.15 or 0.3 ppm	*Macaca radiata*, M, F 2-6 years old, 2.3-9.7 kg	LM and TEM histochemistry for intraepithelial mucosubstances in nasal mucosa. More mucous cells that had dilated granular endoplasmic reticulum. Also changes in mucosubstances.	Dimitriadis (1992)
0.15 0.25	294 490	8 h/day for 7 days	*Macaca radiata* NS	The O_3 concentration is not clear—the abstract states 0.64 ppm, the text mentions only 0.25 ppm. LM morphometry of vocal fold mucosa. Disruption and hyperplasia of stratified squamous epithelium. Epithelium thickened at 12 h and 7 days PE. Connective tissue of lamina propria thickened at 7 days PE. Basement membrane is undulating rather than smooth. Even though thickened, epithelium appeared normal at 7 days PE.	Leonard et al. (1991)
0.2 0.4 0.8	392 784 1,568	22 h/day for 3 days	Rat, M (Wistar RIVM [TOX]) 150-190 g	LM pathology and tritiated thymidine uptake by nasal epithelia. Observations on mixtures are in Section 6.4. Respiratory epithelium: No changes at 0.2 ppm O_3. Loss of cilia and disarrangement at 0.4 and 0.8 ppm. Some epithelia were hyperplastic or metaplastic or both. Thymidine uptake increased in rostral (anterior) portions.	Reuzel et al. (1990)

Table 6-10 (cont'd). Effects of Ozone on Conducting Airways[a]

Ozone Concentration ppm	Ozone Concentration g/m	Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
0.8	1,568	6 h/day for 3 or 7 days	Rat, F (F344/N) 12-14 weeks old	LM morphometry and histochemistry of nasal nonciliated cuboidal epithelium. Other types of nasal epithelia not examined. After 3 days O_3 and 18 h PE, no changes in cell density or in intraepithelial mucus. After 7 days exposure or after 3 days exposure and 4 days PE, hyperplasia and increased intraepithelial mucosubstances with no change in the ratio of acidic to neutral mucosubstances.	Hotchkiss et al. (1991)
0.8	1,568	6 h	Rat, F (F344/N) 12-16 weeks old	LM pathology and DNA synthesis by BrdU uptake by nasal nonciliated transitional epithelium. Ozone did not result in necrosis, exfoliation, or inflammation, but did increase DNA synthesis.	Hotchkiss and Harkema (1992)
0.96	1,882	8 h/night, 7 nights/week for 3 or 60 nights	Rat, M (S-D) 234-263 g	LM morphometry, histochemistry, autoradiography, and SEM and TEM morphometry of tracheal epithelium. Neither 3 nor 60 days exposure altered the cell density of ciliated, serous, basal, brush, migratory, or unidentified cells in tracheal epithelium. 3 days: Damage to cilia and ciliated cells, including necrosis. Thymidine labeling index increased. Serous cell histochemistry unchanged. 60 days: Less evidence of injury than at 3 days, but more damaged ciliated cells than in controls. Complete recovery of the epithelial changes by 42 days PE.	Nikula et al. (1988a)
0.96	1,882	8 h	*Macaca mulata*, M 2.0-8.5 years old 2.1-6.3 kg	LM and TEM morphometry of trachea, bronchi, and RBs. Increased necrotic cells in trachea and RBs at 1 h PE and in bronchi at 12 and 24 h PE. Decreased ciliated and basal cells in bronchi at 1, 12, and 24 h PE. Basal cells in bronchi also decreased at 72 and 168 h PE. Nonciliated bronchiolar cells in RBs increased only at 24 h PE. In bronchi, smooth muscle increased and amorphous matrix decreased at 24, 72, and 168 h PE. In RBs, smooth muscle increased at 24 h, fibroblasts increased at 24 and 72 h, and amorphous matrix increased at 12 h PE.	Hyde et al. (1992)
1.0	1,960	4 h/day for 5 days (examined at 2 weeks)	Sheep New-born	LM morphometry of tracheal epithelium. Also see Section 6.2.3. Percentage of ciliated and mucous cells remained at newborn levels, rather than ciliated cell percent increasing and mucous cell percent decreasing as in control lambs.	Mariassy et al. (1990)
1.0	1,960	4 h/day for 5 days (examined at 2 weeks)	Sheep New-born	LM morphometry of mucosubstances in tracheal epithelium. No evidence of damage or inflammatory changes. Decreased epithelial cell density, decreased ciliated and basal cells. Lectin-detectable intraepithelial mucosubstances did not undergo the maturation changes seen in control lambs.	Mariassy et al. (1989)
1.0	1,960	96 h (in vitro tracheal explants)	Rat, M (S-D) 250-270 g	Tracheal organ cultures exposed in vitro. Filtered air + O_3 resulted in extensive damage to cilia, and intermediate cells were seen. Cultures exposed to 95% O_2 + O_3 had stratified thickened epithelium with metaplastic cells in a middle zone and no ciliated cells at the surface.	Nikula and Wilson (1990)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

Table 6-11. Effects of Ozone on Lung Structure: Short-Term Exposures (< 2 Weeks)[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)[3]	Reference
ppm	g/m				
0.12 0.8 1.5	235 1,568 2,940	Continuous for 6 h	Rat, M (F344/N) 12-18 weeks old	LM histopathology of lungs and LM morphometry of lavaged AMs: No LM histologic effect detected at 0.12 ppm. No LM histologic effect at 0.8 and 1.5 ppm immediately or 3 h PE. At later PE times, there was mild, patchy CAR bronchiolitis and alveolitis. Increase in AMs and PMNs from 18-66 h PE. Progressive thickening of TB walls and CAR AD septa at 18, 42, and 66 h PE.	Hotchkiss et al. (1989b)
0.12 0.8 1.5	235 1,568 2,940	6 h	Rat, M (F344/N) 12-18 weeks old	Observations of nose and CAR. Same rats as in Hotchkiss et al. (1989a). LM histopathology of CAR is the same; new morphometry of PMNs in CAR and nasal mucosa. Emphasis on PMNs in nasal mucosa and nasal lavage compared with PMNs in CAR tissues and BAL at exposure end and at 3-66 h PE.	Hotchkiss et al. (1989a)
0.12 0.5 0.75	250 1,000 1,500	Continuous for 1-7 days	Rat, M (Wistar RIV:TOX) 8 weeks old	LM: Increased AMs in CAR and parenchyma. CAR increase persisted 5 days PE. TEM and SEM: BAL AMs had microvilli and blebs in addition to ruffles characteristic of AMs from controls. Also see Section 6.2.3.	Dormans et al. (1990)
0.15 0.5		Continuous for 3 or 7 days	Rat, M (Wistar RIV:TOX) 8 weeks old	Elastase-induced emphysema and saline control rats. LM histopathology and morphometry for alveolar size. Also see Section 6.2.5. The incidence and severity of CAR LM lesions was the same in elastase- or saline-treated rats exposed to O_3. No change in alveolar size due to O_3.	Dormans et al. (1989)
0.2 0.4	393 784	4 h	Rat, M (S-D) 7 weeks old	LM histopathology. Necropsy 24 h PE. Lung lesions from 0.2 ppm not reported, but 0.4 ppm resulted in increased AMs and increased cellularity of alveolar septa with focal thickening. No increase in DNA synthesis by nasal epithelium. See mixture effects in Section 6.4.	Mautz et al. (1991)
0.2 0.38	393 745	3.75 h, Rest or exercise	Rat (S-D)	LM histopathology and morphometry. No effect from 0.2 ppm at rest. Increased free cells in airspaces at 0.38 ppm at rest. Exposure during exercise resulted in larger areas with free cells and in areas of septa thickened by infiltrating cells.	Mautz et al. (1985b)
0.25 0.8	490 1,568	29 min/day for 2 days, Strenuous exercise	Horse (Thoroughbred) 5-6 years old Gelding	LM and TEM histopathology (see also Section 6.2.5). 0.25 ppm: Lesions limited to vacuoles seen only by TEM in TB ciliated cells. 0.8 ppm: Gross hemorrhage and edema in two of three horses. CAR lesions, visible only by TEM, included edema, necrosis and sloughing of Type 1 cells, slight increase in AMs, shortened cilia, and vacuoles in ciliated and nonciliated bronchiolar cells.	Tyler et al. (1991c)
0.35 0.6	686 1,176	4 h at rest, 3 h with exercise	Rat, M (S-D) 7 weeks old	Examination 48 h PE. Lung: LM morphometry of lesions as a percent of parenchyma section area. No statistical evaluation of groups exposed to O_3 at rest and exercise. The lesion percent of parenchyma appears concentration-dependent and increased by exercise similar to Mautz et al. (1985b). Nasal epithelium: Evaluated percent thymidine labeled cells in respiratory epithelium. No change due to O_3 at rest. Effects of mixtures in Section 6.4.	Mautz et al. (1988)
0.35 0.8	686 1,568	72 h	Rat, F (S-D) 60 or 444 days old	LM morphometry and SEM. V_v of CAR lesions. Exposed adults lost body weight. Adults exposed to 0.8 ppm had smaller fixed lung volumes. Lesion V_v larger in young than adult rats at both concentrations. Free cell (AM) V_v increased in young rats at 0.35 ppm compared to adults. The CAR lesions were similar by SEM, but younger rats had more AMs in the CAR. Younger rats had larger CAR lesions and more AMs, but older rats had greater changes in body weight and fixed lung volume.	Stiles and Tyler (1988)

Table 6-11 (cont'd). Effects of Ozone on Lung Structure: Short-Term Exposures (< 2 Weeks[a])

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m				
0.35 0.5 1.0	686 980 1,960	2.25 h/day for 5 days	Rat, M (F344) 110-120 days old	LM histopathology and morphometry for parenchymal density and alveolar size. No significant changes in these morphometric parameters. Histopathology: Reported only for 0.5 ppm O_3 + CO_2 challenge group, which had maximal CAR tissue damage on Days 4 and 5. Increased AMs on Days 2 and 3 and foci of necrotic TB epithelium. By Day 5, hyperplasia of TB epithelium and increased AMs and other inflammatory cells, which completely filled some CAR alveoli. Morphologic damage continued, whereas pulmonary functional changes attenuated. Also see Section 6.2.5.	Tepper et al. (1989)
0.4	784	Continuous up to 14 days	Rat, M (Wistar Jcl) 5 weeks old	SEM morphometry, immunocytochemistry. See Section 6.2.1 for biochemistry. Number of Clara cells/m^2 increased at 14 days, but not earlier. The length of the Clara cell apical projection was increased after 6 h, decreased at 1 day, and not different at other periods. Cytochrome P-450 was localized to agranular endoplasmic reticulum of Clara cells.	Suzuki et al. (1992)
0.5	980	20 h/day for 1-14 days	Rat, M (F344) 13 weeks old	LM morphometry of thymidine-labeled cells in bronchus-associated lymph node and MLN. Other lung changes not described. Also see Section 6.2.3.	Dziedzic et al. (1990)
0.64	1,254	Continuous, 7 days	Rat, M (S-D) 250-300 g	LM morphometry of CAR (proximal alveolar) lesions. Increased centriacinar lesions. Rats treated with dimethylthioruea (a H_2O_2 scavenger) had smaller lesion volumes.	Warren et al. (1988)
0.7 0.9	1,372 1,764	20 h/day for 4 days	Mouse, F (CD-1 Crl:CD1/ (CR)BR) 20-22 g	LM morphometry for areal density (e.g., volume density) of lesions. No comparison of O_3 and air exposures. Also see Section 6.2.3.	Dziedzic and White (1987b)
0.75	1,470	Continuous for 3 days	Rat, M (Wistar) 200-250 g	LM histopathology. Ozone-exposed rats gained less body weight. Increased cells in TB and CAR AD septa. Number of cells diminished by Day 4 PE, but foci of AMs remained.	Bassett et al. (1988a)
0.8 0.4	1,568 784	Continuous for 7 days	Rat, M (Wistar) 8 weeks old	LM: Clara cell numbers/mm of TB basement membrane unchanged. Also see Section 6.2.3. Cell isolation: Although the number of isolated Clara cells/10^6 cells isolated/lung was increased, the percent Clara cells in the isolate was not changed. The percent Type 2 cells in the isolate was increased. No morphologic observations at 0.4 ppm.	Van Bree et al. (1989)
0.8	1,568	Continuous for 3 h	Rat, M (S-D) 250-300 g	By LM, PMNs in alveolar septa increased three times at 4 h PE. Number of septal PMNs peaked at 8 h PE and then rapidly declined. Free cells, septal thickening, and cellularity increased with increasing time PE. Also see Section 6.2.2.	Bhalla and Young (1992)
0.82	1,600	Continuous for 7 days	Rat, M (Wistar RIV:TOX) 8 weeks old	LM morphometry of histochemically identified Type 2 cells. Increased number of Type 2 cells than in controls.	Dormans (1989)

Table 6-11 (cont'd). Effects of Ozone on Lung Structure: Short-Term Exposures (< 2 Weeks)[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m				
0.97	1,901	Continuous for 7 days	Rat, M 225-275 g	Smaller body weights. No other statistical comparison of controls and O_3 alone (see Sections 6.2.6 and 6.4).	Last et al. (1986)
1.0 2.0	1,960 3,920	4 h/day for 5 days	Dog, M, F (Beagle) 6 weeks old	LM morphometry for alveolar size. Mean linear intercepts larger (indicating larger alveoli) in 1.0- but not 2.0-ppm group.	Phalen et al. (1986)
1.0 2.0	1,960 3,920	Continuous for 3 h (Isolated perfused lungs)	Rat, M (S-D) 300-380 g	LM and TEM morphology. Necrosis and sloughing of airway epithelial cells of bronchi and larger bronchioles. TB had less severe lesions, including fewer necrotic cells and less damage to cilia. Fragmentation of some Type 1 cells with some areas of bare basal lamina.	Pino et al. (1992a)
1.0	1,960	Continuous for 8 h	Rat, M (S-D) 10 weeks old	LM and TEM morphometry. Also see Section 6.2.2. Rats received either normal rat serum or rabbit anti-rat PMN serum before the exposure. At exposure end, both exposed groups had a smaller volume of ciliated cells per unit area of epithelial basal lamina (V_s) compared with filtered air controls with similar serum. Ciliated cell V_s was also smaller at 4 and 16 h PE.	Pino et al. (1992b)
1.0	1,960	4, 6, 8, and 24 h	Rat, M (S-D) 63 days old	TEM morphometry. Also see Section 6.2.2. Volume of necrotic cells per area basal lamina (V_s) in the TB larger than controls at the end of 4- and 24-h exposure, but not at other exposure or PE times. With increasing exposure time, there was a shift from necrotic cells on the basal lamina to necrotic cells free in the TB lumen. The V_s of necrotic alveolar cells was increased after 4, 6, and 24 h of exposure. Viable undifferentiated cell V_s in TBs was increased after 6-h exposure followed by 18 h PE, 8-h exposure followed by 16 h PE, and after a 24-h exposure. In alveoli, viable Type 1 cell V_s was increased after a 24-h exposure. Total connective tissue V_s changes only increased in TBs after 8-h exposure followed by 4 h PE and in alveoli at the end of 8-h exposure. The V_s of migratory cells in TB interstitium was only increased 4 h after a 6-h exposure. In alveoli, the V_s of capillaries was increased after 8-h exposure.	Pino et al. (1992c)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

Table 6-12. Effects of Ozone on Lung Structure: Long-Term Exposures (> 2 Weeks[a])

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	µg/m³				
Base 0.06; spike to 0.25	Base 118; spike to 490	Base 13 h/day, 7 days/week; Ramped spike 9 h/day, 5 days/week (1, 3, 13, and 78 weeks)	Rat, M (F344) 60 days old	TEM morphometry, data expressed as volume per unit epithelial basement membrane (V_s). Acute response in the CAR (proximal alveolar) region included increased AMs, interstitial edema, and interstitial cell hypertrophy. These changes subsided by 3-weeks exposure. Changes at 13 and 78 weeks include increased V_s, total alveolar tissue, and epithelium. Type 1 cell increases include V_s at 13 weeks; number at 13 weeks, 13 + 6 weeks PE, and 78 weeks; and decreased surface area at 78 weeks. Type 2 cell V_s increased at 78 weeks and after 78 + 17 weeks PE, and number increased at 78 weeks. Total interstitium was increased at 78 weeks, and noncellular interstitium (collagen and basement membrane) was increased at 13 and 78 weeks. Thickened basement membrane had crystalline deposits. No bronchiolization or centriacinar airway remodeling. Changes in terminal bronchiolar cells include a decrease in surface area of ciliated and nonciliated at 78 weeks. No changes in bronchiolar cell numbers. All changes diminished, except the increased volume of Type 2 cells and the thickened basement membrane, 17 weeks PE.	Chang et al. (1992)
A: 0.25 B: Base 0.06; spike to 0.25	490; 118 to 490	6 weeks 3 weeks 13 weeks 12 h/day 22 h/day	Rat (F344) 7 weeks old	Compared effects of A and B exposure regimens. Cumulative doses for A (Barry et al., 1985) were 60.5 and 126.0 ppm-h and, for B, were 45.3 ppm-h at 3 weeks and 196.0 ppm-h at 13 weeks. The B regimen alone (Chang et al., 1992) is described earlier in this table. TEM morphometry of CAR. The pattern of exposure did not affect the degree of injury.	Chang et al. (1991)
0.1	196	2 h/day, 5 days/week for 1 year	Rabbit, M (NZW) 3-3.5 kg	LM morphology and morphometry of intrapulmonary conducting airways. (Also see Section 6.4). No difference in number of airways/area or in distribution of airway size. ESCs in the smallest conducting airways (< 0.30 mm) increased at 4, 6, and 12 mo of exposure to O_3 and decreased at 6 mo PE. ESCs in the next larger airways (0.31-0.49 mm) only increased after 4 mo of exposure and decreased at 6 mo PE. No effect on airways > 0.50 mm.	Schlesinger et al. (1992a)
0.12 0.25	235 490	12 h/day for 6 weeks	Rat, M (F344) 1 day or 6 weeks old	TEM morphometry of proximal alveolar region (CAR). Type 1 epithelial cells increased in number and thickness, but decreased in luminal and basal lamina surface area. Some bare basement membrane where Type 1 cells sloughed, but not significantly increased. At 0.25 ppm, Type 2 epithelium increased in number, but not in volume, thickness, or surface area. Interstitium increased in thickness in adults at 0.25 ppm, but not at 0.12 ppm or in juveniles. AMs increased by 0.25 ppm at both ages, but only older rats had increased interstitial AMs. No differences due to age.	Barry et al. (1985)
0.12 0.5 1.0	235 980 1,960	6 h/day, 5 days/week for 20 mo	Rat, M (F344) 6-8 weeks old	LM morphometry of CAR remodeling. Thickened tips of alveolar septa lining ADs (alveolar entrance rings) 0.2 mm from TB in rats exposed to 0.12 ppm and to 0.6 mm in rats exposed to 1.0 ppm. Interstitial changes accompanied these epithelial changes.	Pinkerton et al. (1995)
0.12 0.5 1.0	235 980 1,960	6 h/day, 5 days/week for 20 mo	Rat, M (F344) 6-8 weeks old	Laser scanning confocal LM immunohistochemistry for CC10 in nonciliated bronchiolar (Clara) cells. Clara cells from rats exposed to 1.0 ppm, but not to 0.12 ppm, had increased cell volume of granule-based CC10, increased CC10 concentration within the granules, and increased number of granules per Clara cell profile.	Dodge et al. (1994)
0.12 0.25 0.5	235 490 980	20 h/week, 7 days/week for 2 years (Examined at 4, 12, 26, 52, 78, and 104 weeks)	Rat, M (F344 CrlBR) 42 days old	Rats exposed to 0.5 ppm had smaller BW after 7-weeks exposure. LM histopathology. Nose: At ≥0.25 ppm, mucous cell respiratory epithelium hyperplasia; no lesions in mainstem or large bronchi. CAR: ≥0.25 ppm, TB epithelium hyperplastic and hypertrophic; bronchiolarization and airway remodeling. No changes in 0.12-ppm group after 26 weeks of exposure. Peribronchiolar tissue and AD walls thickened by eosinophilic material after 12 weeks at 0.5 ppm and after 26-weeks at 0.25 ppm. Collagen found in these areas using special stains. Increased AMs at ≥0.25 ppm.	Smiler et al. (1988) Wright et al. (1989, 1990)

Table 6-12 (cont'd). Effects of Ozone on Lung Structure: Long-Term Exposures (>2 Weeks[a])

Ozone Concentration ppm	Ozone Concentration g/m	Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
0.15 0.3	294 588	8 h/day for 6 or 90 days	*Macaca radiata*, F, M 2-6 years old	TEM, SEM, and LM morphometry. First generation RBs had epithelial hyperplasia, and alveoli opening into these RBs had increased AMs. RB epithelium thickened, but no difference due to either exposure time or concentration. RB interstitium was thickened in all exposed monkeys, but both cellular and acellular compartments were individually thickened only after 90-days exposure to 0.3 ppm. No differences due to age or gender. No evidence of epithelial cell necrosis nor of inflammatory cell infiltration other than the increased AMs.	Harkema et al. (1993)
0.25	490	12 h/day for 6 weeks	Rat, M (F344) 1 day or 6 weeks old	TEM morphometry of the TBs. Luminal surface area covered by cilia decreased, as did the luminal surface of Clara (nonciliated bronchiolar) cells. Number of brush cells decreased. No differences due to age.	Barry et al. (1988)
0.25	490	8 h/day, 7 days/week, "daily" for 18 mo or "seasonal" O_3 odd months, filtered air even months for 18 mo (9 mo of O_3)	*Macaca fascicularis*, M 6 mo old	LM morphometry. Also see Section 6.2.5. Low-grade respiratory bronchiolitis in both exposed groups. Compared with controls, both groups of exposed monkeys had increased V_v of tissue other than parenchyma and V_v of RBs and their lumens. Both V_v and V of RB wall increased in the "daily" group but not in the "seasonal" group. The only significant morphometric difference between the two exposed groups was the V_v of cells, mostly AMs, free in airspace lumens. This difference and the difference in significance of the RB wall thickness was presumed due to the difference in time after the last O_3 exposure and necropsy. Daily group necropsied the day after the last exposure, whereas seasonal group necropsied after a month of filtered air. Seasonal group had an amount of morphological changes similar to the daily group.	Tyler et al. (1988)
0.25	490	8 h/night, 7 nights/week, "daily" for 18 mo or "seasonal" O_3 odd months, filtered air even months for 18 mo	Rat, M (S-D) 22 days old	LM morphology and morphometry. Monkey data from Tyler et al. 1988 (above) compared with rats exposed to a similar regimen. Rats: Estimated the extent of centriacinar remodeling by counting the number of junctions of bronchioles with ADs per area of lung section (B/A J/cm^2). At the end of the exposure, both exposed groups had more B/A J/cm^2 than filtered air controls. Recovery by 30 days PE. No difference between the two exposed groups, even though the daily group was exposed twice as many days as the seasonal group.	Tyler et al. (1991a)
0.3	588	7 h/day, 5 days/week for 6 weeks	Mouse, M (Swiss-Webster) Newborn	LM morphometry of histochemically identified Type 2 cells. Type 2 cells tended to be larger (longer linear intercepts), and the number per microscope field tended to be greater, but the p values were >0.05 in final data in which the images were edited electronically. However, these values were significant ($p < 0.05$) in unedited data. Exposed mice had larger ($p < 0.05$) body weights at both 3 and 6 weeks.	Sherwin and Richters (1985)

Table 6-12 (cont'd). Effects of Ozone on Lung Structure: Long-Term Exposures (>2 Weeks[a])

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
ppm	g/m				
0.35	686	4.5 h/day, 5 days/week for 4 weeks, 380 mmHg (5,400 m) or sea level	Mouse, M (Swiss-Webster) 32 g	Automated LM morphometry of stainable elastin in alveolar walls. Simulated high altitude (5,400 m) with O_3 (SHA-X) or without O_3 (SHA-C) resulted in larger lung volumes than sea-level controls (SL-C), but not different from each other. Unlike most studies, sea-level, O_3-exposed mice had the smallest lung volumes. Alveolar wall areas, after adjustment to SL-C lung volumes, were increased only in the SHA-X group. Alveolar wall elastin area, adjusted to the SL-C lung volumes, increased in both high-altitude groups compared to SL-C and also were different from each other with the largest amount of elastin area in the SHA-X group. However, if the elastin areas were not adjusted for differences in lung volumes, there were no differences between the groups.	Damji and Sherwin (1989)
0.4 0.64	784 1,254	8 h/day, 7 days/week for 90 days	*Macaca radiata*, M 5-8 years old	LM and TEM morphometry with emphasis on RBs. Respiratory bronchiolitis and peribronchiolar inflammation. RB walls thicker with smaller lumens. Increased wall thickness due both to thicker epithelium (significant only at 0.64 ppm) and interstitial components. Epithelial changes in both O_3 groups include increased nonciliated bronchiolar epithelial cells and decreased Type 1 cells. Interstitial changes in both O_3 groups included increased smooth muscle cells, mast cells, and fibers. Components increased at 0.64 ppm, but not at 0.4 ppm, included interstitial AMs, PMNs, and amorphous ground substance.	Moffatt et al. (1987)
0.5	980	6 h/day, 6 days/week for 2, 3, 5, and 12 mo	Rat, M (Wistar) 100 g	LM, TEM, and LM morphometry of collagen fibers. Bronchitis, peribronchitis, CAR remodeling, and increased stainable collagen in bronchioles. Rats apparently had intercurrent respiratory disease, as 10 of 44 exposed rats died of pneumonia or pulmonary edema. In addition, it appears that only one set of 12 rats maintained in room air served as controls for all exposed groups, even though controls and exposed rats would be of significantly different age and size.	Hiroshima et al. (1989)
0.5	980	20 h/day, 7 days/week for 52 weeks	Rat (F344)	LM histopathology. 6-mo exposure: Inflammation, mononuclear cells, and fibroblasts in AD walls and walls of adjacent CAR alveoli. TB not involved. 12-mo exposure: Similar to 6 mo with possibly a slight increase in AMs, some increased thickening of centriacinar AD and alveolar walls, and a few foci of bronchiolization (CAR remodeling). 12-mo exposure + 6 mo PE: Slight dilation of ADs, minimal inflammatory reaction, slight thickening of AD and CAR alveolar walls, and a few foci of bronchiolization.	Gross and White (1987)
0.5	980	Continuous for 120 days	Mouse, F (Swiss) 20-23 g	LM morphometry and histopathology. Also see Section 6.2.3. More tissue, primarily inflammatory cells, at Day 9. Little change from Days 10 to 120. Thickened airway walls with increased collagen. Increased collagen in alveolar walls along the ADs.	Jakab and Bassett (1990)
0.64 0.96	1,254 1,881	8 h/night, 7 nights/week for 42 nights, "pair" fed	Rat, M (S-D) 28 days old	LM morphometry and SEM. Also see Section 6.2.5. End of 42-night exposure: No difference in BW, hemoglobin, or total serum proteins. At 0.96 but not 0.64 ppm, larger fixed and saline-filled lung volumes, lung volume/BW ratios, and volumes of parenchyma. At both concentrations, increased V_v and V of RB and RB walls and their ratios to BW. By SEM, remodeling of CAR airways with the formation of RBs and thickened CAR septa at both ppm. After 42 days PE: Fixed lung volume at 0.96 ppm increased. V_v and V of RB walls and ratio to BW increased at 0.96 ppm, as did ratios of volumes to BW for parenchyma, alveoli, total RB, and RB wall. SEM revealed persistence of CAR remodeling and thickened septa.	Tyler et al. (1987)

Table 6-12 (cont'd). Effects of Ozone on Lung Structure: Long-Term Exposures (>2 Weeks[a])

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
ppm	g/m				
0.64	1,254	8 h/day for 12 mo	*Macaca facisclaris*, M 6-7 mo old	LM morphometry of CAR airway remodeling. Both V_v and V of RBs, and their walls and lumens, increased at end of exposure and 6 mo PE. RB internal diameters were smaller at exposure end, but not at 6 mo PE. V_v free cells, mostly AMs, increased only at exposure end. No differences in BW or fixed lung volumes.	Tyler et al. (1991b)
0.7	1,372	20 h/day for 4 weeks	Rat, M (F344) 14 weeks old	LM histopathology. Also see Section 6.2.5. Exposure end: CAR inflammation of TB, AD, and CAR (proximal) alveoli characterized by edema with mononuclear and leukocyte infiltration. 4 weeks PE: Few inflammatory foci, edema decreased, and interstitial mast cells. Slight thickening of ADs and septa. 9 weeks PE: Inflammation cleared, TB walls slightly thickened by amorphous matrix.	Gross and White (1986)
0.95	1,862	8 h/day for 90 days	Rat, M (S-D) 61 days old	LM and TEM morphology. RB: Increased volume of total RB and of RB wall and lumen. RB walls thickened by interstitial inflammation with edema, hyperemia, fibrosis, and hypertrophied smooth muscle and by interstitial mononuclear cells, granulocytes, and plasma cells. Epithelial and vascular basal lamina fused. TB: Smaller internal diameter and smaller luminal volume, but no change in total TB volume or in wall volume. Proximal AD: Most severe cell damage and inflammation at alveolar septal tips (alveolar entrance rings). Epithelium at these tips was frequently necrotic or missing, leaving bare basement membrane. Duct walls thicker due mainly to increased interstitial edema, fibrosis, and cellular infiltrates. Basal lamina thickened, split or duplicated, and had granular deposits. Site of most severe injury shifted progressively distally as new segments of RB were formed.	Barr et al. (1988)
0.95	1,862	8 h/day for 90 days or 5-day episodes followed by 9 days PE	Rat, M (S-D) 52 days old	LM and TEM morphometry. Both groups examined at exposure end. The lesions were as previously described by Barr et al. (1988). Both groups had CAR airway remodeling with the formation of new RBs. The only morphometric difference in RBs between the groups was the volume of RB wall, which was greater in the daily group, but both groups were greater than controls. The volume of the total RB and of RB lumen was increased in the daily group. RB epithelium of the daily group was more differentiated. TB interstitium was increased in the episodic group. Alveolar duct/sac lumen volume was increased in both groups with the increase in the episodic group significantly greater than the daily group. Alveolar volume was decreased in the episodic group. The total amount of CAR damage was not different for both episodic (35 exposures) and daily (90 exposures) groups.	Barr et al. (1990)
0.96	1,882	8 h/night, 7 days/week for 3 or 60 nights	Rat, M (S-D) 234-263 g	LM morphometry, histochemistry, autoradiography, and SEM, and TEM morphometry. Neither 3 nor 60 days of exposure altered the cell density of ciliated, serous, basal, brush, migratory, or unidentified cells in tracheal epithelium. 3 days: Damage to cilia and ciliated cells, including necrosis. Thymidine labeling index increased. Serous cell histochemistry unchanged. 60 days: Less evidence of injury than at 3 days, but more damaged ciliated cells than in controls. Complete recovery of the epithelial changes by 42 days PE.	Nikula et al. (1988a)
0.98	1,921	8 h/day, 7 days/week for 90 days	Rat, M (S-D) 65 days old	LM morphometry and SEM of CAR. Remodeling of CAR. Increased thickness of septal edge (tips) of alveoli, which form the walls of ADs (alveolar entrance rings) up to 0.6 mm from TB. Alveolar septa thickened by replacement of Type 1 cells by Type 2 and bronchiolar cells to 0.6 mm from TB.	Pinkerton et al. (1992)

Table 6-12 (cont'd). Effects of Ozone on Lung Structure: Long-term Exposures (> 2 Weeks)[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s) [3]	Reference
ppm	g/m				
1.0	1,960	6 h/day, 5 days/week for 20 mo	Rat, M (F344) 6-7 weeks old	LM morphometry, SEM, confocal microscopy, immunocytochemistry, and conventional histochemistry. Remodeling of CAR. Former ADs were converted to RBs. Bronchiolar epithelium in these former ADs consisted of well-differentiated ciliated and nonciliated bronchiolar (Clara) cells.	Pinkerton et al. (1993)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

Harkema et al. (1987) exposed bonnet monkeys to 0.15 or 0.30 ppm O_3, 8 h/day for 6 or 90 days. They sampled four regions of the nasal cavity and nasopharynx. Changes were limited to the respiratory and transitional epithelium in the two most rostral (anterior) of the four sections. No changes were reported in the caudal (posterior) two sections, the last of which included the nasopharynx. The respiratory epithelium of the rostral nasal cavity had both qualitative and quantitative changes. Quantitative changes included decreased density of ciliated cells characterized qualitatively by multifocal loss of cilia, necrotic ciliated cells, ciliated cells with attenuated cilia, and others with only microvillar surface. The respiratory epithelium also had an increased density of SMG cells, presumably related to repair processes. Monkeys exposed to 0.30 ppm O_3 for 90 days also had increased abnormal cells with intracytoplasmic lumens containing both cilia and microvilli. Qualitative changes were also seen in mucous (goblet) cells, which appeared to have fewer secretory granules and dilated endoplasmic cisternae. Ozone exposure resulted in more nonciliated cells with secretory granules and with dilated cisternae of the endoplasmic reticulum. Like the respiratory epithelium, the transitional epithelium had an increased density of SMG cells. In both epithelia, inflammatory cells were increased only in the monkeys exposed to 0.15 ppm O_3 for 6 days. Most of the morphometric changes in the respiratory but not the transitional epithelium were as large after 6 days of exposure to 0.15 ppm O_3 as after 90 days of exposure to either 0.15 or 0.30 ppm. The histochemistry and cytochemistry of the nasal epithelia from these monkeys were studied by Dimitriadis (1992). This investigator reported changes in the intraepithelial mucosubstances and the presence of mucous cells with dilated cisternae in the granular endoplasmic reticulum.

Acute changes in nasal epithelia from rats exposed to O_3 concentrations of 0.12 to 1.0 ppm for 6 h to 7 days have been studied extensively (Table 6-10). In general, short-term exposure to $\leq$0.2 ppm O_3 results in either no changes detectable by LM or in mild hyperplasia. Higher concentrations for up to 7 days can result in damaged cilia, hyperplasia, and increased stored intraepithelial mucosubstances. Several studies document the hyperplasia using morphometry or DNA synthesis and document the stored mucosubstance by histochemistry and morphometry. In one study, the increased stored intraepithelial mucosubstances reached their largest quantity 7 days postexposure (Harkema et al., 1989). Details of individual studies follow.

Exposure to 0.12, 0.8, or 1.5 ppm O_3 for 6 h followed by postexposure periods up to 66 h resulted in inflammatory changes characterized by increased PMNs, but without LM evidence of necrosis, ciliary loss, or hyperplasia (Hotchkiss et al., 1989a). Hotchkiss and Harkema (1992) reported similar LM findings in rats exposed to 0.8 ppm O_3 for 6 h. They also reported increased DNA synthesis by bromodeoxyuridine (BrdU) uptake in nasal nonciliated transitional epithelium. Exposure to 0.8 ppm O_3 6 h/day for 3 or 7 days, or for 3 days with 4 days postexposure, resulted in hyperplasia of the nasal nonciliated cuboidal (transitional) epithelium with increased intraepithelial mucosubstances without significant changes in histochemical staining characteristics (Hotchkiss et al., 1991). In that study, no changes were reported for rats exposed for 3 days and examined 18 h postexposure.

Reuzel et al. (1990) exposed rats to 0.2, 0.4, or 0.8 ppm O_3, 22 h/day for 3 days. They did not report changes in rats exposed to 0.2 ppm, but those exposed to 0.4 or 0.8 ppm had loss of cilia and disarrangement of the epithelium with hyperplasia and metaplasia. Cell proliferation, as measured by radiolabeled thymidine, was increased at the two higher concentrations. The influence of O_3 C $\times$ T on epithelial cell proliferation in the nasal anterior

maxilloturbinates was measured by BrdU uptake (Henderson et al., 1993). Rats were exposed to 0.12, 0.24, and 0.48 ppm O_3 for 3, 6, 12, and 24 h, resulting in six C × T products. Exposure to 0.12 ppm or C × Ts of 0.72 or 1.44 ppm·h did not cause effects. For a given C × T between 2.88 and 11.52 ppm·h, the increased DNA synthesis was similar; the response did not increase linearly with increasing C × Ts. Generally, above 0.12 ppm O_3 there was a linear increase with increasing C but not T. Thus, exposure duration apparently was responsible for the lack of C × T linearity. Johnson et al. (1990) also used BrdU to study DNA synthesis in rats exposed to 0.12, 0.27, or 0.8 ppm O_3, 6 h/day for 3 or 7 days, and examined 3 or 7 days postexposure. Rats exposed to 0.8 ppm O_3, but not to the lower concentrations, had increased DNA synthesis in the nonciliated cuboidal (transitional) epithelium at 3 and 7 days and increased numbers of labeled cells in the ciliated respiratory epithelium and the olfactory epithelium only at 3 days. No changes were found in squamous epithelia except a decrease in labeled cells 7 days postexposure to 0.8 ppm O_3. Johnson and co-workers reported no LM changes in the ciliated respiratory, olfactory, or squamous epithelia, but hyperplasia occurred in the cuboidal transitional epithelium.

Epithelial mucosubstances were studied in rats exposed to 0.12 or 0.8 ppm O_3, 6 h/day for 7 days or 7 days postexposure (Harkema et al., 1989). They reported no LM pathology in the nasal or nasopharyngeal airways from rats exposed to 0.12 ppm, with the exception of an increase in secretory cells in ciliated epithelium. Rats exposed to 0.8 ppm had attenuation of cilia in the lateral walls of the nasopharynx; 7 days postexposure, an increase in stored intraepithelial mucosubstances was observed. The 0.8-ppm group also had hyperplasia of the nonciliated transitional epithelium accompanied by an increase in PMNs in the lamina propria. Seven days postexposure, rats in the 0.8-ppm exposure group had more stored intraepithelial mucosubstances in some areas of ciliated respiratory and nonciliated transitional epithelia.

In rats exposed to 0.12, 0.25, or 0.5 ppm O_3, 20 h/day for 2 years, Smiler et al. (1988) reported hyperplasia, especially of mucous cells, in the respiratory epithelium over the rostral portion of the nasoturbinate of rats in the 0.25- and 0.5-ppm groups. The respiratory epithelium lining other parts of the nasal cavity were less affected, and no changes were found in the squamous and olfactory epithelia.

Harkema et al. (1994) reported no changes in the amount of mucosubstances in conducting airways, including the nasal cavity, of rats exposed to 0.12 ppm O_3, 6 h/day, 5 days/week, for up to 20 mo. After exposure to 0.5 and 1.0 ppm O_3, however, mucous flow rates were slower and mucous cell metaplasia was evident over the lateral wall and turbinates of the proximal third of the nasal airways. Exposure to 0.5 and 1.0 ppm O_3 also caused epithelial hyperplasia in nasal transitional epithelium, an increase in eosinophilic globules in the surface epithelium lining the distal nasal airways, and mild-to-moderate inflammatory cell influx in the nasal mucosa of the proximal and middle nasal passages.

Larynx

Leonard et al. (1991) reported disruption and thickening of the stratified squamous epithelium over the vocal folds of bonnet monkeys (*Macaca radiata*) exposed to O_3, 8 h/day for 7 days. The basement membrane appeared undulating rather than smooth. At 7 days postexposure, the epithelium appeared thickened, but otherwise normal. The O_3 concentration to which the monkeys were exposed is not clear because different concentrations appear in the summary and text sections of the publication. However, these larynges were from bonnet

monkeys that also were studied by Harkema et al. (1987) and Dimitriadis (1992) and, therefore, most likely were exposed to 0.15 ppm.

Trachea and Bronchi

Several investigators studied effects of 0.96 or 1.0 ppm O_3 on the tracheas of monkeys, rats, and sheep during and after short-term (very brief) or long-term exposures. Hyde et al. (1992) studied the trachea, bronchi, and RBs of rhesus monkeys exposed to 0.96 ppm O_3 for 8 h and examined them at 1, 12, 24, 72, and 168 h postexposure. Although the primary objective of the study concerned inflammation (see Section 6.2.2), the study also provided much new morphometric information concerning reactions to O_3 of tracheal, bronchial, and RB epithelia and their interstitium. Both epithelial and interstitial data were determined as volume per surface area of epithelial basal lamina (V_s). At 1 h postexposure, the major change in the tracheal and RB epithelia was an increase in necrotic cells, whereas in the bronchial epithelium, there were fewer ciliated and basal cells. There were no other changes in tracheal epithelial cell V_s at any of the postexposure times examined. At 12 and 24 h postexposure, the V_s of necrotic cells was increased in bronchi but not in the trachea or RBs. The V_s of ciliated and basal cells was smaller in the bronchial epithelium but not in the trachea. Basal cells in bronchi also were increased at 72 and 168 h postexposure. Respiratory bronchioles had smaller V_s of Type 1 alveolar epithelial cells at all times except 1 h postexposure. In RBs, nonciliated bronchiolar cells were increased only at 24 h postexposure. Epithelial cell DNA synthesis was studied in the filtered air controls and at 1 and 12 h postexposure by radiolabeled thymidine incorporation. The only increase was observed in the bronchial epithelium at 12 h postexposure. Changes in the interstitial components of the trachea were minimal, with a decrease in the amorphous matrix at 24 h postexposure. Bronchi had increased V_s of smooth muscle and decreased amorphous matrix at 24, 72, and 168 h postexposure. Collagen fibers in the bronchial interstitium were decreased at 168 h. In RBs, the arithmetic mean thickness was increased at 12 and 24 h, but not at other times. In RBs, smooth muscle V_s was increased at 24 h, V_s of fibroblasts was increased at 24 and 72 h, and V_s of the amorphous matrix was increased at 12 h postexposure.

Nikula et al. (1988a) exposed rats to 0.96 ppm O_3, 8 h/night for 3 or 60 nights or for 60 nights followed by 7 or 42 days postexposure, and examined the tracheas using LM, TEM morphometry, SEM, LM mucosubstance histochemistry, and DNA synthesis by radiolabeled thymidine incorporation. Ciliated cells with short or damaged cilia were increased after 3 and 60 nights of exposure; cells with short cilia were increased after 60 nights of exposure and 7 days postexposure. Intermediate cells, presumed to be immature ciliated cells, were increased only after 3 nights of exposure. However, the numeric density of total ciliated cells, basal cells, total serous cells, brush cells, and total migratory cells was not different from controls. There were no changes in LM histochemistry for mucosubstances at any time. The only increase in thymidine labeling occurred after 3 days of exposure. Recovery was complete 42 days after 60 nights of exposure.

Mariassy et al. (1989, 1990) exposed newborn lambs to 1.0 ppm O_3, 4 h/day for 5 days, and studied controls at birth and controls and exposed lambs at 2 weeks of age. Tracheal mucous velocity was decreased at 2 weeks and at several additional postexposure times (see Section 6.2.3). In control lambs, the percent of ciliated cells increased and mucous cells decreased in the tracheas from birth to 2 weeks of age. This normal change in cell populations did not occur in the exposed lamb tracheas. In the more detailed morphological

study (Mariassy et al., 1989), epithelial cell density (cells per millimeter), rather than differential cell counts, was reported. In tracheas from control lambs, the density of mucous cells decreased from birth to 2 weeks of age. Ozone exposure resulted in decreased total epithelial cell density, with decreased densities of ciliated and basal cells. Mucous cell density remained at newborn levels. Ozone exposure also prevented the normal maturational changes of lectin-detectable mucosubstances but not of tinctorially stained mucosubstances.

The most comprehensive study of the effects of long-term O_3 exposure on conducting airways of rats is that by Plopper et al. (1994a). They used LM morphometry and tinctorial histochemistry to study conducting airways from the trachea to centriacinar alveoli following two "short" and one "long" pathway by airway dissection of fixed lungs. In rats exposed to 0.12, 0.5, or 1.0 ppm O_3 for 6 h/day, 5 days/week for 20 mo, the investigators did not find differences due to O_3 exposure in tracheal or bronchial epithelial thickness, cell populations, or stored glycoconjugates. However, they did find a concentration-dependent loss of stored glycoconjugates in the tracheas and in the caudal long-path bronchi but not in the cranial or central short-path bronchi. Although not significantly different from controls, there was a concentration-dependent thinning of the epithelium in caudal long-path bronchi. Terminal bronchioles from rats exposed to 0.5 and 1.0 ppm O_3 had increased volume fraction (V_v) of nonciliated bronchiolar (Clara) cells, and the epithelium was thicker in TBs from rats exposed to 1.0 ppm. In all exposed rats, the mass (V_s) of nonciliated bronchiolar cells was increased in TBs that had long pathways (caudal) but not in TBs with short pathways (cranial and central).

Centriacinar Region

As described in the previous criteria document (U.S. Environmental Protection Agency, 1986) and in the summary of it above, the CAR varies with the species. By common usage (Weibel, 1963; Schreider and Raabe, 1981; Weibel, 1983; Rodriguez et al., 1987; Haefeli-Bleuer and Weibel, 1988), the acinus consists of a TB, RBs when present, and the ADs and alveoli supplied by that TB. In some species (e.g., humans, monkeys, dogs, and cats), several generations of RBs are found between the TB and ADs. In other species (e.g., rats, mice, guinea pigs, and rabbits), RBs either are absent or very poorly developed and limited to a single, very short generation (Tyler, 1983; Tyler and Julian, 1991). The CAR consists of the TB, RBs if present and alveoli that open directly into RBs, and the initial portions of ADs. Acini that do not have RBs have a smaller volume than those that do. Rodriguez et al. (1987) estimated the acinar volume in rat lungs to be 1.86 mm^3, and Haefeli-Bleuer and Weibel (1988), using the same methods, estimated the acinar volume in the human lungs at 187.0 mm^3. Mercer and Crapo (1989) and Mercer et al. (1991) found that variation in acinar size within an individual lung is an important determinant of the intensity of lesions due to inhaled reactive gases. Thus, the intensity of CAR lesions may vary when animals with differing size acini are compared (Plopper et al., 1991).

The CAR lesion, both in animals with small acini (e.g., rats) and animals with large acini (e.g., monkeys) has been well described, both in the original reports and in the 1986 document (U.S. Environmental Protection Agency, 1986). Some of the reports published since that document contain additional details concerning cellular and interstitial responses in the CAR to short- or long-term O_3 exposure and are presented in this section (Chang et al., 1992; Pino et al., 1992c; Harkema et al., 1993). Most of these studies need TEM levels of resolution and magnification and employ morphometric methods. In other reports,

morphometric estimates of the volume of the CAR lesion were used to study factors that might alter the intensity of the lesion or evaluate the intensity of reaction to specific exposure regimens (Mautz et al., 1988; Warren et al., 1988; Stiles and Tyler, 1988). Due to the size and definition of the CAR lesion, this approach can use LM morphometry to estimate lesion volume. In other studies, the cumulative effect of O_3 on the CAR is estimated by LM morphometry of one of the components, distal airway remodeling, which results in the formation of new RBs (Barr et al., 1988; Tyler et al., 1988; Pinkerton et al., 1993). Examples of each type of study will be presented.

Pino et al. (1992c) exposed rats to 1.0 ppm O_3 for 4, 6, 8, or 24 h, followed by postexposure periods in filtered air for up to 20 h, so that the total exposure and postexposure period did not exceed 24 h. Some of the rats were used for BAL (see Section 6.2.2), others for TEM morphometry. The morphometric data are expressed as V_s values. After a 4-h exposure, necrosis was the dominant morphologic feature, with increases in V_s of necrotic cells in the TB epithelium (ciliated cells) and in CAR alveoli (Type 1 cells). With increasing time of exposure or postexposure, the volume of necrotic cells in TBs shifted from the epithelium to the lumen, with this change being significant at 24 h. In CAR alveoli, increased V_s of total necrotic cells occurred at 4, 6, and 24 h of exposure and at 24 h in the epithelium. Healing in the TBs, evidenced by increased V_s of undifferentiated cells, was underway 18 h after a 6-h exposure, 16 h after an 8-h exposure, and immediately after 24 h of exposure. The only significant change in viable alveolar cells was an increase in V_s of Type 1 cells after 24 h of exposure. This increase appeared predominantly due to swelling of individual Type 1 cells. Increased V_s of total TB interstitium occurred 4 h after an 8-h exposure. In CAR alveoli, total interstitium was increased after 8 h of exposure, with much of the increase due to an increase in capillary volume.

Chang et al. (1992) used TEM morphometry to evaluate cellular and interstitial responses in the CAR, TB, and alveoli (proximal alveoli) of rats exposed to a 9-h peak slowly rising to 0.25 ppm O_3 superimposed on a 13-h background level of 0.06 ppm (the background was 7 days/week, the peak was 5 days/week). Chang and co-workers examined rats after 1, 3, 13, and 78 weeks of exposure; 6 weeks after a 13-week exposure; and 17 weeks after a 78-week exposure. Centriacinar region alveoli had a larger volume of total tissue and total epithelium per area of basement membrane (V_s) only after 13 or 78 weeks of exposure, and these values were not different after postexposure periods. Type 1 cells had a larger volume only at 13 weeks of exposure, increased numbers at 78 weeks, and increased numbers after 13 weeks of exposure plus 6 weeks postexposure. Type 2 cell V_s was increased only after 78 weeks of exposure and 78 weeks of exposure plus 17 weeks postexposure. Macrophages in the CAR alveoli were increased only after 1 week of exposure. In CAR alveoli, both interstitial cells and matrix were increased after 1 week of exposure, and the matrix increased again after 13 and 78 weeks of exposure. This difference was no longer significant after either postexposure period. Although the data are not in the tables or figures in the article, the text indicates that both epithelial and endothelial basement membranes were thickened after 13 and 78 weeks of exposure and after the 17-week postexposure period. Crystalline deposits in the basement membrane are demonstrated in Figure 6-3. In TBs, the luminal surface area of Clara cells was reduced at 1 week of exposure, and both ciliated and Clara cells had smaller luminal surface areas after 78 weeks of exposure; these returned to control values during the postexposure period. However, increased V_s of Type 2 cells persisted for the 17-week postexposure period that followed the

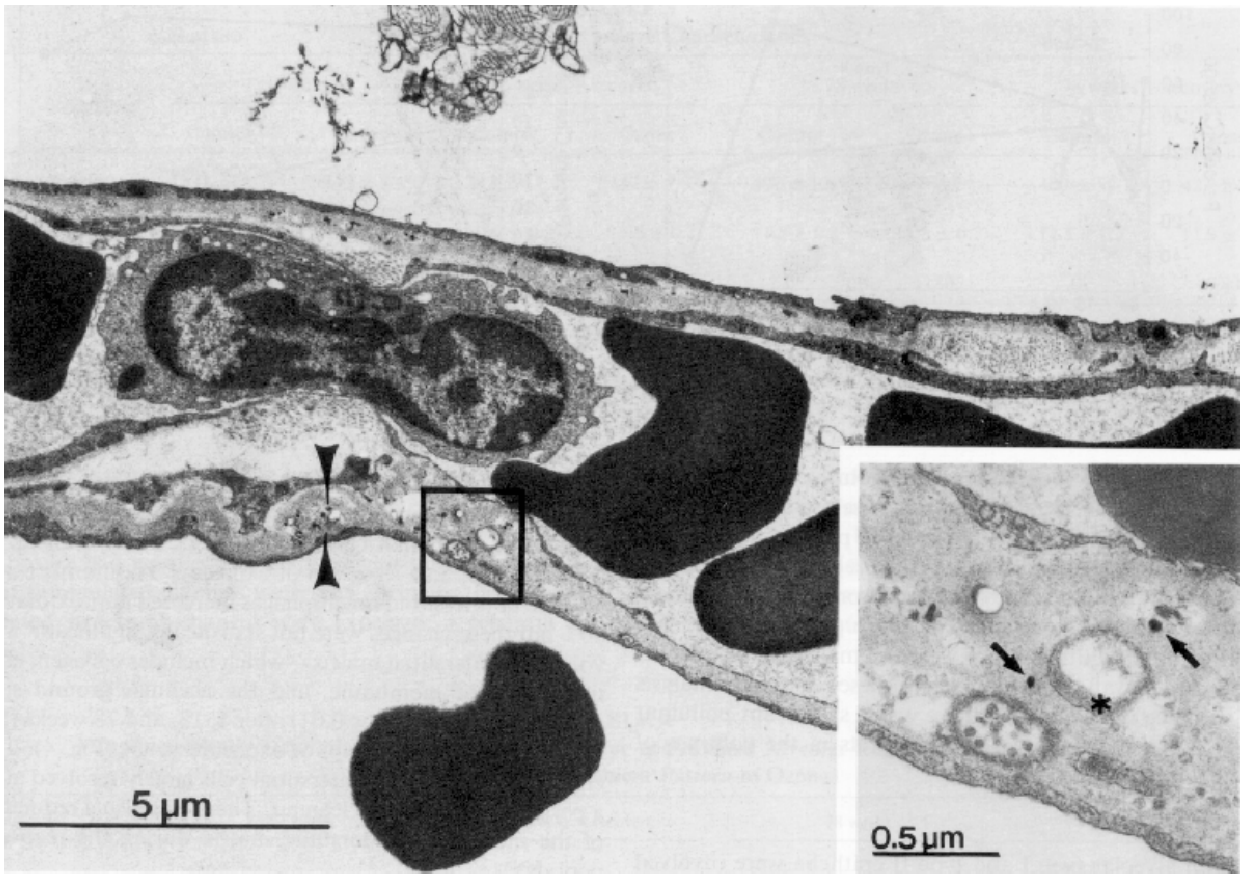

Figure 6-3. Electron micrograph of alveolar septa in the centriacinar region of the lungs from laboratory rats exposed to a simulated pattern of ambient O_3 for 78 weeks, showing thickened basement membrane (arrow heads). Inset micrograph shows dense crystaline deposits (arrows) and cellular extensions (*).

Source: Chang et al. (1992).

78 weeks of exposure. Chang et al. did not find significant bronchiolization of alveoli (i.e., distal airway remodeling). This observation may be due to the way the investigators sampled the CAR proximal alveoli and the strict orientation of the airways to obtain exact cross sections. This study complements those of Barry et al. (1985, 1988), who used similar TEM morphometric methods, by extending the exposure period and adding postexposure periods.

Cellular and interstitial changes were studied in nonhuman primates exposed 8 h/day for 90 days by both Moffatt et al. (1987) and Harkema et al. (1993), using TEM and morphometry. The study by Harkema et al. (1993) is reported here because the concentrations used, 0.15 and 0.3 ppm O_3, were lower than those used by used Moffatt et al. (1987), which were 0.4 and 0.64 ppm. Harkema and co-workers also studied reactions after only 6 days of exposure to 0.15 ppm. There were no major differences among the three exposed groups

(i.e., 6 days to 0.15 ppm, 90 days to 0.15 ppm, and 90 days to 0.3 ppm O_3). All exposed monkeys had thicker RB epithelium and thicker RB interstitium. There were more nonciliated cuboidal epithelial cells per millimeter of basement membrane in all exposed groups and increased squamous cells only in the 6-day group. The thickened total interstitium was due to increases in both acellular (matrix) and cellular components, but both compartments were increased individually only in the 90-day O_3 group. There were no differences in RB smooth muscle. Transmission electron microscopy and SEM observations, which were not studied quantitatively, include increased AMs in alveoli opening into RBs and increased "dome"-shaped nonciliated bronchiolar cells, which had more apical cytoplasm, more agranular and granular endoplasmic reticulum, more mitochondria, and more Golgi with secretory granules. With the exception of increased AMs, there was no evidence of necrosis nor of inflammatory cells. No differences due to age or gender were detected.

Harkema et al. (1993) speculate that their finding of a larger percent increase in RB cuboidal cells in monkeys exposed to a lower concentration of O_3 for the same time than that reported by Moffatt et al. (1987) might be due to the difference in sampling methods. Harkema et al. studied only the first generation RBs, whereas Moffatt et al. (1987) studied a random sample of all generations of RBs. The first generation tends to be more damaged than succeeding generations (Mellick et al., 1977; Eustis et al., 1981). The Harkema et al. (1993) sampling procedure also prevented examination for the increased volume density of RBs and decreased RB diameter reported by Fujinaka et al. (1985) and Moffatt et al. (1987).

Remodeling of Centriacinar Region Airways. This is a less well-known sequela of long-term O_3 exposure. Using SEM, Boorman et al. (1980) and, later, Moore and Schwartz (1981) reported the development in rats of an airway with the appearance of RBs between the TB and ADs. This new segment was longer than those occasionally seen in control rats. Respiratory bronchioles in rats either are absent or developed to only a single, very short segment (Tyler, 1983; Tyler and Julian, 1991).

Barr et al. (1988) examined the development of this new segment using LM and TEM morphometry on lungs from rats exposed to 0.95 ppm O_3, 8 h/day for 90 days. They reported a significant increase in the total volume of RB and of RB lumen and wall. The new RBs reached a maximum length of four alveolar opening rings. They also noted that, in some of these RB segments, the capillary and epithelial basal laminae were fused as they are in TBs, rather than separate as in alveoli. Most Type 1 cell necrosis was found at the tips of alveolar septa immediately adjacent to the RB/AD junction. Thus, the most severe epithelial damage did not occur at the most proximal alveolus in the CAR, but rather in the alveolus immediately distal to the newly formed RB.

Recently, Pinkerton et al. (1993) developed a new LM morphometric method to evaluate remodeling of CAR ADs. In rats exposed to 1.0 ppm O_3 intermittently for 20 mo, the investigators reported well-differentiated ciliated and nonciliated bronchiolar epithelium lining CAR airways that would otherwise be ADs. Some of this epithelium extended five alveoli from the TB. Thus, the Type 1 and 2 cells characteristic of ADs were replaced by both types of bronchiolar cells characteristic of RBs when RBs are present in control rats. Pinkerton et al. (1995) used their new morphometric method to study rats exposed to 0.12, 0.5, or 1.0 ppm O_3 for 6 h/day, 5 days/week for 20 mo. They reported significant thickening of alveolar septal tips 200 mm from the TB in rats exposed to 0.12 ppm, which increased with O_3 concentration to 600 □m in rats exposed to 1.0 ppm, but they did not describe the type of

epithelium covering these thickened tips. Several studies that did not find CAR remodeling also used a slightly different procedure (Barry et al., 1985; Chang et al., 1992).

Plopper et al. (1994a) examined CARs from rats exposed to the same regimen, but studied CARs from one cranial short pathway and a caudal long pathway. They found nonciliated bronchiolar epithelial cells in remodeled former ADs in short- and long-pathway CARs from rats exposed to 1.0 ppm O_3, but only in short-pathway CARs from rats exposed to 0.5 ppm. Central, short-pathway CARs were not examined. Using the airway dissection method of selecting CARs to be studied, nonciliated bronchiolar cells were not found in ADs from rats exposed to 0.12 ppm O_3.

The same phenomena apparently occurs in animals with several generations of RBs as increases in V_v and volume (V) of RBs have been reported in all O_3-exposed monkeys examined using morphometric methods to estimate V_v or V of RBs (Fujinaka et al., 1985; Moffatt et al., 1987; Tyler et al., 1988, 1991b). Inflammatory changes and CAR remodeling occur concomitantly, and inflammatory changes in an airway may indicate future remodeling. Mellick et al. (1977) noted that, in monkeys exposed to 0.8 ppm, 8 h/day for 7 days, the inflammatory process extended throughout the RBs and into ADs. Eustis et al. (1981) reported that, in monkeys exposed to 0.8 ppm, 8 h/day for 90 days, all generations of RBs contained aggregates of inflammatory cells. Monkeys exposed to lower concentrations for the same or longer time have increased V_v and V of RBs (Moffatt et al., 1987).

6.2.4.3 Considerations of Exposure Regimens and Methods

Recovery During Postexposure Periods

Evidence of healing occurs soon after short-term O_3 exposures cease. In the studies of Pino et al. (1992c), evidence of healing is provided by the increased V_s of viable undifferentiated cells in TBs detected 16 h after the end of an 8-h exposure to 1.0 ppm O_3.

Chang et al. (1992) reported an increased V_s of Type 2 cells 17 weeks after a 78-week exposure to a simulated urban exposure regimen with a peak O_3 concentration of 0.25 ppm; there were no changes detected 6 weeks after a 13-week exposure. Gross and White (1987) examined rats 3 and 6 mo after a 52-week exposure (20 h/day, 7 days/week) to 0.5 ppm O_3. Using LM pathology, the only changes visible 6 mo after a 12-mo exposure were a few areas of bronchiolization, slight dilation of ADs, and slight thickening of AD walls and adjacent alveolar septa. In an earlier study, less complete healing was reported by Gross and White (1986), who used LM pathology to study rats 4 and 9 weeks after a 4-week exposure (20 h/day, 7 days/week) to 0.7 ppm O_3. Four weeks postexposure, Gross and White reported a slight, unevenly distributed inflammatory reaction with condensed eosinophilic material, presumed to be collagen, in the interstitium. Nine weeks postexposure, some AD walls and TBs were thickened. Rats exposed to 0.96 ppm, 8 h/night for 42 nights and examined 42 days later using LM morphometry had increased V_v and V of the RB wall and SEM evidence of CAR remodeling (Tyler et al., 1987). Collagen content of these lungs increased during the postexposure period (Last et al., 1984b).

Centriacinar region remodeling was more persistent in monkeys exposed to 0.64 ppm O_3, 8 h/day for 12 mo followed by 6 mo postexposure (Tyler et al., 1991b). By LM morphometry, the V_v and V of total RB, RB lumen, and RB walls were increased both at exposure end and at 6 mo postexposure. At exposure end, but not at 6 mo postexposure, RB internal diameters were smaller, and AMs in the CAR increased.

One study concerned postexposure recovery of the trachea (Nikula et al., 1988a). Complete recovery of the trachea (as evaluated by LM morphometry, SEM, and TEM) of rats exposed to 60 nights (8 h/night, 7 days/week) to 0.96 ppm O_3 occurred following a 42-day postexposure period.

Effects of Episodic and Seasonal Exposure Regimens

Many investigators have noted that lesions due to O_3 reach a maximum intensity in a very few days and that, with continued exposure, the intensity of the lesion decreases. Eustis et al. (1981) reported half the number of inflammatory cells in the CAR of monkeys exposed to 0.8 ppm for 90 days as found in monkeys exposed to the same concentration for 7 days. Chang et al. (1992) noted that the acute reactions to the 0.06-ppm background O_3 (7 days/week), with a 9-h peak (5 days/week) slowly rising to 0.25 ppm, that they reported at 1 week of exposure had subsided at 3 weeks of exposure. Harkema et al. (1993) reported no difference in first generation RB epithelial thickness or cell numbers among monkeys exposed to 0.15 ppm O_3 for 6 days or to 0.15 or 0.3 ppm for 90 days.

These and other similar observations prompted Chang et al. (1991) to compare effects of two exposure regimens, which were evaluated using the same TEM morphometric approach. The first regimen was a "square wave", 12-h/day, 7-day/week exposure to 0.12 or 0.25 ppm O_3. The second regimen simulated urban O_3 exposures by exposing rats 7 days/week for 13 h to 0.06-ppm background, with a peak slowly rising to 0.25 ppm over a 9-h period (5 days/week). They calculated cumulative O_3 concentration ($C \times T$) for each exposure regimen and concluded that increases in volume of Type 1 and 2 alveolar epithelial cells were linearly related to increasing $C \times T$. The relationship for Type 1 cells was more robust.

Barr et al. (1990) used TEM and LM morphometry to compare effects of 90 days of daily exposure of rats for 8 h/day to 0.95 ppm O_3 with a regimen that modeled 5-day episodes of O_3 exposure. Each 5-day episode was followed by 9 postexposure days of filtered air. The cycle was repeated seven times so that the "episodic" group was exposed a total of 35 days over an 89-day period, and the "daily" group was exposed for 90 days to the same O_3 concentration. Both groups had CAR remodeling with the formation of RBs. The volume of RBs formed was not different when the two exposure groups were compared. The absolute volume of parenchymal lesion was the same in both groups. The RB epithelial thickness was increased in the daily group but not in the episodic group; conversely, the interstitium of both TBs and ADs was thickened in the episodic group but not in the daily group. Thus, rats exposed to the same concentration of O_3 for 35 days over an 89-day period in an episodic regimen had lesions as severe as those rats exposed daily for 90 days.

Effects of "seasonal" and "daily" exposure of young monkeys to 0.25 ppm O_3 were reported by Tyler et al. (1988). The daily group was exposed every day (8 h/day) for 18 mo, whereas the seasonal group was exposed only during odd months for the 18 mo. Thus, the daily group was exposed twice as many days to the same concentration as the seasonal group. By LM morphometry, both groups had increased V_v of total RB and RB lumen, but RB wall thickness was increased only in the daily group. The only significant morphometric difference between the two groups was an increase in CAR AMs in the daily group. This difference, and the difference in significance of the RB wall thickness in the seasonal group, was presumed due to the daily group being exposed to O_3 the day before necropsy, whereas the seasonal group breathed filtered air for 30 days preceding necropsy. This final 30 days of filtered air

apparently allowed the more acute inflammatory changes in the seasonal group to regress. The seasonal group, but not the daily group, had increased lung collagen (Section 6.2.1) and increased chest wall compliance (Section 6.2.5). Exposure to the same concentration of O_3 for half as many days in a seasonal regimen resulted in morphometric effects similar to daily exposure and in physiological and lung collagen changes not found in the daily group.

Tyler et al. (1991a) exposed rats to seasonal and daily regimens similar to those used for the monkeys described above. The concentration used in both studies was 0.25 ppm O_3 and the total length of exposure was 18 mo. Rats were exposed nights, during their natural period of activity. Both groups of rats were studied at the end of the 18-mo exposure cycle and 30 days postexposure. The lungs were evaluated using a simplified LM morphometric method for CAR airway remodeling, estimating the number of junctions of bronchioles (TB and RB) with ADs per surface area of section. At exposure end, the number of junctions of both exposure groups was increased compared to filtered-air controls; the O_3 groups were not different from each other. Neither group was different from the controls at 30 days postexposure.

Ex Vivo and In Vitro Exposures

Results obtained from studies of isolated perfused lungs and organ culture explants were consistent with some of the findings from in vivo studies (Pino et al., 1992a; Nikula et al., 1988b; Nikula and Wilson, 1990).

6.2.4.4 Considerations of Degree of Susceptibility to Morphological Changes

Species Differences in Degree of Response

Plopper et al. (1991) reviewed data from nonhuman primates and rats that had been exposed to O_3 and evaluated using TEM morphometry. The data were generated in several laboratories and the exposure and evaluation methods were somewhat different, but the data were expressed in similar terms. In the CAR, the results were expressed as total epithelial thickness or numbers of cells per square millimeter of basal lamina. Exposure of rats to 0.25 ppm O_3, 8 h/day for 42 days, resulted in an increase of less than 100% in either parameter compared to controls (Barry et al., 1985, 1988). Exposure of monkeys to 0.15 ppm O_3, 8 h/day for 6 days, resulted in a 230% increase in thickness and a 700% increase in cell number compared to controls (Harkema et al., 1993). As noted earlier (Section 6.2.4.2), the CARs of rats and monkeys are structurally different (Tyler, 1983), and the CAR cells are also different (Plopper, 1983).

There was also a difference when Plopper et al. (1991) compared stored secretory product per square millimeter of basal lamina in the nasal septum and lateral wall of the nasal cavity of O_3-exposed rats and monkeys. Data from the exposure of rats to 0.12 ppm, 6 h/day for 7 days, resulted in a <10% increase in the nasal septum and a <100% increase in the lateral wall. Exposure of monkeys to 0.15 ppm, 8 h/day for 6 days, resulted in a 300% increase in the nasal septum and a 125% increase in lateral wall. As in the CAR, there are major morphological differences in the nasal cavities of these two species (Schreider and Raabe, 1981).

Plopper et al. (1991) also compared collagen metabolism in rats and monkeys exposed to 1.5 ppm O_3, 23 h/day for 7 days, using the uptake of tritium-labeled proline. In rats, there was an increase of 200% above controls, whereas the increase was 800% in monkeys.

From these data, it appears that the respiratory system of monkeys is much more responsive than that of rats to near-ambient concentrations of O_3. The mechanisms responsible for these species differences in response to O_3 remain to be elucidated.

Effects of Age

Several studies published since the previous criteria document (U.S. Environmental Protection Agency, 1986) have addressed the effects of age on the intensity of O_3 morphological changes. The study by Stephens et al. (1978) and the initial report by Barry et al. (1983) were cited. Briefly, Stephens et al. exposed rats ranging in age from 1 to 40 days old to 0.85 ppm O_3 for 24, 48, or 72 h and examined their lungs by LM and TEM. Stephens and co-workers reported that, prior to 20 days of age, they did not find damage to TB-ciliated cells or to CAR Type 1 cells and that the amount of injury increased from 21 to 35 days when a plateau in response was reached.

Barry et al. (1985, 1988) exposed 1-day-old and 6-week-old rats to 0.12 or 0.25 ppm O_3, 12 h/day for 6 weeks. The 1985 study emphasized TEM morphometry of CAR (proximal) alveoli. The investigators did not find differences in response due to age. In both age groups, they found Type 1 cells increased in number and thickness, but decreased in both luminal and basement membrane surface area. They found bare basement membrane where Type 1 cells had been sloughed, but the amount was not increased in exposed groups. In the 0.25-ppm groups, but not in the 0.12-ppm groups, Type 2 cells were increased in density per square millimeter basement membrane but not in volume. Alveolar interstitium was increased only in adults exposed to 0.25 ppm. Macrophages in alveoli were increased in both age groups exposed to 0.25 ppm, but not in adults exposed to 0.12 ppm. Interstitial AMs were increased only in adults exposed to 0.25 ppm. The TEM morphometry of TBs from these rats did not include adults exposed to 0.12 ppm (Barry et al., 1988). There were no differences due to age at start of exposure. In both juvenile and adult rats exposed to 0.25 ppm, Barry and co-workers found that the luminal surface covered by cilia and by nonciliated bronchiolar (Clara) cells was reduced. The number of brush cells was also decreased.

Stiles and Tyler (1988) studied effects in a wider range of ages using LM morphometry and SEM. They exposed 60- and 444-day-old female rats to 0.35 or 0.8 ppm O_3 continuously for 72 h. Body weights of the 444-day-old rats, but not of those 60 days old, decreased during exposure. Fixed lung volumes of 444-day-old rats exposed to 0.8 but not 0.35 ppm were smaller than same-age controls. The V_v of CAR lesions was larger in 60-day-old rats than in the 444-day-old rats exposed to either concentration. The V_v of cells free in lumens (AMs) was increased in young rats exposed to 0.35 ppm compared to the older rats, but was not different for rats exposed to 0.8 ppm. Young rats exposed to either concentration had larger CAR lesions than the older rats, and young rats exposed to the lower concentration had more AMs. Older rats had greater changes in body weight and, in those exposed to the higher concentration, in fixed lung volume.

Effects of Exercise

Exercise increases the dose of inhaled toxicants delivered to sensitive cells (see Chapter 8). Mautz et al. (1985b) studied the effects of 0.2 and 0.38 ppm O_3 on rats at rest and during several treadmill exercise protocols. They found increased percent of lung parenchymal area containing free cells (AMs) in exercised rats exposed to both concentrations compared to

rats exposed at rest. At the higher concentration, there was also an increase in the percent of parenchymal area with thickened ADs and alveolar septa.

Tyler et al. (1991c) exposed thoroughbred horses (trained to a treadmill) to 0.25 or 0.8 ppm O_3 for 29 min on 2 consecutive days using a protocol that included 9 min of graded exercise (3 min at maximum speed) and 20 min of "cool out". During maximal exercise, horses increase their rate of oxygen consumption more than other species. Two of three horses exposed to 0.8 ppm O_3 had significant areas of hemorrhage and edema, and one of them refused the second day's exercise and exposure. By TEM, all horses exposed to 0.8 ppm had CAR lesions including necrosis of Type 1 cells. Lesions in those exposed to 0.25 were limited to CAR ciliated cells. No horses were exposed at rest for comparison.

Elastase-Induced Emphysema

Rats with elastase-induced emphysema and saline-instilled controls were exposed to 0.15 or 0.5 ppm for 3 or 7 days (Dormans et al., 1989). Mean linear intercepts, a measure of alveolar size, were determined using LM. The incidence and severity of CAR inflammatory changes were the same in O_3-exposed elastase-treated and saline-control rats. There were no changes in mean linear intercepts due to the O_3 exposure.

6.2.4.5 Summary

Research since the previous O_3 criteria document (U.S. Environmental Protection Agency, 1986) continues to support the concept that all mammalian species respond to O_3 concentrations < 1.0 ppm in a similar manner, but with significant differences in intensity of reactions among the species studied (Plopper et al., 1991). Dungworth (1989) provided a schematic overview of morphological reactions of the CAR from mammalian lungs to continuous exposure to low concentrations of O_3 as a series of time-response profiles (Figure 6-4). Bronchoalveolar exudative processes are the predominate early response, but the magnitude decreases rapidly with increasing duration of exposure and continues to decline during postexposure periods. Epithelial hyperplasia also starts early and increases in magnitude for several weeks, after which a plateau is reached until the exposure ends. Epithelial hyperplasia declines slowly during postexposure periods. Interstitial fibrosis has a later onset and may not be apparent for a month or more. The magnitude of this response, however, continues to increase throughout the exposure and, at least in some cases (Last et al., 1984b), continues to increase after exposure ends.

Nonhuman primates appear to respond more than rats to O_3 at concentrations < 1.0 ppm. However, the mechanisms responsible for these differences in response have not been elucidated. Differences in cell, tissue, and circulating levels of several antioxidants are being studied, as are differences in in vitro responses to O_3 by cultures of cells from the various species. Basic morphological differences in the structure of the most injured portion of the lung, the CAR, and the size (volume) of the basic structural unit, the acinus, may also be factors in the greater response of monkeys to O_3. Both human lungs and lungs from nonhuman primates have CARs characterized by several generations of RBs, whereas rats have no RBs, or only a single poorly developed generation (Tyler and Julian, 1991). Within an individual lung, acinar volume is directly related to the intensity of CAR lesions (Mercer and Crapo, 1989; Mercer et al., 1991). The volume of individual acini in human lungs is 100 times larger than individual acini in rat lungs (Rodriguez et al., 1987; Haefeli-Bleuer and Weibel, 1988).

Acinar volume of the monkeys used in O_3 studies is not known but, on the basis of the CAR structure, is assumed to be more like that of human lungs than rat lungs.

Another morphological factor that may be responsible in part for the greater response to O_3 of nonhuman primates than of rats may be differences in the complexity of the nasal cavity. Schreider and Raabe (1981) studied the cross-sectional morphology of the nasal-pharynx in rats, beagle dogs, and a rhesus monkey. They concluded that the

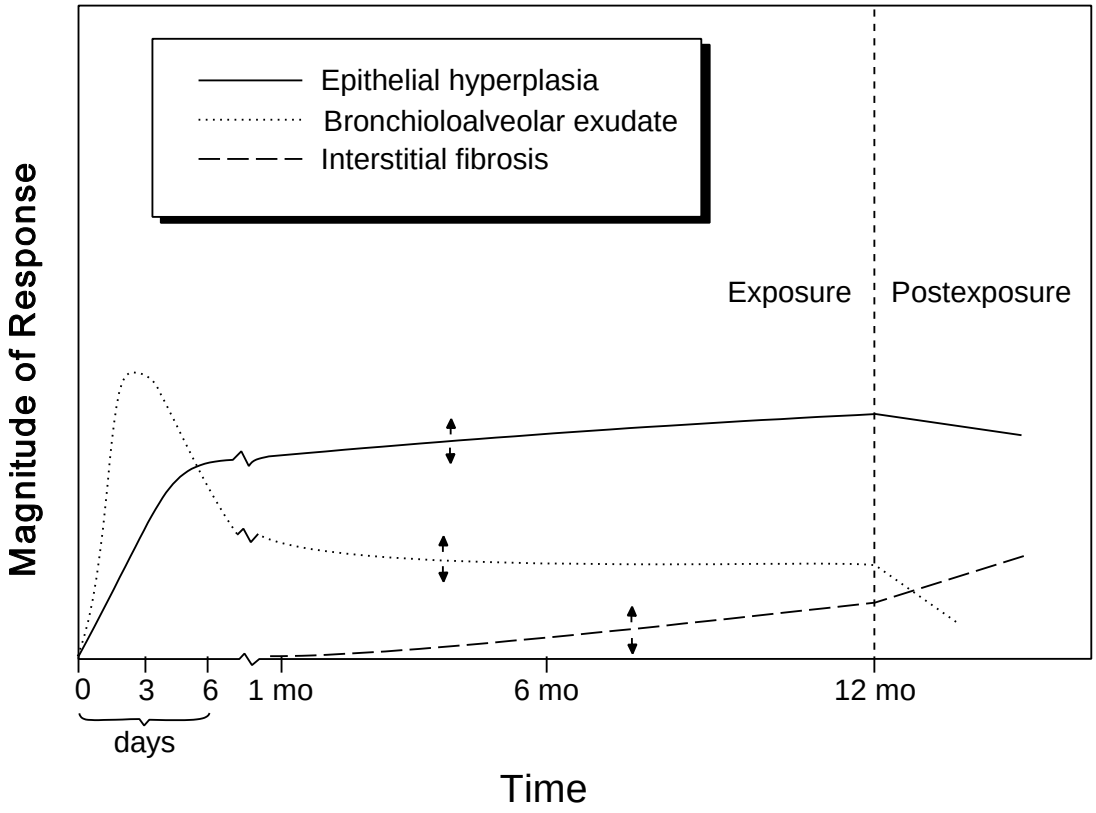

Figure 6-4. Schematic comparison of the duration-response profiles for epithelial hyperplasia, bronchoalveolar exudation, and interstitial fibrosis in the centriacinar region of lung exposed to a constant low concentration of ozone.

Source: Dungworth (1989).

complexity of the nasal cavity, and therefore the "scrubbing" effect (Yokoyama and Frank, 1972; Miller et al., 1979), which reduces the concentration of inhaled O_3 delivered to the lower respiratory tract, would be greater in rats than in monkeys. Schreider and Raabe (1981) proposed that, with appropriate scaling, the monkey could serve as a model for aerosol and gas deposition in the nasopharyngeal region of humans. However, the sensitivity of the nasopharyngeal epithelium may be different because changes in the nasal epithelium that follow O_3 inhalation, like those in the CAR, are more severe in monkeys than in rats (Plopper et al., 1991).

The effect of age at start of exposure on O_3-induced lung injury has not been resolved. Barry et al. (1985, 1988) reported no differences in TEM morphometry of CAR and TB lesions due to age at start of exposure. However, they studied a narrow range of ages, l and 42 days old at the start of a 42-day exposure. Barry et al. (l985, 1988) speculate that much of the CAR response in the rats that began exposure at 1 day of age might have occurred during exposure Days 21 to 42 because, as the earlier studies of Stephens et al. (1978) found,

rats are not sensitive to morphological effects of O_3 until weaning at 21 days of age. Thus, the rats that were 1 day old at the beginning of exposure may have developed the same intensity of lesions during the last 21 days of exposure as the older rats did in 42 days of exposure. In studies using a wider range of ages (60- and 444-day-old rats), Stiles and Tyler (1988) reported larger CAR lesions in the younger rats but greater changes in body weight and fixed lung volume in the older rats following a 3-day exposure to O_3.

The effects of exposure regimen and duration were evaluated in more recently published studies. Exposure of young monkeys to 0.25 ppm O_3 in a "seasonal" regimen (i.e., exposure in odd months and postexposure in even months) for 18 mo resulted in the same quantity of CAR lesions as daily exposure to the same concentration for 18 mo (Tyler et al., 1988). A similar quantity of CAR lesions was reported in rats exposed to 0.95 ppm O_3 in a 35 day, "episodic" regimen (units of 5-day exposures and 9 days without exposure) for a total of 89 days, as those exposed to the same concentration each day for 90 days (Barr et al., 1990). Chang et al. (1991) calculated the cumulative O_3 concentration for a "square wave" exposure to 0.12 or 0.25 ppm for 12 h/day, 7 days/week, with a simulated "urban" exposure regimen of 0.06 ppm for 13 h/day, 7 days/week and then raising that background 5 days/week to a peak of 0.25 ppm over a 9-h period. Using TEM morphometry of the CAR, they found no difference due to the pattern of exposure. Thus, it appears that the pattern of daily exposure does not influence the intensity of CAR lesions, but that episodic and seasonal patterns of exposure, with multiple days of clean air between days of exposure, are equivalent to daily exposure.

It has become clear that remodeling of centriacinar airways is cumulative. Using a stereological approach, Barr et al. (1988) reported an increase in the total volume of RB wall and lumen in rats exposed to 0.95 ppm O_3, 8 h/day for 90 days. Barr and co-workers also reported continuing Type 1 cell necrosis at the tips of alveolar septa (alveolar opening rings) immediately distal to the newly formed RB/AD junction (rather than in the TB/AD junction). It appears that some of the necrotic Type 1 cells were replaced by bronchiolar epithelium, rather than by Type 2 cells as previous studies indicated. This was confirmed by Pinkerton et al. (1993), who reported fully differentiated ciliated and nonciliated bronchiolar epithelium lining alveolar tips along a former AD up to 1 mm from the TB in lungs from rats exposed to 1.0 ppm O_3 for 6 h/day, 5 days/week for 20 mo. Remodeling of centriacinar airways appears to be a general phenomena, as increases in the V_v and V have been reported in lungs from all exposed rats and monkeys examined using stereological or morphometric methods that could detect this change (Fujinaka et al., 1985; Moffatt et al., 1987; Tyler et al., 1987; Tyler et al., 1988; Barr et al., 1990; Pinkerton et al., 1992, 1993). Centriacinar region remodeling has been demonstrated to persist in monkeys 6 mo after a 12-mo exposure to 0.64 ppm O_3 (Tyler et al., 1991b) and in rats 42 days after a 42-night exposure to 0.96 ppm O_3 (Tyler et al., 1987).

Several studies have confirmed and extended the earlier reports of epithelial degenerative changes followed by sloughing (i.e., leaving bare basement membrane that is recovered by other cell types), thus altering epithelial cell populations and increasing cell density (hyperplasia) in TB and centriacinar alveoli (Barry et al., 1985, 1988; Moffatt et al., 1987; Chang et al., 1988, 1992; Harkema et al., 1993). Epithelial replacement, a reparative process, occurs very early (Pino et al., 1992c), even though degeneration and necrosis continues (Barr et al., 1988). In specific airways, these processes appear to reach a maximum early in the exposure, as reported by Harkema et al. (1993), who found no difference in the intensity of lesions in first generation RBs, as measured by RB epithelial cell thickness and

numbers, among monkeys exposed to 0.15 ppm O_3 for 6 days or to 0.15 or 0.3 ppm for 90 days. However, it is important to note that there may have been differences in response if more distal generations of RBs or random generation RBs had been selected for study. The interstitium in the CAR also thickens by the addition of cells and matrix. Thickening of the basement membrane and the presence of granular material in it were reported by Barr et al. (1988) and Chang et al. (1992). Chang et al. (1992), using TEM morphometry, reported that some changes in epithelial cell populations persist in rats for 17 weeks after a 78-week exposure to a model urban profile with a peak of 0.25 ppm O_3. At the LM level, Gross and White (1987) reported that 6 mo following a 12-mo exposure to 0.5 ppm O_3, 20 h/day, CAR inflammation had all but disappeared, and only a slight dilation and thickening of some ADs and adjacent alveoli remained.

The epithelia of the nasal cavity respond rapidly to O_3. In ciliated regions, cilia are attenuated, and intraepithelial mucosubstances increase. Hyperplasia and increased intraepithelial mucosubstances are reported in areas of nonciliated transitional epithelium (Harkema et al., 1989). These effects persisted throughout a 20-mo intermittent exposure of rats to 0.5 or 1.0 ppm O_3, but were not seen in rats exposed to 0.12 ppm for that period (Harkema et al., 1994). After acute exposure, DNA synthesis of the epithelium of the anterior maxilloturbinates of rats increases according to a given C × T product at □2.88 ppm·h, but the increase is not linear with increasing C × T (Henderson et al., 1993). Changes have not been reported in the olfactory epithelium or in the squamous epithelium of the nasal cavity.

Respiratory epithelia in other conducting airways, especially the trachea, appear to react in a manner similar to early necrosis of ciliated cells (Hyde et al., 1992). Cell replacement starts early (Hyde et al., 1992), and, after 60 nights of exposure of rats to 0.96 ppm O_3, numeric density of specific cell types was not different from controls (Nikula et al., 1988a). In newborn lambs exposed to 1.0 ppm O_3, 4 h/day for 5 days and examined 9 days later, the normal change in epithelial cell population that occurs by 2 weeks of age did not occur (Mariassy et al., 1990).

6.2.5 Effects on Pulmonary Function

6.2.5.1 Introduction

Numerous studies have been published on the effects of O_3 exposure on pulmonary function in animal models. This work has been reviewed by the U.S. Environmental Protection Agency (1986) and Tepper et al. (1995). The evaluation of pulmonary function after exposure may help provide a more integrated assessment of the severity of health effects by indicating the magnitude, location, and duration of functional disability. In an attempt to summarize the literature here, only key studies employing multiple concentrations or studies demonstrating a particular functional effect, testing a different species or strain, or showing the relationship with a unique variable such as age or sex will be discussed. Because purely descriptive pulmonary function studies now are rarely reported, newer studies will be discussed within the context of the study hypothesis. To enable discussion of the full range of studies, some exposures greater than 1 ppm O_3 will be discussed. For example, many of the airway reactivity mechanism studies were conducted at higher concentrations.

This section is organized by duration of exposure (brief, acute, repeated, and long-term). Within each of these sections, there are subsections on different types of pulmonary function measures. These subsections include a discussion of ventilatory patterns, breathing

mechanics, airway reactivity, and more extended characterizations of lung function, whenever such data are available.

Ventilation

Evaluation of the sinusoidal breathing pattern includes the measurement of tidal volume (V_T) and frequency of breathing (f) and their product, $\dot{V}_E$. Such measurements have proven to be sensitive indicators of O_3 effects. Numerous animal and human studies have shown that O_3 exposure increases f and decreases V_T (tachypnea) (U.S. Environmental Protection Agency, 1986). Although there is evidence indicating that tachypnea may serve to protect the deep lung from exposure, other evidence indicates that this sign of pulmonary irritation represents deep lung toxicity and is of greater concern than breathing pattern changes indicative of upper airway irritation.

Breathing Mechanics

Measurement of breathing mechanics (dynamic compliance [Cdyn] and total pulmonary resistance [R_L]) in animals has an advantage over simple measures of ventilation in that these parameters can assess the mechanical effort required to breathe and can help localize the site of dysfunction to the airways (resistance) or the parenchyma (compliance). With sufficient O_3 exposure, increases in R_L and decreases in Cdyn have been observed (U.S. Environmental Protection Agency, 1986). Changes in R_L and Cdyn typically reverse rapidly after high ambient O_3 exposures; however, these alterations can signal underlying inflammatory or lung permeability changes.

Airway Reactivity

Increased airway reactivity, an exaggerated response of the lung to an exogenously administered bronchoconstrictor, has been observed with O_3 (U.S. Environmental Protection Agency, 1986). Typically in humans, heightened airway responsiveness is determined using progressively increasing concentrations of aerosolized bronchoconstrictors, such as methacholine or histamine (see Chapter 7). Although bronchoprovocation protocols employing doubling doses of inhaled bronchoconstrictors have been relatively standardized for human experiments, no such standardization exists for animal studies, making comparisons between animal and human studies difficult. Increased airway reactivity is a hallmark of asthma and occurs in many other lung diseases, yet the long-term pathological consequences of hyperreactive airways are unknown.

Extended Functional Characterizations

A more complete assessment of the nature and magnitude of functional changes related to O_3 exposure includes an extended characterization of the lung using a battery of human clinical pulmonary function test analogs. Such tests include measurement of static lung volumes, volume-pressure and flow-volume relationships, as well as evaluation of inhomogeneity of ventilation and problems associated with oxygen diffusion across the epithelial barrier. Although these latter measurements are technically complex, they may contribute to a more in-depth understanding of the nature and severity of the physiological impairment and may provide in vivo evidence to suggest the anatomical localization of the functional abnormality.

6.2.5.2 Brief Ozone Exposures (Less Than 30 Minutes)

Few experiments have evaluated the effects of brief exposures (< 30 min) to O_3. Most of these brief exposure studies have examined changes in regional breathing mechanics through exposures to the lower respiratory tract via a tracheal tube, thus eliminating any scrubbing by the nasal or oropharynx and thereby increasing the effective dose of O_3 delivered to that region of the lung. The relevance of this method of delivering the exposure, as compared to the typical inhalation route, is uncertain. However, positive effects have been observed, indicating that very rapid reflex responses occur with brief, direct O_3 exposure.

Ventilation and Breathing Mechanics

No studies have evaluated the effects of brief O_3 exposure on ventilatory pattern; however, breathing mechanics (Cdyn and R_L) have been evaluated. The previous criteria document (U.S. Environmental Protection Agency, 1986) described two experiments by Gertner et al. (1983a,b,c), which demonstrated increased collateral resistance within 2 min of exposure to 0.1 ppm O_3 in anesthetized dogs exposed via a fiber-optic bronchoscope wedged into a segmental airway. The response rapidly attenuated with exposure to 0.1 but not 1.0 ppm. Atropine or vagotomy blocked the increase in collateral flow resistance to 0.1 ppm, indicating that vagal postganglionic stimulation was involved, but the response to the 1.0-ppm O_3 exposure was blocked only partially.

More recently, Kleeberger et al. (1988), using a technique similar to Gertner et al. (1983a), exposed the segmental airways of mongrel dogs to 1.0 ppm O_3 for 5 min through a wedged bronchoscope (Table 6-13). As previously described, collateral resistance increased, and this increase was reproducible even when four 5-min exposures over a 3-h period were performed. Thus, no immediate tolerance was observed. Furthermore, this response could be blocked partially by administration of a cyclooxygenase inhibitor (indomethacin) and a H_1-receptor blocker (chlorpheniramine), whereas a thromboxane synthetase inhibitor was ineffective. This study suggests that histamine or cyclooxygenase products released from resident cells directly or via the parasympathetic nervous system may mediate the increase in collateral resistance. However, because collateral resistance probably makes up only a small proportion of pulmonary resistance, these results may not be generalizable to more prolonged exposures and to larger airway responses.

Airway Reactivity

Baboons were exposed via an endotracheal tube to 0.5 ppm O_3 for 5 min after a baseline methacholine inhalation challenge test (Fouke et al., 1988) (Table 6-14). Lung resistance increased with O_3 exposure, and the baboons showed an enhanced response to methacholine. This enhanced methacholine response was due almost exclusively to the post-O_3 increase in R_L and, thus, resulted in no change in the provocative dose that increased R_L by 50%. The experiment was repeated 5 to 14 days later, except that before O_3 exposure, cromolyn sodium was administered. In the presence of cromolyn, baseline R_L after O_3 exposure was less (not significant), but the response to methacholine challenge was significantly lower. In a follow-up study (Fouke et al., 1990) using a similar O_3 exposure protocol (no methacholine challenge), cromolyn partially blocked the O_3-induced increase in R_L; however, post-O_3 exposure analysis of BAL indicated that cromolyn did not affect the level of several measured prostanoids (6-keto $PGF_{1\alpha}$, PGE_2, TXB_2, or $PGF_{2\alpha}$), suggesting that these mediators were not related to the change in R_L.

Table 6-13. Effects of Ozone on Pulmonary Function[a]

Ozone Concentration						
ppm	μg/m³	Exposure Duration	Drugs	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
Base 0.06 spike 0.25	Base 118 spike 490	Base 13 h/day, 7 days/week; ramped spike 9-h/day, 5 days/week, for 1 week, 1, 3, 12, and 18 mo		Rat, M (F344) 60 days old	Increased expiratory resistance observed at all time points, but mostly at 78 weeks.	Tepper et al. (1991)
0.13 0.22 0.45	255 431 882	3 h Through tracheal tube	Pentobarbital Gallamine	Dog (Foxhounds) 16-20 kg	Positron camera indicated nonuniform distribution of ventilation in small airways; no change in R_L, Cdyn, or forced expiratory flow.	Morgan et al. (1986)
0.2- 0.8	392- 1,568	3 h		Rat, M (S-D) 7 weeks old	Maximum O_2 consumption decreased at 0.2 ppm, tachypnea observed at 0.4 ppm, and ventilation and core temperature decreased at 0.6 ppm.	Mautz and Bufalino (1989)
0.25 0.5 1.0	490 980 1,960	2 h; 2, 4, 6, and 8% CO_2 alternating 15 min		Rat, M (F344) 90 days old	Concentration response-related increase in f and flow at zero pleural pressure, decrease in V_T, no change in $\dot{V}_E$, R_L, or Cdyn.	Tepper et al. (1990)
0.25 0.5 1.0	490 980 1,960	3 h for 5 days	Pentobarbital	Mouse, F (CD-1) 3-4 weeks old	O_3 alone had no effect, but, in combination with virus, a decreased DL_{CO}, N_2, and lung volumes were observed more often than in virus alone at 6, 9, and 14 days PE.	Selgrade et al. (1988)
0.5	980	2 h	Chloralose	Dog (Mongrel) 15 ± 0.9 kg	Increased R_L, decreased Cdyn. No change in BAL prostanoids.	Fouke et al. (1991)
0.35 0.5 1.0	686 980 1,960	2 h/day for 5 days, 8% CO_2 alternating 15 min		Rat, M (F344) 110 days old	Attenuation of tachypnea with consecutive exposures; BAL antioxidants and protein did not adapt with exposure, histopathology increased in severity.	Tepper et al. (1989)
0.5 0.8	980 1,568	2 or 7 h, 8% CO_2 alternating 15 or 45 min/h	Halothane	Rat, M (F344) 90 days old	FVC, DL_{CO}, and N_2 slope all decreased with increasing C × T products. The magnitude of the decrement depended on the both the duration and concentration of O_3 exposure and the measured parameter.	Costa et al. (1989)
0.6	1,176	2 h exercise, muzzle		Dog, F (Beagle) 2-7 years old	Tachypnea, $\dot{V}_E$, O_2 consumption, CO_2 output, and R_L increased; Cdyn decreased.	Mautz et al. (1985b)
0.7	1,372	20 h/day for 28 days	Halothane	Rat, M (F344) 14 weeks old	Decreased forced expiratory flow and DL_{CO} and increased FRC immediately PE, no effect at 4 weeks PE, decrease in forced expiratory flow at 9 weeks PE.	Gross and White (1986)

Table 6-13 (cont'd). Effects of Ozone on Pulmonary Function[a]

Ozone Concentration		Exposure Duration	Drugs	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m					
1.0	1,960	5 min Through tracheal tube	Pentobarbital	Dog, M (Mongrel) 20.2 ± 0.8 kg	Ozone-induced increase in collateral resistance blocked by indomethacin and histamine antagonist, not by thromboxane synthetase inhibitor.	Kleeberger et al. (1988)
1.0	1,960	1 h	Ketamine Xylazine	Guinea pig, M (Hartley) 250-300 g	Decreased TLC, VC, FRC, RV, and R_L. Indomethacin and cromolyn blocked change in FRC and RV at 2 and 24 h PE. DL_{CO} increased, blocked by cromolyn.	Miller et al. (1988)
1.0	1,960	1 h	Ketamine Xylazine	Guinea pig, M (Hartley) 250-300 g	R_L increased at 2 but not 8 h. Lung volumes, DL_{CO}, and alveolar ventilation increased at 8 and 24 h PE.	Miller et al. (1987)
1.0	1,960	2 h Through tracheal tube		Dog (Mongrel) 22-25 kg	Tachypnea with inspiratory time and expiratory time equally shortened. No increase in ventilatory drive 1 and 24 h PE.	Sasaki et al. (1987)
1.0	1,960	6 h/day, 5 days/week for 12 weeks	Ketamine Xylazine	Monkey, M (Cynomolgus) 4.5 ± 0.1 kg	No R_L, Cdyn, or forced expiratory flow changes associated with exposure.	Biagini et al. (1986)
1.0 2.0	1,960 3,920	3 h	In vitro	Rat, M (S-D) 300-380 g	R_L increased and Cdyn decreased in rat isolated perfused lung preparation.	Pino et al. (1992a)
1.0 2.0	1,960 3,920	6 h/day for 7 days (1 ppm) or 3 days (2 ppm)	Pentobarbital	Rabbit, M (Albino) 2.5 kg	At 17 h PE, 1 ppm increased R_L; 2 ppm trapped air, decreased Cdyn and forced expiratory flows, and increased R_L.	Yokoyama et al. (1989a)
1.0	1,960	24 h	Pentobarbital	Rat, M (Wistar) 6 weeks old	Decreased Cdyn, Cst, $\dot{V}_{max50\%TLC}$; increased FRC, RV; no additional effect of elastase pretreatment in O_3 exposed rats.	Yokoyama et al. (1987)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

Table 6-14. Effects of Ozone on Airway Reactivity[a]

Ozone Concentration[b]		Exposure Duration	Challenge[c]		Drugs	Species, Sex (Strain) Age[d]	Observed Effect(s)	Reference
ppm	g/m³		Agent	Route				
0.5	980	2-7 h Intermittent 8% CO_2	Ach	IV	Urethane	Rat, M (F344) 70 days old	No increase in airway reactivity despite use of CO_2 during O_3 exposure.	Tepper et al. (1995)
1.0	1,960	4 h	Mch	INH IV		Rat (S-D)	Reactivity in INH- but not IV-challenged rats.	Uchida et al. (1992)
1.0	1,960	2 h	Mch Ach	INH IV	Urethane	Rat, M (F344) 70 days old	Reactivity in INH- but not IV-challenged rats.	Tepper et al. (1995)
1.0	1,960	8 h	Mch	IV	Urethane	Rat, M (F344, S-D, Wistar)	No reactivity in any of three strains.	Tepper et al. (1995)
1.0	1,960	31 h	Ach	IV	Urethane	Rat, M	Increased reactivity.	Tepper et al. (1995)
4.0	7,840	2 h	Ach	INH	Methohexital Pancuronium	Rat, F (Long-Evans) 190-210 g	Reactivity increased immediately, but not 24 h PE, but not accompanied by PMN influx or vascular protein leakage.	Evans et al. (1988)
0.15	294	4 h/day, 5 days/week for 18 weeks	Hist	INH		Guinea pig, M (Hartley) 4-5 weeks old	Increase in sensitivity, but no change in reactivity.	Kagawa et al. (1989)
0.5	980	2 h	Ach	IV	Urethane	Guinea pig, M (Hartley)	Increased reactivity before PMN influx.	Tepper et al. (1990)
0.8	1,568	2 h	Sulfuric acid	INH		Guinea pig, F, M (Hartley) 2 mo old	No increased reactivity to acid; O_3 alone increased gas trapping.	Silbaugh and Mauderly (1986)
1.0 3.0	1,960 5,880	0.5-2 h	Mch	INH	Propranolol	Guinea pig, F (Hartley) 300-400 g	Increased reactivity with 90 min at 1 ppm and with 30 min at 3 ppm. At 2 h, 3 ppm reactivity occurred at 0 and 5 but not 24 h PE.	Nishikawa et al. (1990)
1.0	1,960	2 h	Hist Ach	INH IV	Urethane	Guinea pig, M (Hartley)	Reactivity in INH Hist, but not IV Ach.	Tepper et al. (1990)
3.0	5,880	0.25 h	Ach	IV	Propranolol	Guinea pig, M (English) 550-700 g	Reactivity blocked by lipoxygenase inhibitors but not by indomethacin.	Lee and Murlas (1985)

Table 6-14 (cont'd). Effects of Ozone on Airway Reactivity[a]

Ozone Concentration[b]			Challenge[c]					
ppm	µg/m	Exposure Duration	Agent	Route	Drugs	Species, Sex (Strain) Age[d]	Observed Effect(s)	Reference
3.0	5,880	0.25-2 h	Mch Hist 5-HT SP	IV INH	Pentobarbitone	Guinea pig, M (Dunkin Hartley) 450-550 g	Mch, Hist, 5-HT, and SP caused increase in reactivity INH, but not IV. Ascorbic acid blocked reactivity to Hist and SP. NEP inhibitors increased control response without changing O_3 response (i.e., O_3 inhibits NEP). Atropine and vagotomy did not or only partially reduced reactivity. Neither cyclooxygenase or 5-lipoxygenase inhibitors affected reactivity.	Yeadon et al. (1992)
3.0	5,880	2 h	Mch	INH	Ketamine Xylazine	Guinea pig, M (Dunkin Hartley) 494 ± 39 g	Platelet activating factor antagonist did not inhibit reactivity or eosinophils.	Tan and Bethel (1992)
3.0	5,880	2 h	Ach	IV		Guinea pig, M (Hartley) 550-750 g	Reactivity occurs with leukocyte depletion using cyclophosphamide.	Murlas and Roum (1985b)
3.0	5,880	2 h	Ach	INH		Guinea pig, M (Hartley) 600-700 g	With increased reactivity, increased lysosomal hydrolase observed in BAL.	Lew et al. (1990)
3.0	5,880	2 h	Ach	INH	Propranolol	Guinea pig, M (Hartley) 600-750 g	In vitro reactivity to SP and Ach, but not KCl. Phosphoramidon blocked SP effect, but not Ach reactivity; no increased reactivity was observed when mucosa was removed.	Murlas et al. (1990)
0.5	980	2 h Trach Tube	Mch	INH	Chloralose	Dog (Mongrel) 15 ± 0.9 kg	Increased reactivity, no change in BAL prostanoids.	Fouke et al. (1991)
1.0	1,960	5 min Bronchoscope	*Ascaris suum*	INH	Pentobarbital Succinylcholine	Dog, M (Mongrel) 21.2 ± 0.5 kg	Increased resistance to flow through the collateral system after antigen challenge attenuated with O_3 exposure 1-3 h and 24 h PE; effect independent of PMNs.	Kleeberger et al. (1989)
3.0	5,880	2 h	Ach	INH	Thiopental Chloralose	Dog (Mongrel) 18-23 kg	Cyclo- and lipoxygenase inhibitor BW755C blocked reactivity.	Fabbri et al. (1985)
3.0	5,880	2 h	Ach FS	INH	Thiopental Chloralose	Dog (Mongrel) 16-25 kg	In vivo but no in vitro reactivity; in vitro, trachea showed reactivity to FS.	Walters et al. (1986)
3.0	5,880	1 h	Ach	INH	Pentothal Chloralose	Dog (Mongrel) 24 ± 9 kg	Reactivity blocked with Ambroxyl, but PMN increased; ambroxyl inhibits arachidonic acid products from PMN.	Chitano et al. (1989)
1.0	1,960	2 h Trach tube	Ach	INH	Pentobarbital	Dog (Mongrel) 15-17 kg	Collateral resistance of small airways showed persistent reactivity 15-h PE; no effect on PMN, monocyte, or mast cell numbers at PE time.	Beckett et al. (1988)

Table 6-14 (cont'd). Effects of Ozone on Airway Reactivity[a]

Ozone Concentration[b]			Challenge[c]					
ppm	μg/m	Exposure Duration	Agent	Route	Drugs	Species, Sex (Strain) Age[d]	Observed Effect(s)	Reference
3.0	5,880	0.5 h Trach tube	FS Cch		Pentobarbital	Dog (Mongrel)	In vitro increases in reactivity suggested pre- and postjunctional inhibition (PGE_2) and postjunctional excitation (TXA_2).	Janssen et al. (1991)
3.0	5,880	0.5 h Trach tube	Ach	INH	Pentobarbital	Dog (Mongrel)	Reactivity was not blocked by TX antagonists.	Jones et al. (1990)
3.0	5,880	0.5 h Trach tube	Ach Hist 5-HT	INH	Pentobarbital	Dog (Mongrel) 18-30 kg	In vitro reactivity not altered by epithelial removal, indicating O_3 did not effect epithelial-derived relaxing factor.	Jones et al. (1988b)
3.0	5,880	0.5 h Trach tube	Ach Hist	INH	Pentobarbital	Dog (Mongrel)	Ganglionic blocker hexamethonium did not alter reactivity.	Jones et al. (1987)
3.0	5,880	0.5 h Trach tube	Cch TX	INH	Pentobarbital	Dog (Mongrel)	Airways not responsive to TX mimetic (U46619) but were to carbachol.	Jones et al. (1992)
3.0	5,880	0.5 h Trach tube	Ach FS KCl	INH	Pentobarbital	Dog (Mongrel) 18-30 kg	In vitro hyperresponsiveness of airway smooth muscle was observed with FS and Ach, but not KCl. FS not associated with increased excitatory junction potentials.	Jones et al. (1988a)
3.0	5,880	0.5 h Trach tube	Ach	INH	Pentobarbital	Dog (Mongrel) 21-27 kg	CD11b/CD18 monoclonal antibody prevented PMN influx, but not reactivity.	Li et al. (1992)
3.0	5,880	20 min Trach tube	Ach	INH	Pentobarbital	Dog (Mongrel) 18-32 kg	Allopurinol and desferoxamine inhibited reactivity without inhibiting PMN influx.	Matsui et al. (1991)
3.0	5,880	0.5-2 h Trach tube	Ach	INH	Thiopental Chloralose	Dog (Mongrel) 26-32 kg	Thromboxane synthase inhibitor blocked reactivity without influencing PMN influx.	Aizawa et al. (1985)
0.5 0.6	980 1,176	5 min	Mch	INH	Ketamine Fluorodiazepam	Baboon, M 25-40 kg	Brief exposure caused increased reactivity that was blocked by cromolyn.	Fouke et al. (1988)
0.5 0.6	980 1,176	5 min	Mch	INH	Ketamine Fluorodiazepam	Baboon, M 25-40 kg	Reactivity partially blocked by cromolyn, but no effect on stable prostanoids.	Fouke et al. (1990)
1.0	1,960	6 h/day, 5 days/week for 12 weeks	Ach/Pt	INH	Ketamine Xylazine	Cynomolgus, M 4.5 ± 0.1 kg	No increased reactivity in O_3-only group, but increase with platinum mixture.	Biagini et al. (1986)
1.0	1,960	2 h, 1/week for 19 weeks	Mch	INH	Pentobarbital	*Rhesus Macaca*, F 5-7 kg	5-lipoxygenase inhibitor blocked the development of reactivity.	Johnson et al. (1988)

[a]See Appendix A for abbreviations and acronyms.
[b]Table ordered according to animal species.
[c]Mch = methylcholine, Ach = acetylcholine, Hist = histamine, 5-HT = 5-hydroxytryptamine, SP = substance P, FS = field stimulation, Cch = carbachol, TX = thromboxane, KCl = potassium chloride, Pt = platinum; Route: IV = intravenous, INH = inhalation.
[d]Age or body weight at start of exposure.

The response to antigen-induced bronchoconstriction, an animal model of allergy, also has been evaluated recently. After a 5-min exposure to 1.0 ppm O_3 via a wedged bronchoscope, collateral resistance in dogs increased for 1 to 3 h (Kleeberger et al., 1989). After the O_3-induced resistance returned to baseline, the typical increase in collateral resistance observed in dogs challenged with *Ascaris suum* antigen, to which the dogs were natively sensitive, was attenuated both 1 to 3 h and 24 h post-O_3 exposure. The attenuated antigen response appeared to be independent of PMNs in the airways. In a follow-up study, the late-phase response to antigen (bronchoconstriction 2 to 12 h postantigen challenge) also was blocked in allergic dogs when O_3 exposure (1.0 ppm, 5 min, via a bronchoscope) preceded antigen challenge (Turner et al., 1989). These studies suggest that, at least in the dog, brief local administration of O_3 to the airways may inhibit allergic responses.

6.2.5.3 Acute Ozone Exposures (Less Than One Day)

Ventilation

Alteration of the ventilatory pattern has long been established as a hallmark of acute O_3 exposure. Several animal studies evaluated tidal breathing changes during and after O_3 exposure (U.S. Environmental Protection Agency, 1986). For most species, a tachypneic response (rapid and shallow breathing) has been observed. For example, Murphy et al. (1964) studied unanesthetized guinea pigs exposed for 2-h to 0.34, 0.68, 1.08, or 1.34 ppm O_3 via nose cones, and measured tidal breathing using a constant volume plethysmograph. A similar experimental preparation was used by Amdur et al. (1978) to evaluate the respiratory response of guinea pigs to 0.2, 0.4, and 0.8 ppm O_3. In both experiments, a monotonic increase in f was observed. In the Amdur et al. (1978) study, decreases in V_T were not observed concomitantly.

Lee et al. (1979, 1980) showed that the tachypneic pattern observed in conscious dogs exposed to 0.56 to 0.85 ppm was not altered by bronchodilator pretreatment or atropine administration. These manipulations would suggest that the rapid, shallow breathing was not caused by bronchoconstriction. The response, however, was blocked by vagal cooling, which was interpreted by the authors to suggest that vagal sensory afferent transmission had been blocked. Thus, the authors suggested that increased vagal afferent impulses produced tachypnea and that the response was independent of vagal efferents (increased smooth muscle tone).

Several new studies evaluating ventilation after acute O_3 exposure have appeared in the literature (Table 6-13). Mautz and Bufalino (1989) measured ventilation ($\dot{V}_E$) as well as oxygen consumption and rectal temperature in awake rats exposed for 3 h to 0.2, 0.4, 0.6, and 0.8 ppm O_3. Concentration-related increases in f were significantly different from controls beginning at 0.4 ppm, with a maximal response observed up to 0.6 ppm. Tidal volume was similarly reduced, whereas $\dot{V}_E$ and rectal temperature were less sensitive to O_3 exposure, showing decreases at 0.6 and 0.8 ppm. Oxygen consumption was decreased at all concentrations tested. The authors concluded that the O_3-induced change in breathing pattern did not cause a decrease in metabolic rate or impose a condition of hypoxia. The changes in ventilation and O_2 consumption appeared coincident or possibly preceded the irritant reflex change in breathing pattern.

Tepper et al. (1990) exposed awake rats to 0.12, 0.25, 0.5, and 1.0 ppm O_3 for 2.25 h in head-out pressure plethysmographs. During exposure, CO_2-stimulated breathing was incorporated to augment ventilation, similar to the use of exercise in human studies.

Frequency increased and V_T decreased monotonically between 0.25 and 1.0 ppm during a 2.25-h exposure. No decrease in $\dot{V}_E$ was observed. This difference from the Mautz and Bufalino (1989) study could be due to their restraining rats in a tightly fitting plastic flow plethysmograph with the face sealed by an aluminum nose cone; in the Tepper et al. (1990) study, the rats were exposed in oversized, steel, head-out plethysmographs and were intermittently challenged with CO_2, which may have overridden the metabolic depressant effect.

Mautz et al. (1985b), using exercising dogs exposed to 0.6 ppm O_3 for 140 min, showed tachypnea, increased $\dot{V}_E$, and elevated ventilation equivalents for O_2 and CO_2 compared to dogs exposed to air while exercising. Pulmonary resistance fell in air-exposed, exercising dogs, but climbed toward the end of the exposure in the O_3-exposed dogs.

In a follow-up study to Lee et al. (1979, 1980), Sasaki et al. (1987) performed similar experiments on two awake dogs that were trained to run on a treadmill. Dogs were exposed to 1.0 ppm O_3 for 2 h and evaluated before O_3 exposure and at either 1 or 24 h postexposure. In all studies with or without exercise, O_3 increased f and decreased V_T, without affecting $\dot{V}_E$. Vagal blockade diminished, but did not abolish, the tachypneic response, indicating that both vagal and nonvagal mechanisms were important. The O_3-induced change in f was due to equal reductions in inspiratory and expiratory times with no, or small, diminution (only during CO_2 rebreathing experiments) of ventilatory drive (V_T/inspiratory time). Additionally, O_3 did not affect functional residual capacity (FRC) or core temperature in resting, exercising, or vagally blockaded dogs. The authors speculate that the change in f is due to a vagally mediated lowering of the volume threshold of the pulmonary stretch receptor for inspiration and expiration with a concomitant increase in flow rate, thus leaving the FRC constant. Furthermore, the authors speculated that increased sensitization of rapidly adapting receptors, C-fiber nerve endings, and nonvagal mechanisms also may be contributors to the tachypneic response.

Two recent studies provide further insight into the mechanism of O_3-induced changes in ventilatory patterns. In the first study, Schelegle et al. (1993) showed that O_3-induced (3 ppm O_3 for 40 to 70 min) tachypnea in anesthetized, spontaneously breathing dogs largely could be abolished by cooling the cervical vagus to 0 but not 7 °C. This would indicate that large myelinated fibers were not involved in this reflex response, but nonmyelinated C fibers, whose activity is decreased only at the lower temperature, are important. In a companion study, Coleridge et al. (1993) measured the responses of five types of single vagal nerve fibers: (1) bronchial C-fibers, (2) pulmonary C-fibers, (3) rapidly adapting receptors, (4) slowly adapting pulmonary stretch receptors, and (5) unclassified fibers. During exposure to O_3, bronchial C-fibers were most affected. Because discharge of these fibers was not immediate with the onset of exposure, but took time to develop, the authors suggested that O_3 may not directly stimulate these receptors and that autacoid mediators released in the lung, which previously have been shown to stimulate these fibers, were probably responsible for fiber activation. Rapidly adapting receptors were shown to play a small part in this reflex response; although, surprisingly, pulmonary C-fibers and slowly adapting receptors were found to be unimportant. Ozone also stimulated several unidentified vagal fibers that may be responsible for residual effects not abolished by 0 °C cooling of the vagus. Thus, early inflammatory changes, as well as direct stimulation of bronchial C-fibers, may be responsible for the tachypnea seen with O_3 exposure in animal experiments. Results from these studies, together with the amelioration of spirometric changes found after

indomethacin in humans (see Chapter 7), suggest that tachypnea, inspiratory pain, and the reduction in forced vital capacity (FVC) could reflect early inflammatory lesions as well as neurogenic stimulation.

Breathing Mechanics

Although changes in breathing mechanics have been observed in laboratory animals, these changes are not observed consistently and tend to be reported more frequently at higher exposure concentrations (U.S. Environmental Protection Agency, 1986; Table 6-13).

The previously discussed studies by Murphy et al. (1964) and Amdur et al. (1978) evaluated breathing mechanics in unanesthetized guinea pigs. The Murphy et al. (1964) study showed an increase in flow resistance only at concentrations >1 ppm O_3. Pulmonary compliance was not measured. Amdur et al. (1978) observed a decrease in Cdyn after exposure to 0.4 and 0.8 ppm O_3, but no significant change in R_L was noted.

In an attempt to expose unanesthetized rats using regimens analogous to human clinical studies, Tepper et al. (1990) observed no significant changes in R_L or Cdyn after a 2.25-h exposure to 0.12, 0.25, 0.5, or 1.0 ppm O_3, in spite of intermittent 15-min periods of exercise-like hyperventilation induced by CO_2. Similarly, no changes in breathing mechanics were observed by Yokoyama et al. (1987) when they evaluated anesthetized rats exposed to 1.0 ppm O_3 for 24 h. However, when Cdyn was normalized for differences in FRC, the resulting specific compliance was decreased compared to air-exposed controls. Furthermore, when the animals were paralyzed and ventilated between 40 and 200 breaths/min, O_3-treated animals showed a frequency-dependent decrease in Cdyn as f increased above 120 breaths/min. The authors conclude that because R_L was not affected, the effect of O_3 was to obstruct peripheral airways.

Pulmonary mechanics were evaluated in anesthetized, paralyzed dogs acutely exposed to 0.12, 0.22, and 0.45 ppm O_3 for 3 h via a stainless steel tracheal tube. No changes in R_L or Cdyn were observed at any concentration (Morgan et al., 1986).

In papers by Miller et al. (1987, 1988), the effect of a 1-h exposure to 1.0 ppm O_3 was evaluated. Two hours after exposure, anesthetized, tracheostomized guinea pigs showed a significant increase in R_L that resolved by 8 h postexposure. Both indomethacin and cromolyn sodium partially blocked the increase in R_L at 2 h postexposure (Miller et al., 1988). These results suggest that eicosanoids produced from an inflammatory response in the lung may be responsible for the increase in R_L. However, plasma levels of $PGF_{2\alpha}$ and 6-keto PGF_{α} were not affected by O_3 or drug treatment, and PGE_1 was not affected by O_3. In an attempt to understand the involvement of eicosanoids in the increase in R_L observed with O_3 exposure, Fouke et al. (1991) showed that exposure to 0.5 ppm O_3 for 2 h caused an increase in R_L and a decrease in Cdyn in anesthetized dogs. Bronchoalveolar lavage fluid from these dogs did not have any increase in 6-keto $PGF_{1\alpha}$, PGE_2, TXB_2, or $PGF_{2\alpha}$, suggesting that these cyclooxygenase products were not involved in the changes in breathing mechanics. Similar findings were observed by these authors after brief exposures to baboons (see Section 6.2.5.2).

Gas trapping in the excised guinea pig lung was evaluated by water displacement in guinea pigs challenged with acid aerosol exposure after a 2 h, 0.8-ppm O_3 exposure (Silbaugh and Mauderly, 1986). Ozone exposure followed by air exposure increased gas trapping to roughly the same extent as O_3 followed by a sulfuric acid challenge (1 h, 12 mg/m^3) when compared to the air-only control response. These data indicate that O_3 causes an acute

peripheral airway obstruction, but no additive or synergistic effect of sulfuric acid aerosol was observed.

Airway Reactivity

Probably, the most extensive amount of laboratory animal research has been conducted on the role of O_3 in producing acute airway injury resulting in an increase in airway reactivity (U.S. Environmental Protection Agency, 1986; Table 6-14). Much of this research has used O_3 exposures that are never encountered in the ambient environment (3 ppm for 30 min); thus, the relevance of these studies may be questioned. However, the studies are the most thorough mechanistic account of such O_3 effects and have shown some agreement with human O_3 exposure studies (Chapter 7); therefore, these studies are summarized briefly here. The literature is focused around five primary issues that in recent years have been more thoroughly evaluated.

Concentration and Peak Response Time. Easton and Murphy (1967) were the first to demonstrate an increased responsiveness in unanesthetized guinea pigs post-O_3 exposure (2 h, 0.5 to 7 ppm). In their study, responsiveness was assessed by increased mortality due to severe histamine-induced bronchoconstriction, as well as by increased R_L and decreased Cdyn. Lee et al. (1977) examined anesthetized dogs exposed to O_3 (0.7 to 1.2 ppm, 2 h) via a tracheal tube and determined that increased airway reactivity to inhaled histamine occurred at 24 but not 1 h postexposure. A similar experiment, done in unanesthetized sheep by Abraham et al. (1980), indicated that airway responsiveness was increased at 24 h, but not immediately after a 2-h exposure to 0.5 ppm O_3. When the exposure was increased to 1 ppm O_3, an increase in baseline R_L was reported, and reactivity increased immediately and at 24 h postexposure. In apparent contradiction, Holtzman et al. (1983a) showed that airway reactivity increased markedly 1 h after dogs were exposed to 2.2 ppm O_3 for 2 h and was less evident at 24 h postexposure. Gordon and Amdur (1980) also reported that airway reactivity in guinea pigs was maximal 2 h after a 1-h exposure to 0.1, 0.2, 0.4, or 0.8 ppm O_3, as defined by a significant increase in R_L or decrease in Cdyn after a single subcutaneous challenge of histamine. The effect on R_L was concentration dependent, but was significant only at 0.8 ppm. For Cdyn, there was no concentration-related response, but all O_3 exposures exacerbated the decrease in Cdyn after histamine relative to the air-exposed group. The site of bronchoconstriction was suggested to be the conducting airways, rather than the parenchyma, because dynamic compliance was affected and static compliance was not (Gordon et al., 1984).

To examine the role of duration of exposure on experimental outcome, Nishikawa et al. (1990) exposed guinea pigs to C × T products of 30 (1 ppm × 30 min), 90 (1 ppm × 90 min), 90 (3 ppm × 30 min), and 360 (3 ppm × 120 min) ppm · min O_3. After exposure, specific airway resistance (SR_{aw}) during an inhaled methacholine challenge was measured in unanesthetized animals at 5 min, 5 h, and 24 h. In all but the 1-ppm, 90-min exposure group, there was an increase in baseline SR_{aw} at 5 min, but the response was neither concentration nor C × T dependent. At 5 min postexposure, no increase in airway responsiveness was observed at 30 ppm · min. Airway hyperresponsiveness was observed at 90 ppm · min, using either exposure scenario (1 ppm for 90 min or 3 ppm for 30 min), and the response to the 360 ppm · min was greater than that observed with 90 ppm · min exposure. Significant increases in airway responsiveness at both 5 and 24 h postexposure were observed only in the 360 ppm ·

min group. The authors concluded that exposure duration was an important determinant of O_3-induced airway hyperresponsiveness.

Uchida et al. (1992) reported increased airway reactivity in rats to inhaled methacholine after a 1.0-ppm (2-h) O_3 exposure. These results conflict with other published studies in rats also using inhaled methacholine, which reported the inability to produce consistent increases in airway reactivity after exposure to less than 4 ppm O_3 (Evans et al., 1988). Tepper et al. (1995) reported that airway hyperresponsiveness in rats challenged with iv acetylcholine occurred only at 1 ppm O_3 or higher. In these latter studies, exposure durations ranged from 2 to 7 h, and, in some tests, CO_2 was added to the exposure to increase ventilation. Although guinea pigs are more responsive than rats, they are not as responsive as humans to O_3-induced increased airway reactivity, even under optimal conditions (Tepper et al., 1995).

Inhaled Versus Intravenous Challenge. In a follow-up study using a similar exposure protocol as described above, Abraham et al. (1984) observed increased responsiveness to iv carbachol in unanesthetized sheep 24 h after a 2-h, 0.5-ppm O_3 exposure; inhaled carbachol did not produce a similar response. The authors interpreted this result to indicate a decreased penetration of the carbachol aerosol in O_3-exposed animals compared with the direct stimulation of smooth muscle by the iv route. Roum and Murlas (1984) observed that O_3-induced hyperresponsiveness was similar for inhaled versus iv acetylcholine or methacholine challenge through 14 h postexposure, but after that time, only iv administration revealed a persistent O_3-related response. In contrast, Yeadon et al. (1992) reported that guinea pigs exposed to 3 ppm for 30 min were hyperresponsive to inhaled histamine, serotonin, acetylcholine, and substance P, but were not hyperresponsive to O_3 after iv administration of the same agonists. Tepper et al. (1995) and Uchida et al. (1992) also showed that rats were more sensitive to inhaled methacholine than to iv administration of the agonist.

Neurogenic Mediation. Lee et al. (1977) reported increased airway responsiveness to histamine in dogs exposed to 0.7 or 1.2 ppm O_3 for 2 h. Atropine and vagal blockade were effective in reducing the O_3-induced hyperresponsiveness to histamine, suggesting that heightened vagal activity was responsible. Katsumata et al. (1990) also showed that in the cat, airway hyperresponsiveness to histamine could be attributed to cholinergic reflex. This is in apparent contrast to the increased O_3-induced (1.0 to 1.2 ppm, 2 h) responsiveness to histamine (subcutaneous) that was not blocked by atropine or vagotomy, indicating minimal vagal involvement in guinea pigs (Gordon et al., 1984). In agreement, Jones et al. (1987) found that hexamethonium, a ganglionic blocker, did not prevent O_3-induced hyperresponsiveness in dogs exposed to 3 ppm O_3 for 0.5 h via an endotracheal tube. Similarly, Yeadon et al. (1992) showed that atropine or bilateral vagotomy only partially reduced the hyperresponsivenss in guinea pigs exposed to 3 ppm for 120 min but did not block the response in animals exposed for only 30 min.

A role for prejunctional muscarinic receptors has been demonstrated by Schulteis et al. (1994). The M_2 receptor, which is inhibitory for acetylcholine release, was shown to be defective immediately after a 4-h, 2-ppm O_3 exposure in guinea pigs. Fourteen days after exposure, M_2 receptor function and vagally stimulated responsiveness were normal. Thus, the role of the cholinergic system in O_3-induced airway hyperresponsiveness has yet to be firmly established.

Peptidergic mediators also have recently been suggested as important modulators of this response. Murlas et al. (1992) demonstrated that phosphoramidon, an inhibitor of neutral endopeptidase (NEP), increased the responsiveness to substance P in air-exposed (but not O_3-exposed [3 ppm, 2 h] guinea pigs). Substance P-induced bronchoconstriction in air-exposed animals was increased after phosphoramidon because NEP degrades substance P. The finding was associated with a decrease in tracheal NEP in O_3-exposed animals. Additionally, the increased airway responsiveness in O_3-exposed animals was reversed by inhalation of partially purified NEP. Taken together, these results suggest that O_3 inactivates NEP, thus increasing the response to endogenous tachykinin release. A similar result was obtained by Yeadon et al. (1992) in guinea pigs exposed to 3 ppm O_3 (30 or 120 min) and challenged with aerosolized substance P after pretreatment with the NEP inhibitors phosphoramidon, thiorphan, and bestatin. Tepper et al. (1995) depleted guinea pigs of substance P, using multiple doses of capsaicin, and found that airway reactivity, after a 2-h exposure to 1 ppm O_3, was partially blocked. However, although tracheal vascular permeability also was blocked by capsaicin pretreatment, protein influx into the BAL and tachypnea were not blocked. On the other hand, Evans et al. (1989) did not find increased tracheal vascular permeability in rats exposed to 4 ppm O_3 for 2 h. These studies suggest, at least for the guinea pig, that enhancement of the substance P response, by inhibition of NEP, may be important in O_3-induced hyperresponsiveness.

Inflammation. Holtzman et al. (1983b) found a strong association between increased airway responsiveness and increased PMNs present in the tracheal biopsy of dogs 1 h after a 2-h O_3 exposure to 2.1 ppm. Fabbri et al. (1984) extended these findings, showing an association between increased airway reactivity and increased lavageable inflammatory cells from the distal airways of dogs. Further support for this hypothesis was engendered by the demonstration that in PMN-depleted dogs (produced by administration of hydroxyurea), O_3-induced airway hyperresponsiveness was blocked. This is in contrast to Murlas and Roum's (1985a) findings in guinea pigs exposed to 3 ppm O_3 for 2 h, which indicate that increased airway reactivity, mucosal injury, and mast cell infiltration occur before PMN influx. The authors speculate that PMN influx is a response to the damage, not a cause of the increased airway reactivity. Furthermore, Murlas and Roum (1985b) showed that PMN depletion in the guinea pig with cyclophosphamide did not prevent O_3-induced airway hyperresponsiveness. Similar results were obtained by Evans et al. (1988), who reported that airway hyperresponsiveness was not accompanied by airway PMN influx in rats, and by Joad et al. (1993), who showed that adding human PMNs to the pulmonary circulation of the rat lung during a 3-h, 1.0-ppm exposure to O_3 did not further enhance O_3-induced airway reactivity. Beckett et al. (1988) evaluated dogs exposed for 2 h to 1 ppm O_3 directly to the peripheral airways via a wedged bronchoscope. Fifteen hours postexposure, the exposed peripheral airway segments were hyperresponsive to aerosolized acetylcholine. However, at the site of increased responsiveness, there was no association with increased PMNs, mast cells, or mononuclear cells. Such studies agree with perhaps the most definitive study of this hypothesis (Li et al., 1992), which used monoclonal antibodies (CD11b/CD18) to prevent PMN influx into the airways. When PMNs were present in the circulation, but prevented from entering the lung, the dogs were still hyperresponsive after a 30-min exposure to 3 ppm O_3. Thus, in three species, it appears that PMN influx may be associated with O_3 exposure but is not necessary for producing airway hyperresponsiveness.

Several studies have suggested that arachidonic acid metabolites may be important in O_3-induced airway hyperresponsiveness. Although the primary source of arachidonic acid metabolites is suspected to be inflammatory cells in the lung, cells other than PMNs could be responsible for the liberation of arachidonic acid metabolites. Only one study in dogs indicates that blockage of cyclooxygenase products with indomethacin can protect animals from developing airway hyperresponsiveness (O'Byrne et al., 1984). However, several, more recent studies have found that cyclooxygenase inhibitors were ineffective in blocking this response (Lee and Murlas, 1985; Holroyde and Norris, 1988; Yeadon et al., 1992). Two papers indicated the importance of LTs, as demonstrated by the inhibition of hyperresponsiveness with prior administration of 5-lipoxygenase inhibitors to guinea pigs (Lee and Murlas, 1985; Murlas and Lee, 1985). In contrast, Yeadon et al. (1992) found that a specific 5-lipoxygenase inhibitor did not block the response in guinea pigs. One study with dogs showed that TX generation may be important in this phenomenon (Aizawa et al., 1985), but, more recently, two papers from the same investigators have dispelled that notion (Jones et al., 1990, 1992). Furthermore, exposure to 0.5 ppm O_3 for 2 h caused a decrease in the provocative dose of methacholine necessary to cause a 50% increase in R_L in anesthetized dogs (Fouke et al., 1991). Bronchoalveolar lavage on these dogs did not show any increase in 6-keto $PGF_{1\alpha}$, PGE_2, TXB_2, or $PGF_{2\alpha}$, suggesting that these cyclooxygenase mediators of inflammation were not involved in the changes in airway reactivity. In summary, the initial hypothesis of the role of PMNs or PMN-derived products in O_3-induced airway hyperresponsiveness is questionable because most newer studies, using more specific inhibitors of PMNs, cyclooxygenase, and 5-lipoxygenase, and studies blocking TX receptors indicate the lack of a protective effect.

Interactions with Antigen and Virus. In mice, Osebold et al. (1980) showed that an increased number of animals became sensitized to ovalbumin after 3 to 5 days of continuous exposures to 0.5 and 0.8 ppm O_3. Matsumura (1970) and Yanai et al. (1990) made similar findings in guinea pigs and dogs exposed acutely to higher O_3 concentrations, suggesting that O_3 may enhance either sensitization or response to antigen. These results appear to agree with recent findings in humans (see Chapter 7).

Ozone (1 ppm, 2 h) also may increase hyperreactivity associated with virus exposure. Tepper et al. (1995) exposed rats to O_3 either before or during an influenza virus infection. Rats exposed to O_3 before virus infection were more hyperresponsive to inhaled methacholine 3 days later (at a time when there was no hyperresponsiveness to O_3 alone) than were rats exposed to only the virus. An additive effect was observed in virus-infected rats when O_3 exposure was immediately before methacholine challenge.

Extended Functional Characterizations

Extended characterizations of pulmonary function in laboratory animals indicate that the general pattern of functional impairment reported in human studies also is observed in animal studies of acute O_3 exposure. Anesthetized and ventilated cats showed a general decline in vital capacity (VC), static lung compliance, or diffusing capacity for carbon monoxide (DL_{CO}) with exposures up to 6.5 h of 0.26 to 1.0 ppm O_3 (Watanabe et al., 1973). Inoue et al. (1979) observed functional evidence of premature airway closure, as indicated by increases in closing capacity, residual volume (RV), and closing volume, after rabbits were exposed to 0.24 or 1.1 ppm O_3 for 12 h. The volume-pressure curve indicated increased lung volume at low

distending pressures; additionally, nonuniform distribution of ventilation was observed. The effects were most prominent 1 day following exposure and had mostly subsided by 7 days postexposure.

Most studies of O_3 in experimental animals make little effort to mimic human study designs, thereby further confounding the extrapolation of their results to humans. Recently, however, rat studies involving periods of intermittent CO_2-induced hyperventilation to enhance the delivered dose of O_3 have attempted to capitalize on the qualitative similarity of the rat and human maximum expiratory flow-volume curves as a potentially sensitive endpoint of toxicity (Costa et al., 1988b; Tepper et al., 1989). In the rat, FVC decreases acutely with O_3 exposure, and this response has been mathematically modeled for O_3 concentrations between 0.35 and 0.8 ppm with exposure durations between 2 and 7 h (Tepper et al., 1989). The magnitude of response is apparently less than that observed in humans (Tepper et al., 1995), although the extent to which anesthesia mitigates the rat response or that there are inherent species differences in dosimetry or sensitivity is not clear from these studies (see Chapter 8).

In addition to changes in the flow-volume curve, changes in lung diffusion also are observed. In a study that examined concentration, duration, and ventilation factors, rats were exposed for 2 or 7 h to 0.5 or 0.8 ppm O_3 with intermittent 8% CO_2 to hyperventilate (□2 to 3 times resting V_E) the animals as an exercise analogue to human exposures (Costa et al., 1988a). The DL_{CO} values were reduced by 10% at both 0.5-ppm time points and by 12% with a 2-h exposure to 0.8 ppm. Exposure to 0.8 ppm for 7 h, however, greatly exacerbated the alveolar effect, with a resultant 40% reduction in the DL_{CO}. Static compliance was affected only at this latter exposure concentration and duration. This O_3-induced reduction in DL_{CO} appeared to correlate with the degree of lung edema in affected animals. Yokoyama et al. (1987) found decreases in rat lung volumes (FRC and RV), static compliance (from the volume-pressure curve), and maximal flow at 50% of VC after a 24-h exposure to 1 ppm O_3.

Flow-volume curves and measurements of regional distribution of ventilation, using a positron camera, were evaluated in anesthetized, paralyzed dogs acutely exposed via a stainless steel tracheal tube to 0.12, 0.22, and 0.45 ppm O_3 for 3 h (Morgan et al., 1986). No changes in the flow-volume curve were observed at any concentration, but a less uniform distribution of ventilation was noticed, with the greatest difference occurring between the central and more peripheral regions. The authors conclude that the initial effect of O_3 appears to be obstruction of the small airways.

Miller et al. (1987, 1988) evaluated the effect of a 1-h exposure to 1.0 ppm O_3 on changes in the lung function of anesthetized, tracheostomized guinea pigs. Decreases in lung volumes were noted at 2 h postexposure and were maximum between 8 and 24 h postexposure, after which time they began to resolve. Alveolar ventilation ($\dot{V}_A$) and DL_{CO} also were decreased by exposure. The initial (2 h postexposure) reduction in DL_{CO} may have been caused by a bronchoconstriction-related decrease in $\dot{V}_A$. After this time, disproportionate ratios of DL_{CO} and $\dot{V}_A$ suggest that different mechanisms were responsible for the decreased DL_{CO}. The authors speculate that this latter response probably involves the development of a peripheral inflammatory response (8 to 24 h postexposure) because plasma concentrations of 6-keto $PGF_{1□}$ and PGE_1 also were elevated in guinea pigs exposed for 1 h to 1 ppm O_3 (Miller et al., 1987). Significant increases in the plasma and BAL concentrations of TXB_2 also were observed following acute exposure of guinea pigs to 1 ppm O_3 (Miller et al., 1987) and humans to 0.4 or 0.6 ppm O_3 (see Chapter 7). Both indomethacin and cromolyn sodium partially

blocked the reduction in lung volumes at 2 and 24 h postexposure (Miller et al., 1988). Indomethacin was ineffective in blocking the O_3-induced decrease in DL_{CO} at either 2 or 24 h postexposure, but cromolyn sodium blocked this O_3 response. Both drugs were effective in blocking the O_3-induced decrease in $\dot{V}_A$ at the same examination periods. These results suggest that eicosanoids produced from an inflammatory response in the lung may be responsible for the observed changes in lung function in guinea pigs. However, as noted above the role of eicosanoid mediators in O_3-induced lung injury is controversial.

6.2.5.4 Repeated Acute Exposure Experiments (More Than Three Days)

To date, few physiological studies have examined the attenuation that occurs in humans repeatedly (3 to 7 days) exposed to O_3 (U.S. Environmental Protection Agency, 1986; Table 6-13), despite the fact that this exposure scenario most closely mimics a high oxidant pollution episode.

Ventilation

In the only laboratory animal study using a similar exposure protocol and an experimental design analogous to human repeated-exposure studies, Tepper et al. (1989) showed that rats displayed an initial pulmonary irritant response (tachypnea) that attenuated after 5 consecutive days of exposure in a manner quite similar to the response pattern of humans (see Section 7.2). Exposures were for 2.25 h and included challenge with CO_2 during alternate 15-min periods to augment ventilation (2 to 3 times $\dot{V}_E$—equivalent to light exercise in humans). The functional changes were largest on Day 1 or 2, depending on the parameter and the O_3 concentration (0.35, 0.5, and 1.0 ppm were evaluated). Additionally, lung biochemical and structural consequences were examined at 0.5 ppm O_3 and indicated that several indices of lung damage increased (histopathology) or did not adapt (lavageable protein), despite the loss of the functional response over the 5-day exposure period. Functional attenuation, however, did not occur in the 1.0-ppm O_3 group; such a nonreversing effect has not been observed in humans. It is likely that this lack of reversal was attributable to the high concentration of O_3 and, thus, may be predictive of the human response under similar conditions.

Breathing Mechanics

In rats exposed to 1.0 ppm O_3 for 6 h/day for 7 days, the only change in breathing mechanics was an increase in R_L (Yokoyama et al., 1989a). Whether attenuation occurred cannot be ascertained because functional measurements were obtained only after the end of exposure.

Extended Characterizations

Selgrade et al. (1988) evaluated mice exposed to 1.0 ppm O_3 for 5 days (3 h/day), with and without the inoculation of influenza virus on Day 2 of exposure. Ozone alone did not cause an untoward effect on lung volumes, volume-pressure, and flow-volume relationships when mice were evaluated 1, 4, and 9 days postexposure. Mice exposed to the combination of virus and O_3 showed a decrease in DL_{CO} that persisted for 9 days.

A portion of the Tepper et al. (1989) study, discussed above, was conducted using groups of animals that were exposed between 1 to 5 days to 0.5 ppm O_3. Changes in the shape of the flow-volume curve (as indicated by the change in forced expiratory flow [FEF] at

25% of VC) were maximal on Day 2 but gradually returned to baseline with further repeated exposures.

6.2.5.5 Longer Term Exposure Studies

The question of degenerative or irreversible lung damage when O_3 exposure is extended over periods of days to years remains paramount to the assessment of health risk. Several new studies since the previous criteria document (U.S. Environmental Protection Agency, 1986) have been published using more integrated approaches (structure, function, and biochemical techniques) for understanding this problem. This is especially true for studies evaluating near-lifetime O_3 exposures in rodent species.

Ventilation and Breathing Mechanics

Tepper et al. (1991) evaluated ventilation and breathing mechanics in rats exposed for 1, 3, 13, 52, and 78 weeks to a simulated urban profile of O_3 (Table 6-13). The exposure consisted of a 5-day/week, 9-h "ramped spike" exposure that had an integrated average of 0.19 ppm O_3 and a maximum concentration of 0.25 ppm. During other periods (13 h/day, 7 days/week), the exposure remained at a 0.06-ppm O_3 background level. Pulmonary function measurements were evaluated after 1, 3, 13, 52, and 78 weeks of O_3 exposure in response to a postexposure challenge with 0, 4, and 8% CO_2. Overall, there was a significant increase in expiratory resistance, but only at 78 weeks was resistance significantly different than the time-matched filtered-air control. At all evaluation times, V_T was reduced compared to control rats; this was especially true during challenge with CO_2. Frequency of breathing was significantly decreased when the analysis included all evaluation times, but at no single evaluation time was the reduction significant.

Other evidence of peripheral airflow abnormalities from extended exposures to O_3 is limited. Costa et al. (1983) exposed rats to 0.2 or 0.8 ppm O_3 for 6 h/day, 5 days/week for 12 weeks and did not find a concentration-related increase in pulmonary resistance measured immediately after exposure. Yokoyama et al. (1984) measured increased central resistance in rats exposed for 30 days to 1.0 ppm, but found increased peripheral airway resistance when exposure was for 60 days to 0.5 ppm. Pulmonary resistance was measured at different elastic recoil pressures. Increased R_L at low distending pressures was interpreted as of peripheral origin, whereas uniform increases across all distending pressures were described as originating from the central airways. These changes were consistent with morphological findings of mucus in the large bronchi of rats exposed to 1.0 ppm compared to the rats exposed to 0.5 ppm. These data also agree with a study by Wegner (1982) that suggests the occurrence of airflow obstruction, revealed in terms of small increases in peripheral airways resistance (as measured by oscillation harmonics) that were observed in monkeys after 1 year of exposure to 0.64 ppm O_3 (8 h/day, 7 days/week).

Airway Reactivity

No studies of airway reactivity after long-term exposures were reported before 1985. Since then, several studies have reported no increase in reactivity with daily O_3 exposure (Table 6-14). Biagini et al. (1986) observed no changes in breathing mechanics, FEF parameters, or methacholine and platinum airway responsiveness in a group of monkeys (cynomolgus) exposed to 1 ppm O_3 for 6 h/day, 5 days/week for 12 weeks in a study designed to examine the effects of combining O_3 exposure with the respiratory sensitizer platinum.

Kagawa et al. (1989) exposed guinea pigs 4 h/day, 5 days/week for 4 mo to 0.15 ppm O_3. Baseline total respiratory resistance and response to increasing concentrations of inhaled histamine were assessed every 3 weeks, but did not change in response to O_3. The only exception is the Johnson et al. (1988) study that evaluated airway responsiveness in female rhesus monkeys just before a 2-h single weekly exposure to 1 ppm O_3 delivered via an endotracheal tube. After 19 weeks of exposure, increased responsiveness to inhaled methacholine was observed compared to the animal's historic control. The hyperresponsiveness persisted approximately 15 weeks after exposures were discontinued. Hyperresponsiveness to O_3 was reinstated after a similar 7-week exposure to the same animals. After this exposure regimen, animals recovered in approximately 9 weeks, but hyperresponsiveness was again reinstated with four, once-per-week exposures. The investigators described the effect of a 5-lipoxygenase inhibitor on certain portions of this sequence; however, the descriptions of methods and results were insufficient for evaluation of the effect of treatment on exposure.

Extended Functional Characterizations

The previous criteria document (U.S. Environmental Protection Agency, 1986) cataloged several investigators that reported marginal increases in total lung capacity (TLC) or its component volumes in rats after intermittent or continuous exposures to □0.25 ppm O_3 for 4 to 12 weeks (Bartlett et al., 1974; Costa et al., 1983; Raub et al., 1983). In contrast to these significant results, Yokoyama and Ichikawa (1974) previously had reported no effects on rat static volume-pressure curves after a 6-week exposure to 0.45 ppm (6 h/day, 6 days/week). More recent studies are summarized in Table 6-13.

Exposures of rats to 0.7 ppm O_3 for 28 days (20 h/day) showed an obstructive-type lung function abnormality characterized by a significant reduction in FEFs, lung volumes, and DL_{CO}, and a significant increase in FRC (Gross and White, 1986). These effects largely reversed after an additional 9 weeks of clean air, but some airflow abnormalities persisted.

Tyler et al. (1988) exposed young monkeys to 0.25 ppm O_3 for 8 h/day, 7 days/week for 18 mo or for alternate months of the 18-mo period and observed increased chest wall compliance (C_w) and inspiratory capacity. Because C_w did not decrease with age, as expected, the authors speculated that perhaps O_3 interfered with respiratory system maturation. This effect was greater in monkeys exposed during alternate months than in animals exposed every month of the 18-mo period.

To address the issue of cumulative exposure over a near-lifetime, several rodent studies have been performed using various exposure concentrations. With exposure of rats to 0.5 ppm O_3 for 52 weeks (20 h/day, 7 days/week), increases in RV and FRC were apparent, as was a fall in DL_{CO} (Gross and White, 1987), suggesting substantial end-airway damage and gas-trapping. After a 3-mo period in clean filtered air, these measurements were not different than similarly treated, but air-exposed control rats. In partial contrast, 12 or 18 mo of exposure to a daily urban profile of O_3 (9-h time-weighted average of 0.19 ppm, 5 days/week; a background of 0.06 ppm for 13 h/day, 7 days/week) resulted in small reduction in lung volumes (RV and VC) and an enhanced nitrogen (N_2) washout pattern consistent with a stiffer, restricted lung (Costa et al., 1995). Interestingly, in spite of mural remodeling of small airways (which was concentration dependent), no evidence of airflow obstruction was apparent in this study. However, in a cohort group of animals exposed at the same time, R_L was increased at all time points in unanesthetized animals, as described previously (Tepper et al.,

1991). Harkema and Mauderly (1994) exposed rats for 6 h/day, 5 days/week for 20 mo to either filtered air or 0.12, 0.5, or 1.0 ppm O_3. Within 3 days of the end of exposure, an extended functional evaluation was performed; O_3 caused little impact on respiratory function. However, RV was decreased (between 21 and 36%), a finding similar, but of greater magnitude, to the Costa et al. (1995) study. The existing morphological data in monkeys at the higher concentrations noted above appear consistent with end-airway remodeling, but no clear functional evidence of obstruction has been described (Eustis et al., 1981; Wegner, 1982).

6.2.5.6 Summary

Alterations in the pulmonary function of laboratory animals after exposure to O_3 have been reported by numerous investigators. These changes appear to be homologous with the changes in pulmonary function observed in humans exposed acutely to O_3 (see Chapter 7). Although there are apparent differences in sensitivity among species, it is not clear whether these differences are due to the use of anesthesia or restraint or variances in tissue sensitivity or dosimetry.

Brief exposures to O_3 of less than 30 min have been shown to produce reflex responses (increased collateral resistance) and airway hyperresponsiveness. In the dog, these changes appear to be related, in part, to parasympathetic stimulation and release of inflammatory mediators. However, the relevance of these studies must be questioned because O_3 was delivered to a specific lung region via a bronchoscope and the contribution of collateral resistance to total lung resistance was small.

With exposures lasting greater than an hour, a wide variety of effects has been observed. Most notably, tachypnea (increased frequency of breathing and decreased tidal volume) has been noted in several species at exposures as low as 0.25 to 0.4 ppm (Tepper et al., 1990; Mautz and Bufalino, 1989). In addition to changes in breathing pattern, changes in breathing mechanics (compliance and resistance) and increased airway reactivity have been observed, but, generally, these effects have been reported at concentrations of 1 ppm or greater. In dogs, R_L increased and Cdyn decreased after a 2-h exposure to 0.5 or 0.6 ppm O_3 (Fouke et al., 1991; Mautz et al., 1985b). Enhanced reactivity to bronchoconstrictors has been reported in guinea pigs at 0.5 ppm O_3 (Tepper et al., 1990). The mechanisms that may be responsible for the O_3-induced increase in airway reactivity have been investigated extensively; however, no firm conclusion can be drawn. The most consistent evidence suggests a role for sensory afferent fibers, their associated mediators (tachykinins), and the enzyme responsible for tachykinin degradation (NEP). However, many studies suggest that the parasympathetic nervous system and inflammatory cells and mediators also may play a role in O_3-induced increase in airway reactivity.

Extended characterizations of pulmonary function indicate that the general pattern of functional impairment seen in humans acutely exposed to high concentrations of O_3 (decreased lung volumes, diffusional disturbances, and inhomogeneity of ventilation) also is observed in animals exposed to high ambient O_3 (0.5 to 2.0 ppm). For example, FVC, DL_{CO}, and N_2 slope decreased with increasing C $\times$ T products (0.5 and 0.8 ppm O_3, 2 and 7 h) in rats (Costa et al., 1989).

With daily repeated exposure to O_3, Tepper et al. (1989) showed attenuation of lung function changes (tachypnea and flow volume curve) over 5 days (2 h/day to 0.35 to 1.0 ppm, with CO_2 stimulation of breathing) similar to what is observed in repeatedly exposed humans (Chapter 7). It is of interest that, in this study, morphological changes showed a progressive

increase in severity, and other biochemical indicators of lung injury (lavageable protein and antioxidants) did not show attenuation of the response over the same time period. The findings from long-term exposures of O_3 to laboratory animals are even more difficult to summarize. Various findings in rats ranged from no or minimal effects (Biagini et al., 1986; Kagawa et al., 1989; Chang et al., 1992; Harkema and Mauderly, 1994) to obstructive (Gross and White, 1986; Tepper et al., 1991) or restrictive (Costa et al., 1995) lung function abnormalities. However, in all cases where recovery was evaluated, no severe lung injury was detected, and the physiological alterations that were observed resolved several months after termination of exposure.

6.2.6 Genotoxicity and Carcinogenicity of Ozone

6.2.6.1 Introduction

Ozone is a very reactive molecule and a strong oxidizing agent that can dissolve in aqueous solutions and generate superoxide, hydrogen peroxide, and hydroxyl radicals and can oxidize and peroxidize cellular macromolecules (reviewed in Menzel, 1970; Hoigne and Bader, 1975; U.S. Environmental Protection Agency, 1986; Mustafa, 1990; Victorin, 1992; Pryor, 1993). Early studies of the effects of O_3 on purines, pyrimidines, nucleosides, nucleotides, and nucleic acids showed that O_3 rapidly degraded these compounds in vitro (Christensen and Giese, 1954; reviewed in Menzel, 1984). Ozone-generated hydroxyl radicals can abstract hydrogen from organic molecules, leading to further complex free-radical reactions (reviewed in Menzel, 1970, 1984; Mustafa, 1990; Victorin, 1992). In addition, O_3 initiates radical reactions, resulting in ozonolysis of alkenes to form ozonides, which decompose on reaction with water to form peroxyl radicals, peroxides, and aldehydes. Ozone also can oxidize amines to amine oxides and react with PUFA to form products of lipid peroxidation (reviewed in Menzel, 1970, 1984, 1992; Mustafa, 1990; Pryor, 1978, 1991, 1993). Ozone also has been shown to cause a reduction in plaque formation by bacteriophage f2, to release RNA from phage particles, to inactivate RNA, and to degrade protein (Kim et al., 1980). Hence, because O_3 generates hydroxyl radicals in aqueous solution and degrades DNA, RNA, protein, and fatty acids in vitro, it poses a potential genotoxic hazard by virtue of its ability to generate reactive intermediates that can oxidize nucleic acid bases (reviewed in Victorin, 1992). However, the precise reactions that occur in living cells exposed to O_3 have not yet been defined completely. As the ensuing discussion shows, the genotoxic potential of O_3 is, at most, weak.

This section reviews the information available on the genotoxicity of O_3 since the last air quality criteria document (U.S. Environmental Protection Agency, 1986) was published, although earlier reports are cited to create a historical and scientific perspective for the reader. The areas covered in this review are the ability of O_3 to induce DNA damage, mutagenesis, cell transformation, carcinogenesis, co-carcinogenesis, and tumor promotion. Although modulation of the tumorigenic response by indirect effects of O_3 on the immune system is theoretically possible, no evidence for such modulation has been reported (see Section 6.2.3). Unfortunately, experimental data to evaluate whether O_3 is genotoxic are very limited. Hence, relevant data on genotoxic effects of O_3 above 1 ppm also have been included to ensure discussion of the full array of effects as they currently are understood. Although data at points far above 1 ppm of O_3 are not directly relevant to human health, such high-concentration data serve to address (1) whether ozone is genotoxic at all, (2) whether

concentration-response relationships exist for the specific genotoxicity endpoint studied, and (3) what maximum sensitivity is required for discovering genotoxic effects.

A further caveat is that in many experiments utilizing in vitro systems, O_3 was added to bacteria or to mammalian cells covered by cell culture medium. In all such experiments, the reactivity of O_3 makes it highly likely that the reaction products of O_3 with culture fluid, not O_3 itself, actually reach and interact with cells. This complicates interpretation of the results, making it extremely difficult to extrapolate in vitro results to in vivo results and making it extremely difficult, if not impossible, to extrapolate in vitro results to potential genotoxicity in humans.

6.2.6.2 Ozone-Induced Deoxyribonucleic Acid Damage

Studies utilizing a wide range of O_3 concentrations (0.1 to 20 ppm) have been performed to determine whether O_3 is genotoxic. Hamelin (1985) showed by a combination of agarose gel electrophoresis and electron microscopy that ozonation at 5 to 20 ppm caused single- and double-strand DNA breaks, nicking, relaxation, linearization, and then degradation of double-stranded plasmid pATl53 DNA molecules in solution. Hamelin also showed that ozonation of plasmid DNA reduced the transforming ability of this plasmid, and that *Escherichia coli* strains with mutations in DNA repair pathways (lexA, ozrA, and recA, but not uvrA) were less able to support the transforming ability of the ozonated plasmid. Hence, the lexA, ozrA, and recA gene products participate in repairing O_3-induced DNA breaks.

Similarly, Sawadaishi et al. (1985) showed that ozonolysis of supercoiled pBR322 DNA resulted in conversion of closed-circular DNA molecules to open-circular DNA and caused single-strand cleavage at specific sites. The concentrations of O_3 employed were not listed. Sawadaishi et al. (1986) further explored the specificity of O_3-induced damage to supercoiled plasmid pBR322 DNA by utilizing DNA sequencing techniques. The mechanistic data obtained, showing preferential degradation of thymine bases, are very interesting chemically, but the O_3 concentrations used were far too high (25,600 ppm) to be useful in assessing biologically relevant effects of O_3.

Exposure of naked DNA from HeLa cells to 2 ppm O_3 for 24 h resulted in the formation of hydroxymethyluracil, thymine glycol, and 8-hydroxyguanine (Cajigas et al., 1994). These results indicate a potential mutagenicity for O_3, although the question of the penetration of O_3 to the DNA of intact cells has not been explored carefully. Mura and Chung (1990) also studied the biological consequences of ozonation of DNA. They exposed phage T7 DNA to 5 ppm O_3 for periods of 5 to 15 min and found that O_3 decreased the template activity of the DNA. Both the rate of initiation of transcription and the length of the RNA chains transcribed were reduced. They concluded that O_3 induced abnormal changes in the structure of the phage T7 DNA and that these changes interfered with the ability of the DNA to be transcribed. In mammalian cells, Van der Zee et al. (1987) demonstrated that ozonation of murine L929 fibroblasts caused DNA strand breaks, DNA interstrand cross-links, and DNA protein cross-links, but the O_3 concentrations used were far too high (615 ppm) to be relevant to the ambient exposures that are the focus of this document.

Kozumbo and Agarwal (1990) conducted in vitro studies in which specific arylamines contained in tobacco smoke (l-naphthylamine, 2-naphthylamine, aniline, p-toluidine, o-toluidine, and m-toluidine) were exposed to 0.1 to 1.0 ppm O_3 for 1 h. When the reaction products were added to human lung fibroblasts and transformed human Type 2 cells in vitro, DNA damage occurred. This raises the possibility that smokers could incur

DNA damage in their lung cells due to the interaction of O_3 with arylamines contained in tobacco smoke, but there are no data on whether such reactions occur in vivo.

A logical consequence of these findings (Table 6-15) is that O_3 could inhibit DNA replication in mammalian cells and induce cytotoxicity to these cells, and this was found by Rasmussen (1986). Rasmussen observed that DNA replication was inhibited in a concentration-dependent manner in Chinese hamster V79 cells by O_3 concentrations from 1 to 10 ppm following a 1-h exposure. These exposure regimens also induced cytotoxicity.

Table 6-15. Effects of Ozone on Deoxyribonucleic Acid Damage[a]

Ozone Concentration		Exposure Duration	Exposure Conditions	Cells	Observed Effects	Reference
ppm	μg/m³					
0.1	196	1 h	15 μM l-napthylamine	Diploid human lung fibro-blasts and transformed Type 2 cells	DNA breaks	Kozumbo and Agarwal (1990)
2.0	3,920	24 h	Phosphate buffered saline	Naked DNA from HeLa cells	Formation of hydroxymethyluracil, thymine glycol, and 8-hydroxyguanine in DNA	Cajigas et al. (1994)
1.0-10	1,960-19,600	1 h	Culture	Hamster (Chinese V79 cells)	Inhibition of DNA replication; cytotoxicity	Rasmussen (1986)
5.0	9,800	5-15 min	DNA at 50 μg/mL; O_3 at 0.5 L/min, room temperature	Ozonated T7 phage DNA	Decreased in vitro transcription	Mura and Chung (1990)
5.0-20	9,800-39,200	5-15 min	10 mM Tris/HC1 1 mM EDTA	Plasmid pAT153	Single-/double- strand breaks in DNA	Hamelin (1985)

[a]See Appendix A for abbreviations and acronyms.

Therefore, the available data show that O_3 causes single- and double-strand breaks in plasmid DNA in vitro, damages plasmid DNA so that its ability to serve as a template for transcription is decreased, and inhibits DNA replication and causes cytotoxicity in Chinese hamster V79 cells (Table 6-15).

6.2.6.3 Induction of Mutation by Ozone

Consistent with its ability to induce DNA damage, the very high concentration of 50 ppm O_3 also induced mutation to streptomycin resistance in *E. coli*, via both direct mechanisms and indirectly by the rec-lex error-prone DNA repair system, by a factor from two- to 35-fold in an exposure-time dependent manner (Table 6-16). However, no statistical analysis was performed on these data (Hamelin and Chung, 1975a,b; L'Herault and Chung, 1984). In assays designed to detect base substitution mutations by gases in *Salmonella typhimurium*, Victorin and Stahlberg (1988a) showed that O_3 alone at concentrations of 0.1 to 3.5 ppm did not induce mutation to histidine auxotrophy in Ames' strains TA100, TA102, or TA104. Ozone concentrations □2 ppm were cytotoxic. Victorin and Stahlberg (1988b) also showed that 0.5 and 1.0 ppm O_3, in combination with 1% vinyl chloride, and 1 ppm O_3, plus 0.1 or 1% butadiene, gave rise to a slight (approximately twofold) increase in mutation frequency. In these in vitro studies, as in many studies reviewed in this section, it must be pointed out that because O_3 is so reactive, placing vinyl chloride and butadiene in the experiments would result in exposure of the bacteria to reactive intermediates and reaction products resulting from the mixture, not exposure of the bacteria to O_3 alone. Further, these increases in mutation were small (no more than twofold) and not statistically analyzed, and the authors did not test strictly for concentration-dependent effects (Table 6-16). Thus, it is not clear that these small effects are reproducible.

More recently, Dillon et al. (1992) studied the ability of O_3 to induce mutation in the Ames' strains of *Salmonella* (Table 6-16). Ozone caused no mutation in *Salmonella* strains TA1535, TA98, TA100, and TA104. These authors found that 0.024- and 0.039-ppm O_3 exposures caused small increases in the mutation frequency in *Salmonella* tester strain TA102, which is uniquely sensitive to detecting mutation induced by oxygen radicals. These increases in mutation frequency were significant; however, the authors did not observe consistent concentration-dependent increases in the mutation frequency. At the higher concentrations of O_3, there appeared to be an inverse dependence for induction of mutation by O_3. The authors indicated that the cytotoxicity of O_3 complicated attempts to obtain a clear concentration response for mutagenicity. The presence of Arochlor 1254-induced rat liver S-9 metabolic activation did not affect the mutational responses in any of the strains tested. These authors did not observe reproducible increases in mutation frequency in *Salmonella* strains TA98, TA100, TA104, or TA1535. Dillon et al. (1992) therefore concluded that O_3 is a weak bacterial mutagen only under specific conditions utilizing noncytotoxic concentrations in TA102. However, because clear concentration-dependent responses for mutation could not be achieved, it is not clear that O_3 is definitively mutagenic in these studies.

Gichner et al. (1992) investigated whether O_3 could induce mutation in two mutagenicity assays in plants. The investigators found no induction of mutation in the *Nicotiana tabacum* leaf color reversion assay or in the *Tradescantia* stamen hair assay at 0.1 to 0.3 ppm O_3 (Table 6-16).

Dubeau and Chung (1979) showed that mutants of the yeast of *Saccharomyces cerevisiae* deficient in repair of single- and double-strand DNA breaks were more sensitive to the cytotoxicity of O_3 than wild-type cells, indicating that O_3 kills cells partly by generating these types of breaks. Dubeau and Chung (1982) also showed that treatment of *S. cerevisiae* with 50 ppm O_3 for 30 to 90 min resulted in (1) an 11- to 14-fold increased frequency of forward mutations, (2) an increase in reversions at six different loci by two- to threefold,

Table 6-16. Summary of Findings on the Mutagenicity of Ozone

Ozone Concentration		Exposure Duration	Exposure Conditions	Cells	Observed Effects	Reference
ppm	μg/m³					
0.024	47	35 min	Culture	*S. typhimurium* TA102	At 0.024 ppm: 2.4-fold increase in mutation frequency	Dillon et al. (1992)
0.039	76	35 min			At 0.039 ppm: 1.6-fold increase in mutation frequency	
0.39	764	35 min			At 0.39 ppm: 1.3-fold increase in mutation frequency; no effects seen in *S. typhimurium* TA98, TA1535, TA100, or TA104	
0.1-0.3	196-588	5 or 11 h/day for 1-15 or 18 days		*Nicotiana tabacam Tradescantia*	No mutation at color locus	Gichner et al. (1992)
0.1-3.5	196-6,860	6 h	Culture	*S. typhimurium* TA100, TA102, or TA104	No mutation with or without metabolic activation	Victorin and Stahlberg (1988a)
0.5 1.0	980-1,960	6 h	Culture 1% vinyl chloride	*S. typhimurium* TA100	170% increase in mutation; statistical analysis not conducted	Victorin and Stahlberg (1988b)
1.0	1,960	6 h	Culture 0.1 or 1.0% butadiene	*S. typhimurium* TA100	170% increase in mutation; statistical analysis not conducted	Victorin and Stahlberg (1988b)
50	98,000	1-20 min	Culture	*E. coli*	Exposure-time-dependent mutation to streptomycin resistance, up to 35-fold increases in mutation frequency	L'Herault and Chung (1984)
50	98,000	30-90 min	Culture	*Saccharomyces cerevisiae*	Forward mutations, reversions, gene conversion, mitotic crossing over; no statistical analysis conducted	Dubeau and Chung (1982)

(3) an increase in gene conversions by two- to threefold, and (4) an increase in mitotic crossover by 1.3-fold. No statistical analysis was performed on these data, nor were concentration-response curves generated by the authors. These authors, therefore, demonstrated that O_3 was a mutagen and a recombination-inducing agent in *S. cerevisiae*. However, they also showed that its genotoxic activity was weak (20- to 200-fold less activity in terms of the frequency of mutants or recombinants induced) compared to the known mutagens ultraviolet light, X rays, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).

In this section, the inclusion of data on bacterial mutation utilizing exposures up to 50 ppm O_3 can be justified in order to help determine whether O_3 is genotoxic at all. The available information shows that O_3 is not mutagenic in four *Salmonella* tester strains and may

cause weak mutagenicity in *Salmonella* strain TA102, but these positive results are weakened by the lack of a concentration-response effect. The data also show O_3 is mutagenic in *E. coli*, is weakly mutagenic in *S. cerevisiae*, but is not mutagenic in *N. tabacam* or *Tradescantia*. Hence, because O_3 is mutagenic in three assays, but not in six others, and is weakly mutagenic in assays where the results are positive compared to known strong mutagens, O_3 should be considered a weak mutagen, at most (also reviewed in Victorin, 1992).

6.2.6.4 Induction of Cytogenetic Damage by Ozone

A number of investigators have studied whether O_3 induces cytogenetic damage. The previous air quality criteria document (U.S. Environmental Protection Agency, 1986) described a number of in vitro and in vivo studies in which O_3 exposure produced cytotoxic effects on cells and cellular components, including genetic material; very few newer studies have been reported. Cytogenetic and mutational effects of O_3 have been reported previously in isolated cultured cell lines, human lymphocytes, and microorganisms (Fetner, 1962; Hamelin et al., 1977a,b; Hamelin and Chung, 1975a,b; Scott and Lesher, 1963; Erdman and Hernandez, 1982; Guerrero et al., 1979; Dubeau and Chung, 1979, 1982). One of the earliest studies by Fetner (1962) demonstrated that in vitro exposure of human KB cells to 8 ppm O_3 for 5 and 10 min induced two- and sixfold increases in the number of chromatid deletions. Shiraishi et al. (1986) found that treatment of Chinese hamster V79 cells with 0.1 to 1.0 ppm O_3 inhibited growth of V79 cells by 10 to 70% and also induced a concentration-dependent increase in the number of sister chromatic exchanges (SCEs) per cell, up to a maximum of fourfold of that in untreated, control cells. The results indicate that if cells in culture are exposed to sufficiently high concentrations of O_3 for sufficiently long periods, chromosome damage will result.

In vivo exposure studies are of greater potential interest. Cytogenetic and mutational effects of O_3 in laboratory animals and humans are controversial. Lymphocytes isolated from animals exposed to O_3 were found to have significant increases in the numbers of chromosome (Zelac et al., 1971a,b) and chromatid (Tice et al., 1978) aberrations, after 4- to 5-h exposures to 0.2 and 0.43 ppm O_3, respectively. Single-strand breaks in DNA of mouse peritoneal exudate cells were measurable after a 24-h exposure to 1 ppm O_3 (Chaney, 1981). Gooch et al. (1976) analyzed the bone marrow samples from Chinese hamsters exposed to 0.23 ppm O_3 for 5 h and the leukocytes and spermatocytes from mice exposed for up to 2 weeks to 0.21 ppm O_3. No effect was found on the frequency of chromosome aberrations, nor were there any reciprocal translocations in the primary spermatocytes. These authors did show that there was a slight, but significant increase in the frequency of chromatid aberrations in human peripheral leukocytes exposed in vitro to 7.2 and 7.9 ppm O_3. The small increases observed in chromatid abnormalities in peripheral blood lymphocytes from humans exposed to 0.5 ppm O_3 for 6 to 10 h were not significant, possibly because of the small number ($n = 6$) of subjects studied (Merz et al., 1975). Subsequent investigations with improved experimental design and more human subjects, however, did not show cytogenetic effects after exposure to O_3 at various concentrations and for various times (McKenzie et al., 1977; McKenzie, 1982; Guerrero et al., 1979). Guerrero et al. (1979) showed no elevation in the frequency of SCEs in circulating lymphocytes of humans exposed to 0.5 ppm of O_3 for 2 h. However, these authors did find that exposure of diploid human fetal lung (WI38) cells to 0.25, 0.50, 0.75, and 1.0 ppm O_3 for 1 h in vitro led to a concentration-dependent increase in SCEs in these cells. In addition, epidemiological studies have not shown any evidence of chromosome

changes in peripheral lymphocytes of humans exposed to O_3 in the ambient environment (Scott and Burkart, 1978; Magie et al., 1982). Evidence now available, therefore, fails to demonstrate any cytogenetic or mutagenic effects of O_3 in humans when the exposure regimens are representative of exposures that the population might actually experience.

Finally, a study conducted by Erdman and Hernandez (1982) showed that treatment of *Drosophila virilis* with 30 ppm O_3 for 2 to 6 h resulted in an exposure time-dependent accrual of dominant lethals.

Therefore, O_3 does induce chromosomal aberrations in cultured cells, but the results in animals exposed to O_3 for chromosomal breakage are, at most, weak and their biological significance is controversial.

6.2.6.5 Induction of Morphological Cell Transformation by Ozone

Ozone has been studied in a number of mammalian cell culture systems to determine whether it can induce cell transformation (Table 6-17). The cell transformation assay in C3H/10T1/2 (10T1/2) mouse embryo cells is a standard assay that has been used by many investigators to detect cell tranformation activity as a potential indicator of carcinogenicity and to study molecular mechanisms of cell transformation induced by organic chemicals, carcinogenic metals, and radiation (reviewed in Landolph, 1985, 1989, 1990, 1994). Syrian hamster embryo (SHE) cells also are widely used to detect cell transformation by many classes of chemical carcinogens and radiation (Borek et al., 1986, 1989a,b). Borek et al. (1986) demonstrated that exposure of SHE cells and 10T1/2 mouse embryo cells to 5 ppm O_3 for 5 min inducted morphological transformation in both cell types. Also, in both cell types, there was a synergistic induction of morphological transformation when the cells were treated with 3 Grays of gamma radiation and 5 ppm O_3. These authors therefore concluded that O_3 acts as a direct cell transforming agent and as a co-cell transforming agent in the presence of gamma radiation. Borek et al. (1989a) also observed an additive amount of transformation when these cell types were treated with 6 ppm O_3 and 4 J/m^2 of ultraviolet light. A further study by Borek et al. (1989b) showed that exposure of 10T1/2 mouse embryo cells to 1 ppm O_3 for 5 min did not result in morphological transformation, but that increasing exposure to 5 ppm increased the transformation frequency by a factor of 15. Ozone and gamma radiation caused a synergistic increase in morphological transformation when O_3 was added to cells after the gamma radiation. When O_3 was added to cells before the gamma radiation, the transformation was not increased over that due to gamma radiation. These authors also showed by DNA transfection experiments that three O_3-induced transformed cell lines possessed dominantly acting transforming genes. In these studies, cells were incubated in phosphate-buffered saline during O_3 treatment and, hence, likely were exposed to reaction products of O_3 rather than O_3 itself.

Thomassen et al. (1991, 1992) exposed rats by inhalation to 0.14, 0.6, or 1.2 ppm O_3 for 6 h/day, 5 days/week, for a total of 1, 2, or 4 weeks. There was no increase in the frequency of preneoplastic transformation in cells removed from the tracheas and subsequently cultured. Cells incubated in serum-free medium and exposed to 0.7 or 10 ppm O_3 for 40 min in vitro also did not show an increased frequency of preneoplastic transformation. When rat tracheal epithelial cells were exposed in vitro to 0.7 ppm O_3,

Table 6-17. Effects of Ozone on Morphological Cell Transformation[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain/Cells) Age[b]	Observed Effects	Reference
ppm	g/m^3				
0.14, 0.6, 1.2	274, 1,176, 2,352	6 h/day, 5 days/week for 1, 2, or 4 weeks	Rat, M (F344/N) 7-9 weeks old	No induction of preneoplastic variants in cultured tracheal epithelial cells	Thomassen et al. (1991)
0.7	1,372	40 min twice weekly for 5 weeks (in vitro)	Rat (Tracheal epithelial cells)	Twofold increase in frequency of preneoplastic variants; additive effects with MNNG	Thomassen et al. (1991)
0.7 10	1,372 19,600	40 min (in vitro)	Rat (Tracheal epithelial cells)	No induction of preneoplastic variants	Thomassen et al. (1991)
1.0 5.0	1,960 9,800	5 min (in vitro)	Mouse (C3H/10T1/2 embryo cells)	At 1.0 ppm: No morphological transformation alone; increased transformation by 0.4-Gray radiation by 1.7-fold like a co-carcinogen. At 5.0 ppm: 15-fold increase in morphological transformation and synergism with 4-Gray gamma radiation transformation.	Borek et al. (1989b)
5.0	9,800	5 min (in vitro)	Hamster (Primary diploid cells) Mouse (C3H/10T1/2 embryo cells)	In both cell lines, induction of morphological transformation and synergism with gamma rays	Borek et al. (1986)
6.0	11,760	10 min (in vitro)	Hamster (Syrian primary embryo cells) Mouse (C3H/10T1/2 embryo cells)	In both cell lines, induction of morphological transformation; additive transformation with UV light	Borek et al. (1989a)

[a]See Appendix A for abbreviations and acronyms.
[b]Age at start of exposure.

two times a week for 5 weeks, there was approximately a twofold increase in the frequency of preneoplastic variants detected. These authors also showed that treatment of rat tracheal epithelial cells with MNNG followed by exposure of cells to 0.7 ppm O_3 twice weekly for 5 weeks resulted in an approximately additive increase in the frequency of preneoplastic variants of the cells. The results of these studies should be interpreted cautiously because the changes are very small and because O_3 exposure followed by MNNG treatment yielded negative results (Thomassen, 1992). In addition, it is likely that the culture conditions may significantly affect these results, particularly the volume of culture medium above the cells,

and this variable has not been explored in sufficient detail. Further, the results of these experiments are somewhat variable. Recently, a system has been developed to culture human tracheobronchial epithelial cells and expose them to consistent and reproducible levels of O_3 (Tarkington et al., 1994).

In all these cell transformation experiments, the reactivity of O_3 makes it likely that secondary reaction products of O_3 formed in the aqueous medium, not O_3 itself, induced the cell transformation. Therefore, O_3 is able to induce morphological transformation in C3H/10T1/2 mouse embryo cells and in SHE cells at high concentrations (1.0, 5.0, and 6.0 ppm) but causes no significant effects in rat tracheal epithelial cells in vitro or in vivo.

6.2.6.6 Possible Direct Carcinogenic, Co-carcinogenic, and Tumor-Promoting Effects of Ozone as Studied in Whole Animal Carcinogenesis Bioassays

To investigate whether O_3 has carcinogenic, co-carcinogenic, or tumor-promoting effects, a number of investigators have conducted in vivo carcinogenesis bioassays with O_3 (Table 6-18). Some of the studies have used strain A mice (reviewed in Mustafa, 1990). The advantages and disadvantages in using strain A mice as a general screen for carcinogens by the ip route have been discussed in the literature (Stoner and Shimkin, 1985; Maronpot et al., 1986; Stoner, 1991; Maronpot, 1991). Strain A mice only rarely have been used in inhalation carcinogenesis assays. In addition, the A/J strain of mice has a high spontaneous incidence of benign pulmonary tumors (adenomas). This strain of mice has been shown to be very sensitive to tumor induction by polycyclic aromatic hydrocarbons (PAHs), carbamates, and aziridines and insensitive to aromatic amines, metal salts, and halogenated organic compounds administered by the ip route (Maronpot et al., 1986). In addition, carcinogenicity results in strain A mice did not correlate well with 2-year mouse and rat carcinogenicity results when the results of chemical testing in strain A/St mice (59 chemicals tested) were compared with strain A/J mice (30 chemicals tested) in a 2-year chronic bioassay (Maronpot et al., 1986). The chemicals chosen were heavily weighted with aromatic amines. The author concluded that "carcinogenicity test data are relevant only to the test model employed since there is no absolute reference for carcinogenicity." Maronpot (1991) also demonstrated a poor concordance between results of testing chemicals in the strain A assay and testing them in 2-year rat and mouse carcinogenicity assays at the National Cancer Institute (NCI). Stoner (1991), using the strain A mouse pulmonary assay, indicated that ip injection of PAHs, nitrosamines, nitrosureas, carbamates, aflatoxin, metals, and hydrazines induces tumors, but that the assay is not responsive to aromatic amines, aliphatic halides, and certain compounds carcinogenic in rodent liver or bladder. In this assay, an increase in lung tumor multiplicity (average number of lung tumors per mouse) caused by a chemical is considered as evidence for the carcinogenicity of a chemical.

Hassett et al. (1985) used inbred strain A/J mice, which are very sensitive to induction of pulmonary adenomas by chemical carcinogens. Exposure of A/J mice to 0.31 ppm O_3 for 103 h per week, every other week, for 6 mo, resulted in a 1.3-fold increase (not statistically significant) in the percent of mice with tumors (tumor incidence) and a statistically significant 1.4-fold increase in the number of tumors per mouse (tumor multiplicity). In this experiment, O_3 did not promote the carcinogenicity of urethane when

Table 6-18. Summary of Results on the Possible Carcinogenicity of Ozone[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effects	Reference
ppm	g/m³				
0.05 (sine wave from 0 to 0.1)	98 (sine wave from 0 to 96)	10 h/day for 13 mo	Rat, M (Wistar) 4 weeks old	Lung tumor response increased from 0% in BHPN- or O_3-treated animals, to 8.3% in animals treated with 0.5 g/kg BHPN + 0.05 ppm O_3 (not significant).	Ichinose and Sagai (1992)
0.31	608	103 h/week, every other week for 6 mo	Mouse, F (A/J) 7 weeks old	1.33-fold increase in percent of mice with adenomas, 1.42-fold increase in number of tumors per mouse. No promotion of carcinogenicity of urethane (2 mg/mouse before O_3).	Hassett et al. (1985)
0.5	980	102 h/first week of each month for 6 mo	Mouse, F (A/J) 7 weeks old	2.11-fold increase in percent of mice with tumors, 3.42-fold increase in number of tumors per mouse, interaction between O_3 and urethane (2 mg/mouse after each O_3 week).	Hassett et al. (1985)
0.4 0.8	784 1,568	8 h/day, 7 days/week for 18 weeks	Mouse, M (Swiss Webster and A/J) 8 weeks old	Urethane treatment before O_3 started. In Swiss Webster mice: No increase in lung tumor incidence; nonsignificant decrease in tumors per lung in urethane-treated animals. In A/J strain: No effect at 0.4 ppm. At 0.8 ppm: Threefold increase in percent mice with tumors, and 4.2-fold increase in number of tumors per mouse. Both 0.4 and 0.8 ppm O_3 decreased yield of tumors per mouse in urethane-treated mice, but had no effect on tumor incidence.	Last et al. (1987)
0.8	1,568	23 h/day 7 days/week for 6 mo	Hamster, M (Syrian Golden) 7-11 weeks old	No tumors observed in animals treated with 0.8 ppm O_3 only. In animals treated with 20 mg/kg DEN sc twice/week, 0.8 ppm O_3 did not increase tumors of lung, bronchus, trachea, or nasal cavity. Tumors of lung were decreased 50% (N.S.)	Witschi et al. (1993a,b)
0.12 0.5 1.0		6 h/day 5 days/week for 104 weeks (2 years)	Rat, M, F (F344/N)	No increase in neoplasms at any concentration tested.	National Toxicology Program (1994) Boorman et al. (1994)
0.5 1.0		6 h/day 5 days/week for 124 weeks (lifetime)	Rat, M, F (F344/N)	No increase in neoplasms at any concentration tested.	National Toxicology Program (1994) Boorman et al. (1994)
0.12 0.5 1.0		6 h/day 5 days/week 105 weeks (2 years)	Mouse, M, F ($B6C3F_1$)	No effects in males. In females: Increase in number of mice with neoplasms at 1.0 ppm (combined alveolar/bronchiolar adenoma or carcinoma in lung).	National Toxicology Program (1994)
0.5 1.0		6 h/day 5 days/week 130 weeks (lifetime)	Mouse, M, F ($B6C3F_1$)	In males: Increase in number of mice with carcinoma at 0.5 and 1.0 ppm, but not significant for change in number of mice with total neoplasms. In females: increase in number of mice with adenomas, but not carcinomas or total neoplasms.	National Toxicology Program (1994)

[a]See Appendix A for abbreviations and acronyms.
[b]Age at start of exposure.

O_3 exposure began 1 week after a single injection of animals with a total dose of 2 mg urethane/mouse. In a second experiment, exposure to 0.50 ppm O_3 (102 h during the first week of every month for 6 mo) caused a nonsignificant 2.1-fold increase in tumor incidence and a 3.2-fold increase in tumor multiplicity (statistics not shown) (Hassett et al., 1985).

These authors reported that exposure to 0.5 ppm O_3, followed by urethane treatment (2 mg after each O_3 exposure set), resulted in an interaction between O_3 and urethane such that there were more animals with more than 16 lung tumors each. These authors concluded that exposure to 0.31 and 0.5 ppm O_3 increased the yield of pulmonary adenomas in A/J mice and that O_3 interacted with urethane to produce more lung tumors than urethane alone when O_3 was added before urethane.

The pulmonary adenomas induced in animals by chemical carcinogens are by definition benign tumors (Stoner, 1991; Maronpot, 1991); however, they do represent abnormal cell growth in the form of tumors and, hence, are significant biologically in that they are early steps in the pathway toward malignancy. It is not known if these tumors can progress to malignant tumors, because they are not as amenable to observation as are mouse skin tumors, where adenomas can be converted into adenocarcinomas at a frequency of approximately 8 to 10%. Similarly, it is not known with certainty whether pulmonary adenomas in humans progress to adenocarcinomas. It is clear, however, that lung tumors in mice are not equivalent to bronchogenic carcinomas in humans. However, because a shift is occurring, in that fewer squamous cell bronchogenic carcinomas and more peripheral adenocarcinomas are occurring in humans, the induction of peripheral adenomas and their progression to peripheral adenocarcinomas in mice may be a useful area for further mechanistic insight.

The study of Hassett et al. (1985) was reviewed extensively by scientists from EPA and the National Institutes of Environmental Health Sciences in 1985 and 1986 (Tilton, 1986). The consensus of these extensive reviews was that (1) the tumor yields in O_3-exposed mice were not statistically significantly different from the control animals, (2) any effects were marginally different from control values, and (3) the strain A mouse has a high spontaneous incidence of tumors, making it difficult to interpret the effects of O_3. Chemical induction of tumors in this assay system did not correlate well with the 2-year NCI carcinogenesis bioassay results. In addition, because Hassett et al. (1985) did not demonstrate a concentration-response effect in animals exposed to O_3, the consensus among the reviewers was that one could not conclude from these experiments that O_3 was a significant carcinogen or tumor promoter, and that rigorous inhalation carcinogenesis bioassays needed to be carried out with O_3-exposed animals to address this issue properly.

Last et al. (1987) also studied whether O_3 exposure could influence the yield of urethane-induced lung tumors in A/J and Swiss-Webster mice. Urethane treatment consisted of a single ip injection (1,000 mg/kg) 1 day before O_3 exposure began. In Swiss Webster mice, exposure to 0.4 or 0.8 ppm O_3 alone not only did not increase the tumor yield but actually decreased the yield of urethane-induced lung tumors per mouse, although the decrease was not statistically significant. In A/J mice, exposure to 0.4 ppm O_3 did not increase the lung tumor yield, but exposure to 0.8 ppm O_3 caused a threefold increase in tumor incidence and a 4.2-fold increase in lung tumor multiplicity. Exposure of urethane-treated mice to 0.4 or 0.8 ppm O_3 decreased lung tumor multiplicity but had no effect on tumor incidence. These differences in the strain A mouse were significant. The authors concluded that O_3 was not a tumor promoter or tumor-enhancing agent.

Ichinose and Sagai (1992) studied the ability of O_3 to interact with *N-bis*(2-hydroxypropyl) nitrosamine (BHPN) in the induction of lung tumors in Wistar rats. A single ip injection of BHPN (0.5 g/kg) did not cause any tumors in the rats. Rats were exposed for 10 h/day for 13 mo to a pattern of O_3 consisting of a sine curve from 0 to

0.1 ppm, with a mean concentration of 0.05 ppm. The 13-mo O_3 exposure started the day after BHPN injection, and the rats were examined 11 mo postexposure. No tumors were observed in the O_3-alone or control groups. When rats were exposed to 0.5 g/kg BHPN plus 0.05 ppm O_3, the lung tumor incidence increased to 8.3% (3/36), but this increase was not statistically significant. The tumors observed in this study cannot be stated definitively to have been induced by the treatment agent.

The data available on O_3 exposure and lung carcinogenesis up until 1988 have been reviewed by Witschi (1988). The chemical reactivity of O_3 and, in particular, its radiomimetic activity (ability to mimic radiation effects, such as causing cell-cycle arrest, chromosome breakage, etc.) also make O_3 a potential risk factor for human lung cancer. Nevertheless, as of 1988, there were no experimental studies conclusively linking lifelong exposure to O_3 with lung tumor induction in any animal species, nor was there conclusive epidemiological evidence to associate O_3 exposure with the development of lung cancer in humans. As of 1988, the only data implicating O_3 as a possible tumorigenic agent were from studies carried out in mice, where the tumors are adenomas derived from Type 2 alveolar cells or from Clara cells (reviewed in Witschi, 1988). In the A and Swiss-Webster mouse strains used to assay the carcinogenicity of O_3, the spontaneous incidence of lung tumors is very high. Hence, results of carcinogenicity experiments conducted on O_3 to date that utilize tumor incidence as an endpoint are not strongly positive, due to this high background. In strain A mice, in which the spontaneous multiplicity is usually less than one tumor per mouse, the tumor multiplicity is considered by many investigators to be a sensitive indicator of a carcinogenic effect. However, even using this indicator, the increase in tumor multiplicity after O_3 exposure is small, raising questions about the biological significance of the effects. In addition, the assay for inhalation carcinogenesis in strain A mice was not fully validated at this time. Whereas Hassett et al. (1985) concluded that O_3 increased the number of pulmonary adenomas in strain A mice, Witschi (1988) concluded that O_3 was not implicated unequivocally as a carcinogen in strain A/J mice, that no classical carcinogen bioassays had been conducted on O_3, and that a definitive judgment could not be made on the carcinogenicity of O_3.

A review (Witschi, 1991) of the available data on the carcinogenicity of O_3 and oxygen in mouse lungs indicated that oxidants can enhance or inhibit mouse lung tumorigenesis, depending on the experimental protocol employed, and the carcinogenicity of O_3 in mouse lung had not been established unequivocally. Exposure of strain A mice to O_3 induces hyperplasia of Type 2 alveolar cells, leading to expansion of the target cell population. It was speculated that this might have resulted in spontaneous transformation of these cells (Witschi, 1991). In lungs of animals treated with a carcinogen such as urethane and then exposed to O_3 before or after carcinogen administration, it was speculated that O_3 may cause cell proliferation and result in fixation of DNA damage (Witschi, 1991). The addition of O_3 after carcinogen exposure leads to a decreased tumor incidence compared to treatment with carcinogen alone; the reasons for this decrease with late O_3 exposure are not clear.

The effects of treating male Syrian Golden hamsters with dimethylnitrosamine (DEN) (20 mg/kg given subcutaneously twice per week) during the course of a 6-mo exposure to 0.8 ppm O_3 were studied by Witschi et al. (1993a; see Table 6-18). The rationale for this study was to test the hypothesis that O_3 acts in a manner similar to hyperoxia in enhancing neuroendocrine lung tumors in this animal model. After exposure ceased, the animals were maintained in air for 1 mo. Ozone exposure did not increase the incidence of lung, bronchus, trachea, or nasal cavity tumors in the DEN-treated hamsters. There was a 50% decrease in the

percent of animals with lung tumors in the DEN-plus-O_3-exposed animals compared to the DEN-plus-air-exposed animals, but this was not statistically significant. Ozone did not affect the incidence of DEN-induced liver tumors. The authors concluded that O_3 did not increase the number of DEN-induced respiratory tumors in hamsters and that O_3 exposure might have inhibited or delayed tumor development. Although reduction in tumor incidence caused by O_3 was not significant in this study alone, overall analysis of these data with other data from a 4-mo exposure of similar design (Witschi et al., 1993b) yielded significant results.

A definitive study of the carcinogenicity of O_3 and its ability to act as a co-carcinogen or tumor promoter was conducted by the U. S. National Toxicology Program (NTP) (National Toxicology Program, 1994; Boorman et al., 1994). Animals were exposed to air or O_3 for 6 h/day, 5 days/week for the number of weeks described below. Male and female F344/N rats were exposed to 0.12, 0.5, and 1.0 ppm O_3 for 104 weeks and to 0.5 and 1.0 ppm for 124 weeks ("lifetime" of the animals). Similar protocols were used for $B6C3F_1$ male and female mice, with the exception that the "2-year" study was 105 weeks and the lifetime study was 130 weeks. This study did not find any evidence of carcinogenic activity in male or female rats. There was a negative trend for mammary gland neoplasms in the female rats in the 2-year study, an effect that was not seen in the lifetime study. The NTP found "equivocal evidence"[1] of carcinogenic activity in O_3-exposed male mice and "some evidence" of carcinogenic activity in female mice exposed to O_3. In co-carcinogenesis experiments, male rats were treated with a known pulmonary carcinogen, 4-(*N*-methyl-*N*-nitrosomino)-1-(3-pyridyl)-1-butanone (NNK) (0.1 and 1.0 mg/kg, subcutaneous injection 3 times a week for first 20 weeks) and exposed to 0.5 ppm O_3 for 6 h/day, 5 days/week for 105 weeks. The NTP found "no evidence" that O_3 enhanced the incidence of NNK-induced pulmonary neoplasms. Table 6-19 shows the tumor incidences in mice. In the discussion to follow, all tumors described were at lung alveolar/bronchiolar sites. There was a decrease in the number of hepatocellular adenomas or carcinomas in female mice exposed to 1.0 ppm O_3 for 2 years and for hepatocellular carcinomas in the lifetime study. There was no statistically significant increase in tumors at any site other than the lung.

In male mice exposed to O_3 for 2 years, there were no statistically significant increases in the mice with alveolar/bronchiolar carcinomas or a combination of adenomas or carcinomas; at 0.5 ppm O_3, there was a small, twofold increase in the incidence of adenomas. In the lifetime studies of male mice, the incidence of mice with carcinomas increased 1.9-fold at 0.5 ppm O_3 and 2.3-fold at 1.0 ppm. The incidence of adenomas in

[1]The NTP evaluates the strength of the evidence for conclusions regarding each carcinogenicity study, under the conditions of that particular study. There are five categories: two for positive results ("clear evidence" and "some evidence"), one for uncertain findings ("equivocal evidence"), one for no observable effects ("no evidence"), and one for experiments that cannot be judged because of major flaws ("inadequate study") (National Toxicology Program, 1994). This approach is very different from the weight-of-evidence approach used by EPA for cancer classification because the EPA approach considers all the available studies.

Table 6-19. Alveolar/Bronchiolar Tumor Incidence in B6C3F$_1$ Mice in the National Toxicology Program's Chronic Ozone Study

	Males			Females		
ppm	Adenomas[a]	Carcinomas[a]	Both[a]	Adenomas[a]	Carcinomas[a]	Both[a]
2-year exposure						
0.0						
0.12	6/50	8/50	14/50	4/50	2/50	6/50
0.5	9/50 (p = 0.3)	4/50 (p = 0.15)	13/50 (p = 0.44)	5/50 (p = 0.55)	2/50 (p = 0.65)[b]	7/50 (p = 0.57)
1.0	12/50 (p = 0.06)	8/50 (p = 0.45)	18/50 (p =0.12)	5/49 (p = 0.52)	5/49 (p = 0.26)	9/49 (p = 0.33)
	11/50 (p = 0.11)	10/50 (p = 0.27)	19/50 (p = 0.10)	8/50 (p = 0.24)	8/50 (p = 0.053)	16/50 (p = 0.02)
Lifetime exposure						
0.0						
0.5	8/49	8/49	16/49	3/50	3/50	6/50
1.0	8/49 (p = 0.61)[b]	15/49 (p = 0.05)	22/49 (p = 0.14)	3/49 (p = 0.63)	5/49 (p = 0.33)	8/49 (p = 0.34)
	9/50 (p = 0.47)	18/50 (p = 0.007)	21/50 (p = 0.15)	11/50 (p = 0.02)	2/50 (p = 0.50)[b]	12/50 (p = 0.10)
Combined						
0.0	14/99	16/99	30/99	7/100	5/100	12/100
0.5	20/99 (p = 0.16)	23/99 (p = 0.08)	40/99 (p = 0.06)	8/98 (p = 0.48)	10/98 (p = 0.14)	17/98 (p = 0.20)
1.0	20/100 (p = 0.14)	28/100 (p = 0.009)	40/100 (p = 0.04)	19/100 (p = 0.01)	10/100 (p = 0.14)	28/100 (p = 0.004)

[a]Number of animals with neoplasm/number of animals necropsied (p [probability] value, logistic regression test).
[b]Lower incidence.

Source: National Toxicology Program (1994).

male mice did not change significantly. In female mice exposed for 2 years, there were no statistically significant changes at 0.12 or 0.5 ppm O_3. However, at 1.0 ppm O_3, there was a fourfold increase in the frequency of female mice with carcinoma and a 2.7-fold increase in combined adenomas plus carcinomas. In female mice exposed for their lifetimes to O_3, 0.5 ppm caused no significant effects. At 1.0 ppm O_3, there was a 3.7-fold increase in the incidence of mice bearing pulmonary adenomas, a nonsignificant change in the frequency of mice with carcinomas, and a twofold ($p = 0.1$) increase in the incidence of combined adenomas and carcinomas.

When the results of the 2-year and lifetime O_3 carcinogenesis studies were combined and analyzed, for male mice, there was no statistically significant increase in the incidence of animals bearing adenomas, and, for carcinomas, there was a marginally significant increase at 0.5 ppm O_3 (1.4-fold increase, $p = 0.08$) and a significant, 1.7-fold increase at 1.0 ppm. The incidence of male mice bearing adenomas or carcinomas showed a marginally significant increase (1.3-fold, $p = 0.06$) at 0.5 ppm O_3 and a 1.3-fold increase at 1.0 ppm ($p = 0.045$). In the combined analysis of the 2-year and lifetime exposure of female mice, there were no statistically significant changes at 0.5 ppm O_3. At 1.0 ppm O_3, there was a 2.7-fold increase in the percent of mice bearing adenomas and a 2.3-fold increase in the frequency of mice with adenomas or carcinomas. There was no statistically significant increase in the carcinoma incidence.

The overall conclusions of the authors of the NTP O_3 inhalation carcinogenesis study were (1) there was no increased pulmonary tumor incidence in male or female F344/N rats exposed to 0.12, 0.5, or 1.0 ppm O_3; (2) male F344/N rats treated with the tobacco carcinogen NNK and exposed to 0.5 ppm O_3 did not have an increase in the pulmonary tumor incidence above that caused by NNK alone; (3) O_3 caused a slightly increased incidence of alveolar/bronchiolar adenoma or carcinoma that yielded equivocal evidence of carcinogenicity

of O_3 in male $B6C3F_1$ mice; and (4) O_3 increased the incidence of alveolar/bronchiolar adenoma or carcinoma in female $B6C3F_1$ mice, yielding some evidence of carcinogenic activity of O_3 in female mice.

Generally, in mice, adenomas appear to progress into carcinomas with time, and, thus, the incidence of mice having both adenomas and carcinomas is probably the more useful indicator of effects. The incidence of tumor-bearing mice was elevated significantly only in female mice exposed for 2 years to 1.0 ppm O_3. When both the 2-year and lifetime exposure studies were combined, there was a increased incidence of tumors in the males at 0.5 and 1.0 ppm O_3 and in females at 1.0 ppm. The NTP designated the data for male mice as equivocal for carcinogenesis because the combined tumor incidence in the 2-year study was within the historical range, and the combined incidence for the lifetime study was not significant, even though the carcinoma incidence was significant in the lifetime study. The evaluation of female mice resulted in NTP's finding of "some evidence of carcinogenic activity" because the combined pulmonary adenoma/carcinoma incidence was significantly increased and outside the range of the historical control tumor rates. When the lifetime and 2-year studies were combined, there were 28/100 adenomas plus carcinomas in the 1.0-ppm O_3 exposure group versus 12/100 in the controls ($p = 0.004$).

In summary, the strongest data on carcinogenicity come from the NTP study, which was ambiguous in male mice and positive only in female mice at high concentrations of O_3 (i.e., 1.0 ppm). This may represent a toxic or irritant effect, giving a nonspecific type of tumor due to mitogenesis. The carcinogenicity data are weak or equivocal in male mice, negative in F344/N male and female rats, and negative for co-carcinogenesis in male rats. Therefore, the potential for animal carcinogenicity is uncertain at the present time.

6.2.6.7 Possible Effects of Ozone on Injected Tumor Cells That Lodge in the Lung and Form Lung Colonies

To date, no rigorous studies have been conducted to examine the effects of O_3 on true lung tumors that would have metastasized. No studies have been conducted in which lung tumor cells detach themselves from primary lung or other tumors growing in organs and invade adjacent tissue, blood vessels, or lymphatics. A few studies have been conducted in which tumor cells are injected intravenously into animals and then lodge in the lung, forming lung colonies (Table 6-20). It must be stressed, however, that this experimental model is not an adequate model for lung tumor cell metastasis.

Kobayashi et al. (1987) showed that exposure of C3H/He mice for 1 or 14 (but not other) days to 0.1 ppm O_3 after mice were injected in the tail vein with the fibrosarcoma cell line (NR-FS) increased the number of metastatic lung tumors. Animals were exposed to O_3 for 14 days, then fibrosarcoma cells were injected into the tail vein of the animals, and pulmonary metastases were scored 14 days later. One day of exposure to 0.8 ppm O_3 gave the maximal enhancement of pulmonary metastases. This enhancement of pulmonary metastasis was concentration-dependent, in the range from 0.4 to 0.8 ppm O_3 from 1 to 14 days, but increases were small. This effect may arise in two ways: (1) by damage to the microvasculature and (2) by the differential sensitivity of various tumor cells to O_3 cytotoxicity (reviewed by Witschi, 1988). Richters (1988) reported that exposure of mice to 0.15 or 0.30 ppm O_3 for 60 days did not increase colonization of the lungs of mice injected iv with B16 melanoma cells.

Table 6-20. Effects of Inhaled Ozone on the Ability of Injected Tumor Cells To Colonize the Lungs of Mice[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effects	Reference
ppm	g/m^3				
0.15 0.3	294 588	60 days	Mouse, M (C57B/6J) 5 weeks old	No increase in lung metastases from iv-injected B16 melanoma cells.	Richters (1988)
0.1 0.2 0.4 0.8	196 392 784 1,568	1-14 days	Mouse, M (C3H/He) 8-12 weeks old	Pulmonary metastases from iv-injected NR-FS fibrosarcoma cells. After 1 and 14 days of 0.1 ppm, 1.3-fold increases. After 5 and 7 days of 0.2 ppm, 1.3 and 2.3-fold increases. After 1 and 5 days of 0.4 ppm, 2.3- and 2.2-fold increases. After 1 day of 0.8 ppm, 4.6-fold increase.	Kobayashi et al. (1987)

[a]See Appendix A for abbreviations and acronyms.
[b]Age at start of exposure.

6.2.6.8 Summary and Conclusions

In summary, there are some weakly positive data and some negative data on the genotoxicity of O_3 (summarized in Table 6-21). Ozone at very high concentrations (5 to 20 ppm) causes DNA strand breakage in plasmid DNA (Hamelin, 1985). Ozone is, at most, weakly mutagenic in some assays and negative in others. Ozone is not mutagenic in *Tradescantia* or *N. tabacam* at concentrations of 0.1 to 0.3 ppm (Gichner et al., 1992); is weakly mutagenic in *E. coli* at 50 ppm, and *S. cerevisiae* at 50 ppm (L'Herault and Chung, 1984; Dubeau and Chung, 1982); and is nonmutagenic in three strains of *Salmonella* and, at most, marginally mutagenic in *Salmonella* strain TA102 at concentrations of 0.024, 0.039, and 0.39 ppm (Victorin and Stahlberg, 1988a,b; Dillon et al., 1992). Despite extensive studies by Dillon et al. (1992), the mutagenicity of O_3 in *Salmonella* TA102 is not conclusive because convincing concentration-dependent mutagenic effects have not yet been demonstrated, possibly due to the strong cytotoxicity of this compound. Ozone causes cytogenetic damage in cultured cells in vitro (e.g., Hamelin et al., 1977a,b; Dubeau and Chung, 1979, 1982), but no effects or small and conflicting effects when animals are exposed in vivo (Zelac et al., 1971a,b; Tice et al., 1978). Cell transformation studies have shown positive results on exposure of cells to O_3, but these studies were conducted with a fluid barrier above the cells that may have resulted in artifacts compared to an in vivo exposure (Borek et al., 1986, 1989a,b).

The in vitro studies are mechanistically interesting, but there are difficulties in the design of many of these studies. First, the concentrations used in these in vitro studies were typically orders of magnitude greater than those found in ambient air. Second, extrapolation of in vitro exposure concentrations to human exposure dose requires special methods that were not used in these studies. Third, direct exposure of isolated cells to O_3 is somewhat

Table 6-21. Summary of Data on the Genotoxicity of Ozone[a]

Assay System in Which Ozone Was Tested	Result[b]	Comments
Mutation to histidine prototrophy in *Salmonella* TA100	−	Small effects obtained, less than twofold; concentration-response effect was not shown at 0.024, 0.039, and 0.39 ppm O_3.
Mutation to histidine prototrophy in *Salmonella* TA102	+/−	Small effects obtained, and there was no direct exposure-response at 0.024, 0.039, and 0.39 ppm O_3.
Mutation to streptomycin resistance in *Eshcherichia coli*	+	Only 50 ppm O_3 was tested.
Mutation in *Saccharomyces cerevisiae*	+	Ozone caused mutation and recombination at 50 ppm, but this was a weak response compared to known strong mutagens (20- to 200-fold less mutagenic than UV light, X rays, and MNNG).
Mutation in *Nicotiana tabacam* in a leaf-color reversion assay	−	0.1 to 0.3 ppm O_3 was tested.
Mutation in *Tradescantia* in a stamen-hair assay	−	0.1 to 0.3 ppm O_3 was tested.
Chromosomal breakage in cultured mammalian cells	+	8 ppm O_3 in human KB cells, and 0.1 to 1.0 ppm O_3 in V79 cells.
Chromosomal breakage in animals	+/−	Results are, at best, weak and controversial; results in this assay are considered ambiguous and not definitively positive at present. Ozone was tested at 0.2, 0.43, 7.3, and 7.9 ppm.
Morphological transformation in C3H/10T1/2 mouse embryo cells and in Syrian hamster embryo cells	+	Experiments need to be conducted without or with only minimal amounts of fluid bathing the cells. Concentrations giving positive results are high (5 and 6 ppm O_3).
Induction of preneoplastic variants in rat tracheal epithelial cells	−	Both in vitro and in vivo exposures give negative or, at most, only twofold increases in cells exposed to 0.14, 0.6, 0.7, 1.2, or 10 ppm O_3.

Table 6-21 (cont'd). Summary of Data on the Genotoxicity of Ozone[a]

Assay System in Which Ozone Was Tested	Result[b]	Comments
Lung tumor induction in whole animals		
(a) Strain A/J mice, Swiss-Webster mice, Syrian Golden hamsters, Wistar rats	+/−	Positive results marginal, not statistically significant; experiments not designed to determine whether a concentration-response exists. Ozone was tested at 0.05, 0.31, 0.4, 0.5, 0.8, and 1.0 ppm.
(b) National Toxicology Program Studies—Male and female F344/N rats	−	No increased incidence of pulmonary adenomas or carcinomas in rats exposed to 0.12, 0.5, or 1.0 ppm O_3 for 2 years or with 0.5 or 1.0 ppm O_3 for animals' lifetimes.
Male B6C3F_1 mice (2-year study)	+/−	No effect at 0.12 ppm O_3, slight increases in the total pulmonary neoplasms at 0.5 and 1.0 ppm O_3, but they were not statistically significant.
Male B6C3F_1 (Lifetime studies)	+/−	Alveolar/bronchiolar carcinoma incidence increased twofold at 0.5 ppm O_3 ($p = 0.05$) and 1.0 ppm O_3 ($p = 0.007$); no increases in total pulmonary neoplasms.
Female B6C3F_1 mice (2-year study)	+	Fourfold increase in alveolar/bronchiolar carcinoma at 1.0 ppm O_3 ($p = 0.053$).
Female B6C3F_1 (Lifetime studies)	+	Fourfold increase in alveolar/bronchiolar adenomas and carcinomas at 1.0 ppm O_3 ($p = 0.02$).
	+	Threefold increase in alveolar/bronchiolar adenomas at 1.0 ppm O_3 ($p = 0.02$).
	+	Twofold increase in bronchiolar/alveolar adenomas and carcinomas at 1.0 ppm O_3, but not statistically significant.

[a]See Appendix A for abbreviations and acronyms.
[b]− = no effect; + = effect.

artifactual because it bypasses all the host defenses that would normally be functioning to protect the individual from the inhaled dose. Direct, in vitro O_3 exposure of isolated cells in tissue culture medium also results in chemical reactions between O_3 and culture media to generate chemical species that may not be produced in vivo. Therefore, for these reasons, the relevance and predictive value of in vitro studies to human health are questionable The most relevant data on the genotoxicity of O_3 should therefore be obtained from in vivo studies.

The earlier studies in whole animal carcinogenesis bioassays must be considered ambiguous at this time (Witschi, 1988, 1991). The NTP study utilized an inhalation model, assayed the carcinogenicity of O_3 in male and female F344/N rats and $B6C3F_1$ mice, and also tested whether O_3 could enhance the tumorigenicity of the tobacco-specific pulmonary carcinogen NNK (National Toxicology Program, 1994; Boorman et al., 1994). This study clearly showed that O_3 was not carcinogenic in female and male rats at 0.12, 0.5, and 1.0 ppm O_3 (6 h/day, 5 days/week for 2 years) or at 0.5 and 1.0 ppm O_3 (6 h/day, 5 days/week, lifetime). Exposure to 0.5 ppm O_3 did not enhance the carcinogenicity of NNK in male rats, leading to the conclusion that O_3 does not act as a co-carcinogen or tumor promoter in these animals. In the male mice, O_3 had equivocal effects at 0.5 and 1.0 ppm O_3 in the 2-year and lifetime inhalation studies. In the female mice, there was some evidence for the carcinogenicity of O_3 at 1.0 ppm (2.7-fold increase in total pulmonary neoplasms [$p = 0.02$] in the 2-year study; and twofold increase in total pulmonary neoplasms [$p = 0.1$] in the lifetime study; and 2.3-fold increase in total pulmonary neoplasms when the 2-year and lifetime study were combined [$p = 0.004$]).

Therefore, the earlier negative animal carcinogenesis studies, the negative carcinogenicity results in inhalation carcinogenesis studies in F344/N male and female rats, the ambiguous data in male $B6C3F_1$ mice, and the weak carcinogenicity of O_3 in female $B6C3F_1$ mice indicate that O_3 is carcinogenic only in female $B6C3F_1$ mice at high concentrations (1.0 ppm). The weak carcinogenicity of O_3 in female mice, the weak/ambiguous results in male mice, and the negative results in male and female F344/N rats point to, at best, a weak carcinogenicity of O_3 at very high concentrations.

6.3 Systemic Effects of Ozone

6.3.1 Introduction

Ozone has long been known to cause effects in organs and tissues outside the respiratory tract. The mechanisms are not known, but it is quite unlikely that O_3 itself enters the circulation (Pryor, 1992). Another possibility is that transported reaction products cause distant effects. Some effects may be secondary to effects on the lung (e.g., aversive behaviors that may result from lung irritation). The relatively few systemic studies reported since the last O_3 criteria document (U.S. Environmental Protection Agency, 1986) are discussed below. Some classes of effects (i.e., reproduction/development, endocrine system) were studied earlier and, hence, are cited briefly here in the introduction.

No reproductive toxicity studies of O_3 were found. Only two developmental studies provided sufficient details in the report to determine the exposures used. The only effect observed by Kavlock et al. (1979) in pregnant rats exposed to 0.44 to 1.97 ppm O_3 for the entire period of organogenesis or the three stages of gestation was an increased resorption of fetuses in rats exposed to 1.49 ppm in midgestation; no terata were found. A follow-up study revealed that pups from dams exposed to 1 ppm O_3 during mid- or late gestation showed lower

body weights 6 days after birth (Kavlock et al., 1980). A higher concentration (1.5 ppm) delivered during late gestation permanently runted 14% of the male pups.

Studies on the effects of O_3 on the endocrine system date to 1959. Generally, the body of work indicates that O_3 can affect the pituitary-thyroid-adrenal axis (U.S. Environmental Protection Agency, 1986). For example, a 1-day exposure to 1 ppm O_3 decreased serum levels of thyroid-stimulating hormone, thyroid hormones, and protein-bound iodine; prolactin levels increased (Clemons and Garcia, 1980a,b). Structural changes occurring in the parathyroid glands after a 4- to 8-h exposure to 0.75 ppm O_3 included hyperplasia of chief cells, but circulating hormone levels were not measured (Atwal and Wilson, 1974).

6.3.2 Central Nervous System and Behavioral Effects

Reports of headache, dizziness, and irritation of the nose, throat, and chest are common complaints that are associated with O_3 exposure in humans (see Chapter 7). Laboratory animal studies have been performed that demonstrate behavioral effects over a wide range of O_3 concentrations (0.08 to 1.0 ppm) and suggest that these behavioral changes may be analogous to the symptoms reported in humans. Although these behavioral changes may be indicative of O_3-induced symptoms, they are not indicative of neurotoxicity. Most of the studies prior to 1986 indicated that behavior could be suppressed with O_3 exposure. For example, Murphy et al. (1964) and Tepper et al. (1982) showed that running-wheel behavior was suppressed, and Peterson and Andrews (1963) and Tepper et al. (1983) showed that mice would alter their behavior to avoid O_3 exposure. Furthermore, Weiss et al. (1981) showed that bar-pressing responses for food reinforcement were suppressed, but greater O_3 concentrations were required to decrease this behavior than the concentrations needed to decrease running-wheel behavior.

Since 1986, several reports have extended the previous findings (Table 6-22). Tepper et al. (1985) compared the effects of a 6-h exposure to O_3 on the suppression of running-wheel behavior in rats and mice. The study indicated that the lowest effective concentration was about 0.12 ppm O_3 in the rat and about 0.2 ppm in the mouse. It also was observed that, with exposure to 0.5 ppm, recovery from O_3 required at least 3 h. In a follow-up study, Tepper et al. (1985) required mice to make a response that turned off the brief delivery (60 s) of O_3 at concentrations between 0.25 to 16 ppm. Mice learned to terminate O_3 exposures at 0.5 ppm. With each of three determinations of the concentration-response curve, mice got better at terminating O_3 exposure, rather than exhibiting an adaptation to exposure. The authors suggest that mice may have learned to use the odor of O_3 as a conditioned stimulus to initiate termination of exposure instead of responding directly to the irritant properties of O_3.

Because free-access wheel-running behavior was suppressed at 0.12 ppm O_3 (Tepper et al., 1982), and lever pressing for food reinforcement was reduced only at 0.5 ppm (Weiss et al., 1981), a series of experiments was performed to evaluate the behavioral determinants of the O_3 response (Tepper and Weiss, 1986). Food deprivation and response contingencies (having to perform a certain response to get a reward) were found to be relatively unimportant determinants of behavior because rats that had to run rather than press a lever to obtain food reinforcement showed behavioral suppression of running at 0.12 ppm. However, in another experiment, suppression of lever pressing was shown to be

Table 6-22. Effects of Ozone on Behavior[a]

Ozone Concentration		Exposure Duration	Behavioral Conditions	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m^3					
0.08 0.12 0.25 0.5	157 980	6 h	Free-access wheel running	Rat, M (Long-Evans) 10 weeks old Mouse, M (Swiss-Webster) 5 weeks old	Mice less responsive than rats. Reduction in free-access wheel running at approximately 0.12 ppm in rats and 0.2 ppm in mice. Recovery from exposure to 0.5 ppm did not occur by 5 h postexposure in either mice or rats.	Tepper et al. (1985)
0.08 0.12 0.25 0.5	157 980	6 h	Wheel running for food	Rat, M (Long-Evans) 300 g	0.12 ppm O_3 decreased wheel running for food reinforcement.	Tepper and Weiss (1986)
0.08 0.12 0.25 0.5	157 980	6 h	Lever pressing for access to the running wheel	Rat, M (Long-Evans) 300 g	0.12 ppm O_3 decreased bar press for access to the running wheel. Two of the four animals were affected at 0.08 ppm.	Tepper and Weiss (1986)
0.12-1.5	235-2,940	6 h	Nose poke response for food	Rat, M (Long-Evans) 275 g	0.5 ppm O_3 decreased nose poking for food reinforcement. Effects were enhanced postexposure.	Tepper and Weiss (1986)
0.1-0.8	196-1,568	7 days continuous	Drinking, eating	Mice, M (ICR) 8-26 weeks old	Drinking, food consumption, and body weight initially decreased, but adapted with continued exposure, starting at 0.2 ppm.	Umezu et al. (1993)
0.25-16	490-31,360	60 s maximum	Nose poking terminated O_3 exposure	Mouse, M (Swiss-Webster) 30 g	At 0.5 ppm, mice learned to terminate O_3 exposure.	Tepper and Wood (1985)
0.4 1.2	784 2,352	13 days continuous	Home cage behavior	Mice	During first hour, rearing, grooming, sniffing, and social interactions increased, crossings and wall climbing decreased. These behaviors did not adapt with continued exposure.	Musi et al. (1994)
0.5 2.0	980 3,920	3 h	Lever pressing to avoid electric shock	Rat (Wistar) 300 g	Suppression of lever pressing began after 45 min of 2.0-ppm exposure and after 90 min of 0.5- or 1.0-ppm exposures.	Ichikawa et al. (1988)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

equally sensitive to O_3 exposure when pressing a lever allowed rats to have access to a running wheel. The authors concluded that increased physical activity, either used as the response to obtain reward, or as the reward, was an important behavioral variable in determining sensitivity to O_3 exposure. Ichikawa et al. (1988) demonstrated that behavior (lever pressing) maintained by the avoidance of electric shock, was even less sensitive to O_3 exposure (3 h, 1.0 ppm) than behaviors maintained by food reinforcement, as described above. Furthermore, the animals recovered quickly after O_3 exposure was terminated (60 to 120 min).

In mice exposed to O_3 continuously for 13 days (0.4 to 1.2 ppm), both increases and decreases in measured behaviors were observed (Musi et al., 1994). During the first hour of exposure to 0.8 or 1.2 but not 0.4 ppm O_3, increases in rearing, grooming, sniffing, and social interactions were observed, but locomotion and bar holding declined. With continued exposure (measurements on Days 3, 7, and 10), grooming and rearing were still increased but crossings and wall climbing remained depressed. The affected behaviors did not show adaptation. However, drinking, food consumption, and body weight were initially depressed, but abated with continued exposure, a finding previously reported in mice at O_3 concentrations as low as 0.2 ppm (Umezu et al., 1993).

In summary, the behavioral data indicate that transient changes in behavior occur in rodent models that are dependent on a complex interaction of factors such as (1) the type of behavior being measured, with some behaviors increased and others suppressed; (2) the factors motivating that behavior (differences in reinforcement); and (3) the sensitivity of the particular behavior (e.g., active behaviors are more affected than more sedentary behaviors).

6.3.3 Cardiovascular Effects

Several reports have demonstrated that O_3 exposure causes dramatic effects to the cardiovascular system in the rat (Table 6-23). Uchiyama et al. (1986) initially reported that heart rate (HR) and mean arterial blood pressure (MAP) were decreased by 53 and 29%, respectively, during a 3-h exposure to 1.0 ppm O_3. Arrhythmias, including atrioventricular block and premature atrial contractions, also were observed frequently. The effects appeared to be age- but not sex-dependent, with 11-week-old rats showing a greater response than did 8- or 4-week-old rats. Yokoyama et al. (1989b) showed that recovery from the effects of the 3-h, 1.0-ppm O_3 exposure was not complete by 5 h and that, with three consecutive daily exposures, both the HR and MAP responses were attenuated. Further investigations by the same group of authors (Uchiyama and Yokoyama, 1989) showed that, with exposures to 0.5 ppm O_3 for 6 h, HR and MAP decreased by 32 and 18%, respectively. A 4-week continuous exposure to 0.2 ppm initially resulted in a 12% decrease in HR, but this response was attenuated on Day 2 and was almost eliminated by Day 3. No further effects were observed during the rest of the 4-week exposure period. When these same animals were subsequently challenged with 0.8 ppm O_3 for 1.5 h, they also had an attenuated response when compared to rats that were O_3 naive. Additionally, some rats were instilled intratracheally with elastase to create an animal model of emphysema. This pretreatment, however, did not affect the outcome of either the HR or MAP responses to O_3 in any of the experiments, except in the 0.8-ppm challenge experiment. In this experiment, elastase-treated, O_3-exposed rats challenged with O_3 had a similar response to O_3 challenge as did O_3-naive rats, suggesting that the elastase treatment affected the ability of the rats to develop an adaptive lung response. In contrast, Tepper et al. (1990) did not observe an alteration in

Table 6-23. Effects of Ozone on the Cardiovascular System[a]

Ozone Concentration ppm	Ozone Concentration µg/m	Exposure Duration and Conditions	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
0.1 0.2	196 392	5 days continuous	Rat, M (Wistar) 8 weeks old	0.1 ppm O_3 caused bradyarrhythmia up to 3 days of exposure; broadycardia occurred at 0.2 ppm during first 2 days of exposure. No effects on sleep-wakefulness patterns.	Arito et al. (1990)
0.2 0.5 1.0	392 980 1,960	4 weeks continuous 6 h 3 h	Rat, M (Wistar) 13 weeks old	At 0.2 ppm a 12% decrease in HR; response attenuated by 3 days. At 0.5 ppm, HR and MAP decreased by 32 and 18%, respectively.	Uchiyama and Yokoyama (1989)
0.25 1.0	490 1,960	2 h 18-20 °C 30-32 °C	Rat, M (F344) 13-16 weeks old	0.37 ppm O_3 caused bradycardia and bradyarrhythmia, ambient temperature of 30-32 °C blocked response.	Watkinson et al. (1993)
0.5 1.0	980 1,960	6 h 3 h	Rat, M (Wistar) 10 weeks old	0.5 and 1.0 ppm O_3 caused bradycardia and bradyarrhythmia. 1.0-ppm response was partially blocked by atropine.	Arito et al. (1992)
0.5 1.0	980 1,960	6 h 3 h, 3 days	Rat, M (Wistar) 10-11 weeks old	1.0 ppm O_3 caused bradycardia, bradyarrhythmia, and decreased MAP. The response to 1.0 ppm lasted > 5 h postexposure and was attenuated with 3 consecutive daily exposures.	Yokoyama et al. (1989b)
1.0	1,960	3 h	Rat, M, F (Wistar) 4, 8, and 11 weeks old	1.0 ppm O_3 caused bradycardia, bradyarrhythmia, and decreased MAP. Older animals (11 weeks) were more affected than younger ones. No sex-related differences were noted.	Uchiyama et al. (1986)
1.0	1,960	135 min	Rat, M (F344) 90 days old	Ventilation stimulated with CO_2. No effect on mean blood pressure.	Tepper et al. (1990)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

blood pressure of rats exposed to 1.0 ppm O_3 for 135 min, even though their ventilation was increased by CO_2.

Arito et al. (1990) demonstrated bradycardic responses at 0.2 ppm O_3 during the first 2 days of a continuous 5-day exposure; bradyarrhythmia occurred during the first 3 days of a 0.1-ppm exposure. Simultaneously, these authors measured the sleep/wakefulness of the rats during exposure and found that more bradyarrhythmias occurred during wakefulness than during slow-wave or paradoxical sleep. Sleep/wakefulness patterns were not altered by this O_3 exposure. At high O_3 concentrations (1 ppm for 3 h), wakefulness and paradoxical sleep were suppressed, the amplitude of the electroencephalogram (EEG) was lowered, and slow-wave sleep was increased (Arito et al., 1992). These EEG changes appear to be temporally associated with the decrease in behavioral activity previously discussed (Tepper et al., 1982). Atropine sulfate blocked the suppression of wakefulness and bradycardia in a concentration-related manner and decreased slow-wave sleep, suggesting that some of the O_3 effects are parasympathetically mediated. The effects of O_3 on paradoxical sleep and the EEG amplitude were not affected by atropine administration. Watkinson et al. (1993) extended these findings by showing that the core temperature of rats was also reduced when HR fell at O_3 exposure concentrations between 0.37 and 1.0 ppm (2 h). Increasing ambient temperature to 30 to 32 °C attenuated the 1.0 ppm O_3-induced reduction in HR and core temperature.

In an attempt to synthesize the results from these studies, Watkinson and Gordon (1993) questioned the relevance of these parameters in the rat as compared to the human. Rats have different thermoregulatory responses than humans and typically respond to toxic insult by lowering core temperature. This response has been shown to increase survival value (Watkinson et al., 1989). Similar changes in core temperature and HR have not been reported in humans. This may be because of the large, and thus stable, thermal mass of humans, or, alternatively, these effects have not been observed because they were not measured, and because most O_3 exposure experiments are done using exercise, which may mask these responses. In support of this latter idea, Coleridge et al. (1993) reported that stimulation of bronchial C-fibers produces bradycardia. Ozone preferentially stimulates bronchial C-fibers and, as a result, induces bradycardia and tachypnea in the anesthetized, open-chest dog model. Furthermore, the tachypnea produced by O_3 exposure is inhibited by atropine administration (the effect on HR was not reported).

6.3.4 Hematological and Serum Chemistry Effects

Hematological effects reported in laboratory animals and humans after inhalation of O_3 indicate that the gas or, more likely, some reaction product can cross the blood-gas barrier. The effects of in vivo O_3 exposure in animals were summarized in the previous O_3 criteria document (U.S. Environmental Protection Agency, 1986). The hematologic parameters most frequently used to evaluate O_3 toxicity were morphologic and biochemical effects on erythrocytes (RBCs). These studies reported alterations in RBC morphology, increased RBC fragility, increased hemolysis, and decreased survival. The biochemical studies reported variable results, depending on the O_3 exposure concentration and the RBC enzyme under investigation.

More recent studies have stressed serum effects of O_3 exposure (Table 6-24). Exposure of rats for 2 h to 0.1 ppm O_3 increased plasma creatinine kinase activity, whereas no such effect was observed when exposure was to 0.05 and 0.25 ppm O_3 (Veninga and

Table 6-24. Hematology and Serum Chemistry Effects[a]

Ozone Concentration		Exposure Duration	Species, Sex (Strain) Age[b]	Observed Effect(s)	Reference
ppm	g/m^3				
0.05 0.1 0.25	100 200 500	2 h	Rat, M (Wistar) 200 g	Increased plasma creatine kinase activity at 0.1 but not 0.05 and 0.25 ppm.	Veninga and Fidler (1986)
0.1 0.2 0.4 0.6	196 392 784 1,176	3 h	Rabbit, F (NZW) 2.5-3.5 years old	No change in plasma retinol, ascorbic acid, and -tocopherol concentrations.	Canada et al. (1987)
0.4	784	Continuous for 14 days	Rat, M (Wistar) 20 weeks	Decrease in serum retinol concentration.	Takahashi et al. (1990)
0.8	1,568	18 h	Rat, M (F344)	Decrease in plasma lactic dehydrogenase isoenzyme activity.	Nachtman et al. (1988)
1.0	1,960	1 h	Guinea Pig, M (Hartley) 250-300 g	Increases in plasma concentrations of TXB_2, 6-keto-PGF_{1}, and PGE_1.	Miller et al. (1987)
1.0	1,960	1 h	Guinea Pig, M (Hartley) 250-300 g	Increases in plasma concentrations of TXB_2, 6-keto-PGF_{1}, and PGE_1.	Miller et al. (1988)
1.0	1,960	23 h/day for 2 weeks	Rat, M (CD) 400 $\pm$ 25 g	Heat-inactivated plasma increases DNA synthesis by lung fibroblasts and pneumocytes.	Tanswell et al. (1989, 1990) Tanswell (1989)
1.0	1,960	4 h	Mouse, M (CD-1) 8 weeks old	Inhibition of RBC deformability.	Morgan et al. (1988)

[a]See Appendix A for abbreviations and acronyms.
[b]Age or body weight at start of exposure.

Fidler, 1986). Decreased serum retinol concentrations were observed following continuous exposure of rats for 14 days to 0.4 ppm O_3 (Takahashi et al., 1990), but no changes in plasma retinol, ascorbic acid, and □-tocopherol were observed following exposure of rabbits for 3 h to O_3 ranging from 0.1 to 0.6 ppm (Canada et al., 1987). In similar studies, a decrease in plasma lactic dehydrogenase isoenzyme activity also was observed following exposure of rats for 18 h to 0.8 ppm O_3 (Nachtman et al., 1988).

Miller et al. (1987, 1988) investigated the effect of a 1-h exposure of guinea pigs to 1.0 ppm O_3 on plasma eicosanoid levels and observed increases in TXB_2, 6-keto-$PGF_{1□}$, and PGE_1. These data suggest that some of the systemic effects of O_3 exposure, such as impairment of peritoneal AM phagocytosis (Canning et al., 1991), may be mediated by the immunosuppressive effects of the prostanoids (Oropeza-Rendon et al., 1979). Heat-inactivated plasma from rats exposed for 23 h/day for 2 weeks to 1.0 ppm O_3 also increases DNA synthesis by lung fibroblasts (Tanswell et al., 1989) and lung pneumocytes (Tanswell et al., 1990).

6.3.5 Other Systemic Effects

Previous studies suggest that O_3 has effects on the xenobiotic metabolism of the liver (U.S. Environmental Protection Agency, 1986). This effect has been observed in mice, rats, and hamsters as a prolongation of pentobarbital sleeping time (Graham et al., 1981). The effect appears to be sex dependent, with females having greater responses than males. Canada and Calabrese (1985) performed a similar experiment in both young (3- to 4-mo-old) and older (2-year-old) rabbits exposed for 3.75 h/day to 0.3 ppm O_3 for 5 consecutive days. They observed significant prolongation of the elimination of theophylline in older rabbits, but not in young rabbits, and the effect was more pronounced in females than in males. In a follow-up study, Canada et al. (1986) could not demonstrate increased pentobarbital sleeping in young (2.5-mo-old) mice or rats of comparable age to the study by Graham et al. (1981). However, effects were observed in older (18-mo-old) female mice and rats. Two other studies (Heng et al., 1987; Zidenberg-Cherr et al., 1991) from the same group of investigators indicate that liver antioxidant enzymes (Cu/Zn- and Mn-SOD and GSHPx) are decreased commensurate with the increase in these enzymes that is observed in the lung.

6.3.6 Summary

Several reports recently have appeared that extend previous observations in laboratory animals that indicate that ambient levels of O_3 can affect animal behavior. These effects are interpreted as analogous to O_3-induced symptoms in humans, rather than as evidence of neurotoxicity. The behavioral changes are transient but may persist several hours after acute exposure. Different types of behaviors appear to be variably sensitive to O_3 exposure, with active behaviors showing suppression at lower O_3 concentrations than do more sedentary behaviors or behaviors maintained by electric shock (Ichikawa et al., 1988; Tepper et al., 1985; Tepper and Weiss, 1986). For example, a 6-h exposure of rats to 0.12 ppm suppressed running-wheel behavior (Tepper et al., 1985). Furthermore, animals will respond to terminate a 1-min exposure to 0.5 ppm O_3, thus directly implicating the irritant properties of O_3 (Tepper and Wood, 1985). It appears that with additional training, animals can learn to terminate exposure using conditioned stimuli rather than relying directly on the aversive properties of O_3 (Tepper et al., 1985).

Ozone has been found to decrease HR, MAP, and core temperature profoundly in rats (Watkinson et al., 1993; Arito et al., 1990; Uchiyama and Yokoyama, 1989). During exposure, arrhythmias frequently occur. After a 3-h exposure to 1.0 ppm O_3, these effects appear to occur more in adult rats (11 weeks old) than in younger animals (4 and 8 weeks old), especially when the rats were awake (as measured by EEG) during the exposure (Uchiyama et al., 1986). The lowest exposures causing bradycardia in rats was 0.2 ppm for 48 h; 0.1 ppm for 24 h caused bradyarrhythmia (Arito et al., 1990). Similar effects have not been observed in humans or other species. In part, this may be because they have not been systemically examined or that human studies have been carried out during concurrent exercise, which may mask these effects. More likely, these effects represent species differences related to the magnitude and localization of reflex responses and differences in thermal mass.

6.4 Interactions of Ozone with Other Co-occurring Pollutants

6.4.1 Introduction

Most of the toxicological data for O_3 are derived from studies using O_3 alone. However, it is also important to evaluate responses to inhalation of typical pollutant combinations because ambient exposures involve mixtures. Such mixtures provide a basis for toxicological interactions, whereby combinations of chemicals may behave differently than would be expected from consideration of the action of each separate constituent. This section discusses toxicological studies of pollutant mixtures in which O_3 is one component. Discussions of many of these studies addressing the effect of O_3 alone on various organs or systems appear elsewhere in this chapter.

Evaluating the role of O_3 in observed responses to inhaled mixtures is not easy. In spite of the myriad of interpretative difficulties, it is essential to attempt to understand the potential for interactions because O_3 does not exist alone. One of the problems involves definitions of terms, and the study of toxicant interaction is complicated by the dilemma of attempting to characterize the effects from exposure to two or more chemicals. Most studies have employed a statistical definition, but this merely provides a description, and tells nothing about the mechanism of any interaction. But, in many cases, the mechanism of action of the individual components may not be understood fully, and information concerning the types of interactions may provide useful beginnings for studying mechanisms of action of the mixture components. Furthermore, any conclusion of interaction is highly dependent on the specific type of model used. Because the purpose of this chapter is to provide a toxicologic background for effects of O_3 in terms of public health significance, interaction will be defined as a departure from the additivity model (i.e., interaction is considered to occur when the response to a mixture is significantly different from the sum of the responses to the individual components). A less than additive interaction is antagonism, whereas synergism is an interaction that is more than additive. A subclassification of synergism, termed potentiation, is often used to describe an interaction in which response to a mixture is greater than the sum of the responses to the individual components, but where only one component produced a response different from control when administered alone. In many instances, however, potentiation and synergism have been used interchangeably. Although some synergistic interactions actually may serve to stimulate repair processes, or otherwise reduce the harmful

effects of O_3, and some antagonistic interactions eventually may increase the risk of disease development, synergism, as currently used, generally implies greater risk, and antagonism implies lesser risk. However, such assumptions eventually may be proven to be invalid in some instances. Also, interactions with the large number of natural air pollutants, such as microbes, spores, and dusts, that can produce considerable responses alone are not included in this section.

In most cases, the interaction of O_3 with other pollutants has been studied using mixtures that contained only one other copollutant (i.e., simple or binary mixtures). In such studies, the role played by each pollutant in eliciting measured responses can be elucidated with the appropriate experimental design, but most of the database involves exposures to the mixture and O_3 only, with no exposure to the copollutant alone. Although the O_3 concentration may have been varied among exposure groups or was present in one group and not in another (so its relative influence could be assessed to some extent), it cannot be determined in such cases whether the response to the mixture involved actual interaction or was merely additive.

The ambient atmosphere in most environments is generally a mixture of a number of pollutants, and assessing effects of such multicomponent atmospheres may serve to provide some indication of biological responses under conditions that better mimic ambient exposure. However, very few studies have used realistic combinations of pollutant concentrations when assessing interaction.

The ability to discern the contribution of O_3 to observed responses becomes even more difficult when such complex mixtures are studied. Even when binary mixtures are used, they often do not mimic the ambient pattern (e.g., NO_2 levels peak before O_3 levels do) or ambient concentrations (as absolute values or as ratios). Rarely are concentration-response mixture studies performed. This raises the possibility that an unrealistic experimental design may lead to masking the effect of a copollutant or to identifying a response that may not occur in the real world.

Another problem in assessing responses to mixtures involves the statistical basis for the conclusion of significant interaction. For example, a number of studies determined interaction by comparison of the response from exposure to only one component of the mixture with that from exposure to the complete mixture. On the other hand, some studies used statistical approaches specifically designed to indicate interactions. As another example, it may be relatively straightforward to study interactions when one exposure concentration of each of two pollutants is used, but it becomes much more difficult when there are multiple concentrations used, and even more difficult still when more than two pollutants are involved. Because variable criteria for conclusions of interaction have been used, the available database is one in which the statistical significance for determination of interaction varies in terms of its robustness.

6.4.2 Simple (Binary) Mixtures Containing Ozone

Tables 6-25 and 6-26 outline studies performed since publication of the last O_3 criteria document (U.S. Environmental Protection Agency, 1986) in which experimental animals were exposed to atmospheres containing O_3 with only one other copollutant. These tables provide the experimental details for the discussion that follows.

Table 6-25. Toxicological Interactions of Ozone and Nitrogen Dioxide[a]

Concentration[b] ppm	Concentration[b] µg/m³	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.05 0.04 0.05 0.4	98 75 98 752	O_3 NO_2 O_3 NO_2	NO_2: 24 h/day; O_3: Intermittent during hours 9-19/day following sine curve from 0-0.1 ppm (0.05 avg); total duration: 5-22 mo	Rat, M (Wistar) 7 weeks old	Lung protein content; lung lipid peroxides; antioxidant enzymes (G6PD, 6PGD, GR, GST, GSHPx, SOD)	0 PE	Protein: no change; peroxide: increase between 5 and 9 mo, return to control at >9 mo (greater effect with 0.4 ppm NO_2); enzymes: no change.	Protein: none (no effect of O_3 or NO_2 alone); peroxide: synergism (no change with O_3 or NO_2 alone for 9 mo); enzyme: none (no effect of O_3 or NO_2 alone).	Sagai and Ichinose (1991)
0.05 0.4	98 752	O_3 NO_2	O_3: Concentration ranged from 0 to 0.1 with sine curve over 9-19 h (0.05 avg); NO_2: 24 h/day; both 13 mo, 11 mo recovery	Rat, M (Wistar) 6 weeks old	Development of lung tumors from exposure (ingestion) of carcinogen, *N-bis*(2-hydroxypropyl) nitrosamine prior to O_3 and NO_2	0 PE	Increased tumor incidence (compared to air-exposed control).	Suggested synergism: no increase with O_3 alone (NO_2 alone not done).	Ichinose and Sagai (1992)
0.1 0.5 1.2 4.0 0.1 0.3 1.2 2.5 0.05 0.1 0.5 1.0 0.05 0.1 0.05 0.1	196 980 2,256 7,520 196 588 2,256 4,700 98 196 940 1,880 98 196 94 188	O_3 (baseline) O_3 (peak) NO_2 (baseline) NO_2 (peak) O_3 (baseline) O_3 (peak) NO_2 (baseline) NO_2 (peak) O_3 (baseline) O_3 (peak) NO_2 (baseline) NO_2 (peak) O_3 (baseline) O_3 (peak) NO_2 (baseline) NO_2 (peak)	15 day, continuous exposure to basal level; peaks: 1 h, twice daily, 5 days/week beginning after 64 h of continuous exposure	Mouse, F (CD-1) 4-6 weeks old	Bacterial infectivity (to *Streptococcus zooepidemicus* given after pollutant exposure)	Bacterial challenge given 0 or 18 h PE	No effect at low level; increased mortality at other levels.	Synergism: at 0.05 O_3 + 0.5 (1.0) NO_2, 0.1 O_3 + 1.2 (2.5) NO_2; marginal synergism at 0.1 O_3 + 1.2 (4) NO_2. Both O_3 and NO_2 increased mortality at two highest levels; only NO_2 increased at two lowest levels.	Graham et al. (1987)
0.1 0.3	196 564	O_3 NO_2	NO_2: 24 h/day; O_3: 8 h/day; 1, 3, 6, 18 mo	Rat, M (F344) 5 weeks old	Histopathology (LM, TEM)	0 PE	Connective tissue edema, Type 2 cell hypertrophy and enlarged lamellar bodies.	Changes more marked than with O_3 alone, NO_2 affected response to O_3 (no quantitation performed).	Terada et al. (1986)

Table 6-25 (cont'd). Toxicological Interactions of Ozone and Nitrogen Dioxide[a]

Concentration[b] ppm	Concentration[b] µg/m	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.15 0.35	294 564	O_3 NO_2	7 h/day, 5 days/week for 12 weeks	Mouse, M (C57Bl/6J) 5 weeks	Colonization of lung by melanoma cells (injected after pollutant exposure)	Inject melanoma cells 0 PE and sacrifice 3 weeks PE	Increase in number of colonies/lung (compared to air control).	Not specified: no change with O_3, but previous study showed effect with NO_2.	Richters (1988)
0.2 4.0	392 7,520	O_3 NO_2	Continuous 1-2 mo	Rat, M (Wistar) 22 weeks old	Pulmonary xenobiotic metabolism, lung protein (homogenate)	0 PE	No effect on protein content, increase in selected enzymes.	Suggested antagonism for xenobiotic enzymes: O_3 induced increase in selected enzymes is lowered by addition of NO_2.	Takahashi and Miura (1989)
0.2 3.6 0.4 7.2 0.6 10.8 0.8 14.4	392 6,768 784 13,536 1,176 20,304 1,568 27,072	O_3 NO_2 O_3 NO_2 O_3 NO_2 O_3 NO_2	24 h/day for 3 days 12 h/day for 3 days 8 h/day for 3 days 6 h/day for 3 days	Rat, M (S-D) 10-12 weeks old	Protein (lavage), lavaged cells (epithelial PMN)	0 PE	Increased protein and cells.	Synergism: protein at 0.6 and 0.8 ppm O_3 mixtures, lavaged cells at 0.4-0.8 ppm O_3; others additive.	Gelzleichter et al. (1992a)
0.2 3.6 0.4 7.2 0.6 10.8	392 6,768 784 13,536 1,176 20,304	O_3 NO_2 O_3 NO_2 O_3 NO_2	6 h/day for 3 days	Rat, M (S-D) 10-12 weeks old	Protein (lavage), lavaged cell counts (epithelial, PMN, AM), DNA content of cell pellet	0 PE	Increased protein and cells, depending on concentration.	Synergism: cell counts at ≥0.4 ppm O_3 mixture, protein additive.	Gelzleichter et al. (1992b)
0.2 3.6 0.4 7.2 0.6 10.8 0.8 14.4	392 6,768 784 13,536 1,176 20,304 1,568 27,072	O_3 NO_2 O_3 NO_2 O_3 NO_2 O_3 NO_2	24 h/day for 3 days 12 h/day for 3 days 8 h/day for 3 days 6 h/day for 3 days	Rat, M (S-D) 250-275 g	Airway labeling index	4 days PE	Increased index in peripheral airways (TBs opening into ADs) and large airways at three highest doses, increased alveolar index at 0.8 + 14.4 ppm only.	Synergism for peripheral airways at highest dose and large airways at three highest doses only.	Rajini et al. (1993)
0.3 1.2	588 2,256	O_3 NO_2	Continuous for 3 days	Rat, M (S-D) 3 mo old	Lung enzymes (G6PD, 6PGD, ICD, GSHPx, GR, DR, GDT, NADPH-CR)	0 PE	Increased activity.	Synergism: 6PGD, ICD, GR, SOD; additive: GP, DR; others: effect same as O_3 only.	Lee et al. (1990)

Table 6-25 (cont'd). Toxicological Interactions of Ozone and Nitrogen Dioxide[a]

Concentration[b] ppm	Concentration[b] µg/m	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.3 3.0	588 5,640	O_3 NO_2	2 h Nose-only	Rabbit, M (NZW) 4.5 mo old	Pulmonary eicosanoids (lavage) (PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TXB_2, LTB_4)	0 or 24 h PE	Increases in PGE, $PGF_{2\alpha}$, and TXB_2 immediately PE (compared to air control).	Synergism: PGE_2, $PGF_{2\alpha}$; TXB_2 effect similar to O_3 alone.	Schlesinger et al. (1990)
0.3 3.0	588 5,640	O_3 NO_2	2 h/day for 14 days Nose-only	Rabbit, M (NZW) 4.5 mo old	Pulmonary eicosanoids (lavage) (PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TXB_2)	0 PE after 7 or 14 exposures, or 24 h PE after 14 exposures	Decrease in PGE_2 (compared to air control) after 7 and 14 days and 24 h PE; 6-keto-$PGF_{1\alpha}$ decreased 24 h postexposure.	None: effects additive or similar to NO_2 alone.	Schlesinger et al. (1991)
0.4 0.4	784 752	O_3 NO_2	Continuous for 2 weeks	Mouse, M (ICR); Hamster, M (Golden); Rat, M (Wistar); Guinea pig, M (Hartley) all 10 weeks old	Lung lipid peroxides, antioxidant content, phospholipids, and fatty acids	0 PE	Variable increases to no effect, depending on species.	Not determinable: compared mixture vs. air control, no measure of single pollutants performed.	Sagai et al. (1987)
0.4 0.4	784 752	O_3 NO_2	24 h/day for 2 weeks	Mouse, M (ICR); Hamster, M (Golden); Rat, M (Wistar); Guinea pig, M (Hartley) all 10 weeks old	Lung lipid peroxides, antioxidant enzymes, total protein (homogenate)	0 PE	Increases, which were species dependent.	Not determinable: compared mixtures vs. air control, no measure of single pollutants performed.	Ichinose et al. (1988)
0.4 0.4	784 752	O_3 NO_2	Continuous for 2 weeks	Rat, M (Wistar) 10 weeks old; Guinea pig, M (Hartley) 10 weeks old	Lipid peroxides, lung antioxidants, and antioxidant enzymes	0 PE	Increased peroxides in guinea pig but not rat; increased antioxidants in rat but not guinea pig; enzymes increased or decreased in guinea pigs, increased to no change in rat.	Synergism for some endpoints, additive to no interaction for others; species dependent.	Ichinose and Sagai (1989)

Table 6-25 (cont'd). Toxicological Interactions of Ozone and Nitrogen Dioxide[a]

Concentration[b] ppm	Concentration[b] µg/m	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.4 0.4	784 752	O_3 NO_2	Continuous for 2 weeks	Rat, M (Wistar) 10 weeks old; Guinea pig, M (Hartley) 10 weeks old	Lipid peroxides; lung antioxidants, and antioxidant enzymes	0 PE	Increased peroxides in guinea pig but not rat; increased antioxidants in rat but not guinea pig; enzymes increased or decreased in guinea pigs, increased to no change in rat.	Synergism for some endpoints, additive to no interaction for others; species dependent.	Ichinose and Sagai (1989)
0.45 4.8	882 7,520	O_3 NO_2	8 h/day for 7 days	Mouse, M (Swiss Webster) 2 mo old	Lung protein, DNA, sulfhydryl and nonsulfhydryl content; GR, GST, G6PD, 6PGD, ICD activities	0 PE	No change in protein or DNA; increase in activity of ICD, G6PD, 6PGD.	Synergism: enzyme activity.	Mustafa et al. (1985)
0.6 2.5 0.35 0.6	1,176 4,700 686 1,128	O_3 NO_2 O_3 NO_2	4 h (rest) 3 h (exercise)	Rat, M (S-D) 7 weeks old	Parenchymal histopathology	2 days PE	Increased focal lesions.	Synergism: ascribed to production of HNO_3 in exposure atmosphere.	Mautz et al. (1988)
0.6 2.5	1,176 4,700	O_3 NO_2	2 h (rest and exercise)	Rat, M (S-D) 47-52 days old	Epithelial permeability (tracheal, bronchoalveolar) (measured 1 and 24 h PE)	0, 1, 2 days PE	Rest: increased bronchoalveolar permeability at 1 and 24 h PE; exercise: increased bronchoalveolar permeability at 1 and 24 h PE (effects greater than with O_3 alone, no effect of NO_2).	Enhanced magnitude and duration of response (suggested potentiation).	Bhalla et al. (1987)
0.8 4.0	1,568 7,520	O_3 NO_2	Continuous for 3-56 days	Mouse, M (BALB/c) 8-10 weeks old	Antibody response to T-cell dependent and independent antigens in spleen	0 PE	Inconsistent pattern of increases and decreases of lung weight, thymus weight, or plaque formation.	Most responses similar to O_3 only; mixture affected some time points not affected by O_3 alone: implied nonadditive interaction, but specifics not determinable.	Fujimaki (1989)
0.8 14.4	1,568 27,072	O_3 NO_2	6 h/day for 3 days (concurrent) or sequential O_3 pre-NO_2; NO_2 pre-O_3; 6 h each	Rat, M (S-D) 10-12 weeks old	Protein (lavage); lavaged cell counts (epithelial, PMN, AM), DNA content of cell pellet	0 PE	Increased protein for concurrent or sequential, increase in cell counts for concurrent.	Synergism: protein and cell counts for concurrent, protein additive or antagonistic for sequential.	Gelzleichter et al. (1992b)

Table 6-25 (cont'd). Toxicological Interactions of Ozone and Nitrogen Dioxide[a]

Concentration[b]		Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
ppm	µg/m								
0.8 14.4	1,568 27,072	O_3 NO_2	6 h/day for 45-79 days	Rat, M (S-D) 10/12 weeks old	Various biochemical and histological endpoints	0 PE	Increased lung DNA, protein, collagen, elastin; some deaths with mixture only at >55 days; decreased hydroxypyridinium.	Suggested synergism for hydroxyproline and hydroxypyridinium.	Last et al. (1993b)

[a]See Appendix A for abbreviations and acronyms.
[b]Grouped by pollutant mixture.
[c]Age or body weight at start of exposure.

Table 6-26. Toxicological Interactions to Binary Mixtures of Ozone with Acids and Other Pollutants[a]

Concentration[b] ppm	Concentration[b] µg/m	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.1	196 125	O_3 H_2SO_4 (0.3 µm)	2 h/day, 5 days/week for up to 1 year Nose-only	Rabbit, M (NZW) 4.5 mo old	Tracheobronchial mucociliary transport, bronchial tree epithelial secretory cell numbers	Mucociliary transport during exposure; secretory cells 3 days after 4, 8, and 12 mo of exposure	Normal to accelerated clearance, increase in secretory cell numbers at early time points (4-mo exposure).	Clearance: No interaction; secretory cell numbers: synergism at 4 mo, antagonism at 8 and 12 mo.	Schlesinger et al. (1992a)
0.1 0.3 0.6 	196 588 1,176 50 75 125	O_3 O_3 O_3 H_2SO_4 (0.3 µm) H_2SO_4 (0.3 µm) H_2SO_4 (0.3 µm)	3 h Nose-only	Rabbit, M (NZW) 4.5 mo old	Lavage cell counts; lavage LDH, PGE_2, $PGF_{2\alpha}$; AM phagocytosis; superoxide production; TNF activity	0 PE	No effects on lavage cell counts or LDH, PGE_2, $PGF_{2\alpha}$ or increase or decrease in TNF and phagocytosis depending on exposure concentration; no change in superoxide.	Antagonism: Phagocytosis, at all combinations; antagonism: Superoxide at 0.1 and 0.3 ppm O_3 and 75 and 125 µg/m³ H_2SO_4; synergism: TNF at 125 µg/m³ H_2SO_4 and 0.3 and 0.6 ppm O_3.	Schlesinger et al. (1992b)
0.12-0.64	235-1,254 40-1,000	O_3 H_2SO_4	6 h for 7 days (23.5 h/day)	Rat, M (S-D) 250-300 g	Lavageable protein	0 PE	Increase (compared to air control).	Synergism at ≥100 µg/m³ H_2SO_4 and 0.2 ppm O_3 for 3 days.	Warren and Last (1987)
0.12-0.64	235-1,254 40-1,000	O_3 H_2SO_4	23.5 h/day for 5-9 days	Rat, M (S-D) 250-300 g	Lung tissue protein	0 PE	Increase (compared to air control).	Synergism at 1,000 µg/m³ H_2SO_4 and 0.64 ppm O_3, ≥100 µg/m³ H_2SO_4 and 0.20 ppm O_3.	Warren and Last (1987)
0.12-0.64	235-1,254 40-1,000	O_3 H_2SO_4	23.5 h/day for 7 days	Rat, M (S-D) 250-300 g	Rate of collagen synthesis	0 PE	Increase (compared to air control).	Synergism at ≥200 µg/m³ H_2SO_4 and 0.64 ppm O_3, ≥500 µg/m³ H_2SO_4 and 0.2 ppm O_3 (with suggestion at < 500 µg/m³ H_2SO_4).	Warren and Last (1987)
0.15	294 300	O_3 H_2SO_4 (0.09 µm)	1 h to H_2SO_4, 2 h rest, then 1 h to O_3 Head-only (acid), whole-body (O_3)	Guinea pig, M (Hartley) 260-325 g	Pulmonary function	0 PE	Acid-induced decrease in DL_{CO} not affected by O_3.	None: O_3 did not alter acid effect.	Chen et al. (1991)

Table 6-26 (cont'd). Toxicological Interactions to Binary Mixtures of Ozone with Acids and Other Pollutants[a]

Concentration[b] ppm	Concentration[b] µg/m	Pollutant [3]	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.15	294 84	O_3 H_2SO_4 (layered on ZnO)	1 h to H_2SO_4, 2 h rest, then 1 h to O_3 Head-only (acid), whole-body (O_3)	Guinea pig, M (Hartley) 260-325 g	Pulmonary function	0 PE	Greater decrease in DL_{CO}, VC after O_3; no change in alveolar volume, TLC with mixture.	Suggested synergism (greater than additive) for DL_{CO}, but not VC.	Chen et al. (1991)
0.15	294 24	O_3 H_2SO_4 (layered on ZnO)	H_2SO_4 3 h/day for 7 days, O_3 on Day 9 Head-only (acid), whole-body (O_3)	Guinea pig, M (Hartley) 260-325 g	Pulmonary function	0 PE	Decrease in TLC, VC, DL_{CO} enhanced by O_3.	Suggested synergism (greater than additive).	Chen et al. (1991)
0.2	392 1,000	O_3 H_2SO_4	23.5 h/day for 7 days	Rat, M (S-D) 250-300 g	Rate of collagen synthesis	0 PE	Increase.	Possibly synergism: Effect different from O_3 alone.	Warren et al. (1988)
0.2	392 1,000	O_3 H_2SO_4	15 or 30 days	Rat, M (S-D) 250-300 g	Lung protein content	0 PE	Increase only at 15 days.	Suggested synergism.	Last (1991b)
0.64	1,254 1,000	O_3 H_2SO_4	23.5 h/day for 7 days	Rat, M (S-D) 250-300 g	Protein content (lavage)	0 PE	Increase.	None: Effect same as O_3 alone.	Warren et al. (1988)
0.64	1,254 1,000	O_3 H_2SO_4	23.5 h/day for 7 days	Rat, M (S-D) 250-300 g	Proximal acinar lesion volume	0 PE	Increase.	None: Effect same as O_3 alone.	Warren et al. (1988)
0.64	1,254 1,000	O_3 H_2SO_4	23.5 h/day for 7 days	Rat, M (S-D) 225-275 g	Lung protein and free proline content	0 PE	Increase with H_2SO_4 and O_3 only.	Synergism.	Last et al. (1986)
0.8	1,568 1,200	O_3 H_2SO_4 (0.63 µm)	O_3 for 2 h, followed by H_2SO_4 for 1 h	Guinea pig, F, M (Hartley) 1.5-2 mo old	Airway constriction (measured by trapped gas volume)	0 PE	Increase compared to air.	No interaction: Effect same as O_3 alone; H_2SO_4 had no effect.	Silbaugh and Mauderly (1986)
0.2	392 5	O_3 $(NH_4)_2SO_4$	23.5 h/day for 7 days	Rat, M (S-D) 250-300 g	Rate of collagen synthesis	0 PE	Increase.	Synergism: Effect greater than O_3; sulfate had no effect.	Warren et al. (1986)
0.2	392 5	O_3 $(NH_4)_2SO_4$	23.5 h/day for 2 days	Rat, M (S-D) 250-300 g	Lavageable protein	0 PE	Increase.	Synergism: Effect greater than O_3; sulfate had no effect.	Warren et al. (1986)
0.2	392 5	O_3 $(NH_4)_2SO_4$	23.5 h/day for 3 days	Rat, M (S-D) 250-300 g	Lavageable protein	0 PE	Increase.	No interaction: Effect same as O_3 alone.	Warren et al. (1986)

Table 6-26 (cont'd). Toxicological Interactions to Binary Mixtures of Ozone with Acids and Other Pollutants[a]

Concentration[b] ppm	Concentration[b] μg/m³	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.2-0.64	392-1,254	O_3	23.5 h/day for 7 days	Rat, M (S-D) 250-300 g	Lung DNA content; tissue protein content; lavage LDH, acid phosphatase, N-acetyl-β-D-glucosaminidase	0 PE	No change in DNA, increase in tissue protein, increases in all enzyme levels.	No interaction: Effect on protein and enzymes same as O_3 alone.	Warren et al. (1986)
	5	$(NH_4)_2SO_4$							
0.64	1,254	O_3	23.5 h/day for 7 days	Rat, M (S-D) 225-275 g	Lung protein and free proline content	0 PE	No change.	None.	Last et al. (1986)
	1,000	$(NH_4)_2SO_4$							
0.96	1,882	O_3	23.5 h/day for 7 days	Rat, M (S-D) 225-275 g	Lung protein content, proline content, apparent collagen synthesis rate, fibroblast numbers in lesions, lesion volume	0 PE	Increase.	Synergism: Effect greater than O_3 alone; H_2SO_4 had no effect (previous study).	Last et al. (1986)
	5,000	$(NH_4)_2SO_4$							
0.3	588	O_3	5 h/day for 3 days Head-only	Sheep, F Adult (31 kg)	Tracheal mucus velocity	0 and 24 h PE	Decrease in velocity (compared to air control).	Not determinable: no measure of single pollutants performed.	Abraham et al. (1986)
3.0	7,860	SO_2							
0.15	294	O_3	4 h/day for 4 days Nose-only	Rat, M (F344) 250 g	Lavage cell population; protein (lavage); AM respiratory burst, LTC_4; elastase inhibitory capacity (lavage)	18 h PE	No change in any endpoint (compared to air control).	None.	Nadziejko et al. (1992)
0.1	250	HNO_3							
0.6	1,176	O_3	4 h Nose-only	Rat, M (F344) 250 g	Lavage cell population; protein (lavage); AM respiratory burst, LTC_4; elastase inhibitory capacity (lavage)	18 h PE	Increased protein, PMN number, elastase inhibiting capacity; no effect on other endpoints (compared to air control).	Less than additive for protein, PMN number, elastase inhibitory capacity; no interaction for other endpoints.	Nadziejko et al. (1992)
0.4	1,000	HNO_3							
0.4	784	O_3	4 h Nose-only	Rat, M (S-D) 7 weeks old	Breathing pattern, fatty acid composition of surfactant, nasal epithelium and parenchymal lesions, lavage protein (24-48 h PE)	Pulmonary function during exposure, others 23 h PE	Rapid breathing, increased protein, decreased fatty acid content, focal lesions with thickened alveolar septa and cellular infiltration in parenchyma.	None: Effect similar to O_3 alone.	Mautz et al. (1991)
	380	HMSA[d] (0.32 μm)							

Table 6-26 (cont'd). Toxicological Interactions to Binary Mixtures of Ozone with Acids and Other Pollutants[a]

Concentration[b] ppm	Concentration[b] μg/m³	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.2 0.4 0.8 1.0	392 784 1,568 1,230	O_3 O_3 O_3 HCHO	22 h/day for 3 days	Rat, M (Wistar) 150-190 g	Nasal epithelial cell turnover, histopathology (LM)	Thymidine administered 2 h PE, sacrifice 4 h PE	Increased turnover (but some conditions produced decrease due to change in ventilation).	Synergism for turnover with 0.4 ppm O_3 and 1-3 ppm HCHO, depending on anatomical site (greater than the sum of individual responses to O_3 and HCHO); microscopic lesions similar to O_3 and/or HCHO alone.	Reuzel et al. (1990)
0.4 0.3 1.1 3.3	784 369 1,353 4,059	O_3 HCHO HCHO HCHO	22 h/day for 3 days						
0.6 10	1,176 12,300	O_3 HCHO	3 h (rest)	Rat, M (S-D) 7 weeks old	Nasal epithelial cell turnover, parenchymal histopathology	2 days PE	Increased focal parenchymal lesions with exercise, but no effect at rest; increased nasal cell turnover at rest or exercise.	Parenchyma: Synergism with exercise, antagonism at rest. Nasal: Synergism.	Mautz et al. (1988)
0.6 10	1,176 12,300	O_3 HCHO	3 h (exercise)						
1.0 1.0	1,960 1,960	O_3 Cigarette smoke O_3 Cigarette smoke	O_3: 0.5 h, then 5 puffs smoke (sequential) O_3: 1.5 h, then 10 puffs smoke (sequential) O_3: chamber, smoke: head-only	Guinea pig, F (Hartley) 350-400 g	Airway responsivity (to metacholine challenge), tracheal vascular permeability, 0-24 h PE	0, 5, or 24 h PE	Increased responsivity and permeability immediately PE at both doses (magnitude, but not duration of effect).	Suggested synergism: No effect of O_3 or smoke alone at low dose; high O_3 increased responsivity and permeability; high smoke increased responsivity.	Nishikawa et al. (1992)
0.8	1,568 2,000- 50,000	O_3 Silica (instilled)	Silica instilled on Day 1 followed by O_3 for 6 h/day, 5 days/week for 37 days beginning Day 4	Rat, M (S-D) 17 weeks old	Pulmonary fibrosis	24 h PE (last O)	No change in lung DNA, protein, or hydroxyproline content; increase in ratio of hydroxyproline to DNA, protein, or wet weight (compared to air control) at 50,000 μg.	None: No biological significance.	Shiotsuka et al. (1986)

Table 6-26 (cont'd). Toxicological Interactions to Binary Mixtures of Ozone with Acids and Other Pollutants[a]

Concentration[b]									
ppm	μg/m³	Pollutant	Exposure Duration	Species, Sex (Strain) Age[c]	Endpoints	Time of Endpoint Measurement	Response to Mixture	Interaction	Reference
0.8 1.5 -	1,568 2,940 10,000	O_3 O_3 Carbon black	4 h	Mouse, F (Swiss) 20-23 g	Cell counts in lavage; AM phagocytosis	20 h PE	Increase PMN counts compared to O_3 alone; greater depression of phagocytosis than O_3 alone.	At 0.8 ppm: No intereaction; at 1.5 ppm: Suggested synergism.	Jakab and Hemenway (1994)

[a]See Appendix A for abbreviations and acronyms.
[b]Grouped by pollutant mixture.
[c]Age or body weight at start of exposure.
[d]HMSA = hydroxymethanesulfonate.

6.4.2.1 Nitrogen Dioxide as Copollutant

The most commonly studied copollutant in binary mixtures with O_3 is NO_2. Studies discussed in the previous O_3 criteria document indicated that, although interaction may occur between these two pollutants, in general, O_3 often masked the effects of the NO_2 or accounted for most of the response. This is because, on a mole-to-mole basis, O_3 is considerably more toxic than NO_2, and the relative contribution of O_3 and NO_2 to pulmonary injury is driven by the exposure ratio of the two pollutants. Commonly studied endpoints for assessing effects of these mixtures were lung morphology, biochemistry, and resistance to bacterial infection.

To put the exposure concentrations of NO_2 into some perspective, short-term, 24-h averages are generally □0.17 ppm, and 1-h averages are generally □0.4 ppm in major metropolitan areas. However, hourly averages in most regions often exceed 0.2 ppm at least once during the year (Schlesinger, 1992).

An earlier study noted that the morphological response of the rat lung alveolar epithelium following 60 days of exposure to O_3/NO_2 mixtures (0.25 ppm O_3 + 2.5 ppm NO_2, or 0.9 ppm O_3 + 0.9 ppm NO_2) was due to the O_3 (Freeman et al., 1974). However, the duration of exposure may affect the contributory role of the copollutant. Thus, for example, Terada et al. (1986) exposed rats to O_3 alone, or to a mixture of 0.1 ppm O_3 + 0.3 ppm NO_2, with O_3 administered 8 h/day and NO_2 administered 24 h/day for up to 18 mo. Following 1 mo of exposure, observed lung lesions in the group exposed to the mixture were similar in severity to those noted with exposure to O_3 alone, but as the duration of exposure increased, the morphological changes in interstitial tissue appeared more marked in those animals exposed to the mixture. Edema of pulmonary connective tissue was more pronounced and alveolar Type 2 cells became swollen. Although this study was not quantitative, qualitative observations led the authors to conclude that the lesions were not due to O_3 alone but that NO_2 played some, albeit undefined, contributory role.

The effect of exposure duration on interaction was also noted in studies of Schlesinger et al. (1990, 1991). Rabbits exposed to 0.3 ppm O_3 + 3.0 ppm NO_2 for 2 h showed synergistic increases in certain BAL eicosanoids obtained immediately after exposure, whereas animals exposed to the same mixture for 2 h/day for 14 days showed no interaction for the same parameters.

A number of studies examined other biochemical responses to O_3/NO_2 mixtures (e.g., sulfhydryl metabolism and the activity of certain enzymes). Some of the studies discussed in the previous O_3 criteria document were found to involve synergism (e.g., Mustafa et al., 1984). More recent studies of lung biochemistry also suggest that O_3 and NO_2 interact synergistically. Ichinose and Sagai (1989) exposed rats and guinea pigs to 0.4 ppm O_3, 0.4 ppm NO_2, or a mixture of the two pollutants continuously for 2 weeks. No change in lung peroxide production was observed in rats, but the mixture synergistically increased peroxide levels in guinea pigs. The guinea pigs showed no change in lung antioxidant content following any exposure, whereas the mixture synergistically increased antioxidant levels in rat lung. The conclusion of a significant interaction was based on relative changes from air controls following exposure to the mixture, compared to changes following exposure to each pollutant alone, using the t-test. Synergism was defined as a change greater than the sum of the responses to individual pollutants; no specific test for interaction was performed.

Ichinose and Sagai (1989) also noted that levels of antioxidant enzymes in rat or guinea pig lungs were variously affected by exposure to the above mixture. For example, GST was decreased in both species exposed only to O_3, but the mixture produced a reduction of this

enzyme in guinea pigs and no change in rats. Thus, the occurrence of interaction was dependent on endpoint as well as species. This latter finding likely reflected interspecies differences in biochemical defenses against oxidant pollutants, given the results of a study by Sagai et al. (1987) with four animal species. This study suggested that observed species differences in lipid peroxide formation following exposure were related to the relative content of antioxidants and the specific composition of phospholipids and their fatty acids. The guinea pig was the most sensitive animal, and the hamster was the most resistant.

The effects of exposures to O_3/NO_2 mixtures on lung lipid peroxides and antioxidant activity have been examined in a number of other studies (Ichinose et al., 1988; Lee et al., 1990; Sagai and Ichinose, 1991), and the results generally confirm that noted above (i.e., such mixtures tend to produce synergistic interaction). However, there is also some evidence for antagonism. Takahashi and Miura (1989) examined effects on the pulmonary xenobiotic system of rats exposed for 1 or 2 mo to a mixture of 0.2 ppm O_3 + 4.0 ppm NO_2, as well as to each pollutant alone. Ozone induced an increase in lung cytochrome P-450 content, but the activity of these enzymes was reduced by the addition of NO_2 to the exposure atmosphere; that is, the mixture resulted in levels intermediate between those found with O_3 or NO_2 alone. However, the reduction in enzyme activity induced by NO_2 was restricted to those enzymes that had been increased by exposure to O_3 alone. The authors suggested that antagonism was due to the production of undefined secondary reaction products in the exposure atmosphere. A similar explanation was proposed to explain observed synergism of lung antioxidant activity in another study (Lee et al., 1990). Thus, the response to any secondary product likely depends on the endpoint examined, assuming that the same reaction products were formed in these two studies.

The role of exposure parameters in producing an interaction between simultaneously inhaled O_3 and NO_2 was examined by Gelzleichter et al. (1992a). Rats were exposed to various combinations of O_3 and NO_2 for various durations (6, 8, 12, and 24 h), such that the C × T products were identical for each of four exposure sets. As indicated in Table 6-24, as the exposure duration increased, the exposure concentration of each component of the mixture decreased. Lavaged protein levels and recovered cells were the endpoints. For each exposure combination, the additive response was predicted from the results of exposure to each pollutant alone, and then synergism was indicated when there was deviation from additivity. Responses to exposure to either O_3 or NO_2 alone for 6, 8, or 12 h showed that the product of C × T was a constant for the observed biological effects. However, less severe changes occurred when delivery was at the lowest dose rate (i.e., when the lowest concentration of each pollutant was delivered over the 24-h exposure duration). Exposure at higher dose rates (i.e., 6 to 12 h) increased the magnitude of the response. Thus, the degree of response to each pollutant alone was not a constant function of C × T throughout the entire range of dose rates, but was concentration driven, and was not identical at the highest and lowest rates. Responses following exposure to the mixture did not follow C × T, even over the range of dose rates in which C × T was constant following exposure to the pollutants individually. Thus, interaction, in this case, synergism, appeared to be concentration dependent, in that the response was disproportionately greater at the higher concentrations (higher dose rates) of the constituent pollutants in the mixture. The response following exposure to the mixtures appeared to be a function of peak concentration, rather than of cumulative dose. More recently, Rajini et al. (1993) noted that analysis of all kinetics following similar exposure to mixtures did not reflect a C × T relationship.

All of the studies described above involved simultaneous exposure to O_3 and NO_2. However, ambient exposure to these pollutants has temporal patterns, and exposure to one agent may alter the response to another, subsequently inhaled agent. The realism of these studies is somewhat dependent on their relationship to actual temporal patterns of pollutants in ambient air (i.e., whether one material is the precursor of the other, as is the case for O_3 and NO_2). As described in the previous O_3 criteria document (U.S. Environmental Protection Agency, 1986), Fukase et al. (1978) exposed mice for 7 days to 3 to 15 ppm NO_2 for 3 h/day, followed by 1 ppm O_3 for 3 h/day, and noted an additive effect on the level of lung GSH.

Yokoyama et al. (1980) exposed rats to 5 ppm NO_2 or 1 ppm O_3 for 3 h/day, or to NO_2 for 3 h followed by O_3 for 3 h/day, for various total durations up to 30 days, and assessed lung mechanics in postmortem lungs, lung histology, and enzyme activity in subcellular fractions of lung tissue. The activity of phospholipase A2 in the mitochondrial fraction was increased in those animals exposed to O_3 only or to O_3 after NO_2, and the response in the latter was significantly greater than that in the former. A decrease in activity of lysolecithin acyltransferase in the supernatent fraction was found only in those animals exposed to both NO_2 and O_3. Pulmonary mechanics showed a change in pulmonary resistance (as a function of elastic recoil pressure) in the O_3- and NO_2/O_3-exposed animals. Histologically, the lungs of the animals exposed to both NO_2 and O_3 appeared similar to those exposed to O_3 alone; however, a slight degree of epithelial necrosis in medium bronchi, not found with either NO_2 or O_3 alone, was seen in the animals exposed to both pollutants. In addition, damage at the bronchoalveolar junction appeared to be somewhat more marked in animals exposed to both gases than in those exposed to O_3 alone. This study suggested that sequential exposures produced responses that, in most cases, did not differ greatly from those due to O_3 alone.

Aside from sequential exposures, simulation of ambient exposure scenarios involving NO_2 and O_3 has been performed by examining the effects of a continuous baseline exposure to one concentration of both pollutants, with superimposed short-term peaks to a higher level of one or both gases. The endpoint generally examined in this regard has been bacterial resistance. Studies reported in the previous criteria document (e.g., Ehrlich et al., 1979; Ehrlich, 1983) in which mice were exposed to O_3 under various scenarios of baseline concentrations of NO_2 on which were superimposed daily peak exposures to NO_2 or a combination of NO_2 and O_3 suggested that exposure with peaks can enhance response to pollutant mixtures, and that the sequence of peak exposures was important in producing reduced resistance to infection that was different from that due to exposure to the baseline concentration only.

As a comparison, toxicologic interactions for infectivity involving simultaneous exposure to NO_2 and O_3 discussed in the previous O_3 criteria document were found generally to be additive following acute exposures, with each pollutant contributing to the observed response when its concentration reached the threshold at which the gas would have affected bacterial resistance when administered alone (Goldstein et al., 1974). If the exposure level of either NO_2 or O_3 was below this threshold, then the response was due solely to the constituent inhaled at the more toxic concentration (Ehrlich et al., 1977).

More recently, Graham et al. (1987) examined resistance to respiratory infection (as measured by bacterial-induced mortality) in mice continuously exposed (for 15 days, 24 h/day) to baseline levels of an NO_2/O_3 mixture with two daily, 1-h peaks of the mixture at very high, high, intermediate, and low exposure concentrations (see Table 6-25 for concentrations). Animals were also exposed to the same baseline levels of either NO_2 or

O_3 onto which were superimposed two daily, 1-h peaks of the same single gas in concentrations as above. At the low concentration, only NO_2 increased mortality. At the intermediate exposure level, the mixture was synergistic; NO_2 alone increased mortality and O_3 had no effect. At the high exposure level, the combined exposure was again synergistic; exposure to each gas separately increased mortality. A similar effect was seen at the very high level, although the combined exposure just missed statistical significance for synergism. These results are consistent with those of the earlier studies reported in the previous O_3 criteria document and support the conclusion that response depends on the specific exposure pattern. The results of Graham et al. (1987) are also consistent with those from the earlier studies with simultaneous exposures.

The relationship between exposure and response is very complex and seems to depend on exposure duration, the ratio of O_3 and NO_2 concentrations, and other factors that may include the production of secondary reaction products within the exposure atmosphere. This complexity was highlighted by the study of Gelzleichter et al. (1992b), who examined effects of combined or sequential exposures of rats to mixtures of O_3 and NO_2 at various concentrations ranging from 0.2 to 0.8 ppm O_3 and 3.6 to 14.4 ppm NO_2. Sequential exposures consisted of 6 h of O_3 at night, followed by 6 h of NO_2 during the day, or vice versa; concurrent exposures were for 6 h/day for 3 days. Various endpoints were examined, and it was noted that sequential and concurrent exposures did not result in the same response. Thus, lavage protein levels were increased additively with sequential exposure (in any pollutant order) but were found to be greater than additive with concurrent exposure. An increase in the number of lavaged epithelial cells was additive for the O_3 night/NO_2 day sequence, antagonistic for the NO_2 night/O_3 day sequence, and additive for concurrent exposure. An increase in the number of lavaged PMNs was additive for both sequential conditions and was synergistic for concurrent exposure. It was concluded that production of synergism depended on the concentration of each pollutant within the mixture, and additivity would result for any endpoint when the concentration of each component of the mixture fell below a certain threshold level. However, these threshold concentrations were endpoint specific, with some endpoints being more sensitive than others; it was speculated that the least sensitive assays were based on changes that were reversible, whereas the most sensitive ones were irreversible. The authors also noted that the extent of chemical reaction within the O_3/NO_2 mixture atmosphere was related to the extent of toxicological interaction, suggesting that interaction was due to the production of some secondary reaction product, which, in this case, was suggested to be nitrogen pentoxide. This particular chemical also had been suggested in earlier studies to be responsible for interactions following exposure to NO_2/O_3 atmospheres (e.g., Diggle and Gage, 1955).

The concentrations at which synergism occurred in the study of Gelzleichter et al. (1992b) discussed above ($\sim$0.4 ppm O_3 and $\sim$7.2 ppm NO_2) were higher than those that generally are found in ambient air. However, the threshold concentration for interaction was dependent on exposure dose rate, with higher rates leading to lower threshold concentrations for synergism.

Although interaction is clearly modulated by environmental exposure factors, such as concentration, duration of exposure, or specific exposure regime, host factors also may play a role. Mautz et al. (1988) examined the effect of exercise on rats exposed to mixtures of O_3 and NO_2. Exercise modified the toxic interactions of combined pollutants, resulting in synergistic interaction occurring at lower exposure concentrations of the constituent pollutants

than with exposure at rest. Thus, a similar magnitude of response, an increase in the extent of focal lesions in lung parenchyma, was noted 2 days following a 4-h exposure to 0.6 ppm O_3 + 2.5 ppm NO_2 at rest or with a shorter (3-h) exposure to lower concentrations, 0.35 ppm O_3 + 0.6 ppm NO_2, with exercise. In both cases, the response was different from that due to either pollutant given alone. Furthermore, a greater response was noted with a 3-h exposure to 0.6 ppm O_3 + 2.5 ppm NO_2 with exercise than to the same mixture for the same exposure duration at rest. Thus, exercise also increased the response at similar concentrations compared to rest. The effect of exercise was ascribed to an increase in delivered dose or dose rate, due to increased $\dot{V}_E$. The ability of exercise to enhance response to a pollutant mixture also was noted by Bhalla et al. (1987).

The study of Mautz et al. (1988) above also provided further evidence suggesting that chemical reactions within the exposure atmosphere may play some role in toxicologic interaction. In this case, nitric acid (HNO_3) vapor was noted at concentrations ranging from 0.02 to 0.73 ppm, depending on the concentrations of the primary constituents. As discussed below, acids have been found to interact with O_3. This study also found interaction to occur at a concentration of one of the components, NO_2, that had no effect when administered alone. Although this appears to contrast with the conclusions of Graham et al. (1987) above, the endpoints in these two studies were quite different.

Another aspect of pollutant interaction involves the ability of O_3/NO_2 mixtures to affect the course of other lung changes (e.g., malignant tumor colonization). Richters (1988) exposed mice to a mixture of 0.15 ppm O_3 + 0.35 ppm NO_2 for 7 h/day, 5 days/week for 12 weeks, following which the mice were injected (iv) with viable melanoma cells. The mice were sacrificed 3 weeks later, and the lungs were examined for melanoma colonies. Although exposure to O_3 alone produced no change in the percentage of animals with colonies or in the average number of colonies per lung (compared to air control), exposure to the mixture produced an increase in the latter, suggesting to the authors that the mixture faciliated cancer cell colonization. However, the exact role played by O_3 in the mixture is not clear because a previous study had indicated that NO_2 alone facilitates blood-borne cancer cell spread to the lungs (Richters and Kuraitis, 1981). Furthermore, the experimental model used is not generally accepted as representing metastatic mechanisms.

Ichinose and Sagai (1992) also examined the ability of an O_3/NO_2 mixture to promote primary lung tumor development. Rats were injected (ip) with BHPN and then were exposed for 13 mo to a mixture of 0.05 ppm O_3 + 0.4 ppm NO_2, O_3 alone, or to clean air (chamber control). Although the NO_2 exposure was continuous, the O_3 exposure was intermittent, with the concentration altered between 0 and 0.1 ppm following a sine curve from 9 to 19 h of each day (resulting in a daily mean concentration of 0.05 ppm). One other group of rats served as a room control, maintained in a clean room for 24 mo following injection of BHPN. After an 11-mo recovery period, all animals were autopsied. Compared to clean-air-exposed animals, lung tumor incidence was increased in mice exposed to the mixture; O_3 alone did not increase the tumor incidence. However, the authors noted that tumor incidence in the room control group was not different from that in the group exposed to the mixture, and suggested that the clean air (chamber control) group should be used as a control in interpreting the data from the pollutant-exposed animals. The enhanced incidence in mixture-exposed animals was suggested to be due to synergistic increases in lipid peroxidation, which was noted in other studies (see Table 6-25). A complication in interpreting this study is that a previous study (Sagai and Ichinose, 1991) had suggested tumor development in animals exposed to an

O_3 and NO_2 mixture without BHPN, although this latter study involved a longer exposure duration and somewhat higher pollutant concentrations.

6.4.2.2 Acidic Compounds as Copollutants

Binary mixtures containing acids comprise another type of commonly examined exposure atmosphere. Most of these mixtures included acidic sulfate aerosols as the copollutant. Peak (1-h) ambient levels of sulfuric acid (H_2SO_4) are estimated at 75 $\mu g/m^3$, with longer (12-h) averages about one-third of this concentration (Spengler et al., 1989).

Earlier studies that employed simultaneous single, repeated, or continuous exposures of various animal species to mixtures of acid sulfates and O_3 found responses for several endpoints, including tracheobronchial mucociliary clearance, alveolar clearance, pulmonary mechanics, and lung morphology, to be due solely to O_3 (U.S. Environmental Protection Agency, 1986; Cavender et al., 1977; Moore and Schwartz, 1981; Phalen et al., 1980; Juhos et al., 1978). However, synergism was noted for bacterial infectivity in mice (Grose et al., 1982), for response to antigen in mice (Osebold et al., 1980), and for effects on lung protein content and the rate of collagen synthesis in rats (Last et al., 1983, 1984a; Last and Cross, 1978). More recent studies, performed since publication of the previous O_3 criteria document, support the earlier finding of synergism between O_3 and acid sulfates on lung biochemistry, and provide possible explanations for underlying mechanisms.

Last et al. (1986) exposed rats for 7 days to O_3 alone (at 0.96 ppm) and to mixtures of O_3 with one of three aerosols, sodium chloride, sodium sulfate, or ammonium sulfate [$(NH_4)_2SO_4$] (all at 5 mg/m^3); only the $(NH_4)_2SO_4$ was acidic. Lung protein content, proline content, collagen synthesis rate, fibroblast numbers in parechymal lesions, and the volume of parenchymal lesions were examined following exposure. Mixtures of O_3 with sodium chloride or sodium sulfate produced changes that did not differ from those found with O_3 alone. On the other hand, mixtures of $(NH_4)_2SO_4$ with O_3 resulted in increases in all of the measured parameters, and the increases were greater in magnitude than those due to O_3 alone; synergism was concluded, although there has been some question concerning the statistical approach used (Last, 1991a). These results suggested that acidity was necessary for synergism of the aerosols with O_3. This conclusion was further supported by demonstrating that significant interaction of O_3 with H_2SO_4, which is much more acidic than $(NH_4)_2SO_4$, occurred at lower concentrations than was noted for mixtures of O_3 and $(NH_4)_2SO_4$ (Warren and Last, 1987); interaction was suggested with H_2SO_4 concentrations as low as 40 $\mu g/m^3$ (with 0.2 ppm O_3) for some lung biochemical endpoints. The studies above did not use any specific statistical test for interaction, and conclusions of interaction were based on findings of significant differences from responses following exposure to O_3 alone and the absence of detectable responses following exposure to H_2SO_4 aerosols at the same and higher concentrations.

Warren et al. (1986) found synergistic interaction with the above endpoints following 7 days of exposure to 0.2 ppm O_3 + 5 mg/m^3 $(NH_4)_2SO_4$. However, exposure for only 3 days produced responses that were not different from those noted with O_3 alone. This seems to indicate that the duration of exposure is a factor affecting the occurrence of any interaction. However, exposure duration may also affect the type of interaction. In a study by Schlesinger et al. (1992a) in which rabbits were exposed to a mixture of 0.1 ppm O_3 + 125 $\mu g/m^3$ H_2SO_4 for 2 h/day, 5 days/week, a synergistic increase in bronchial epithelial secretory cell number was noted after 4 mo of exposure, whereas antagonism was noted following 8 mo of continued exposure.

The mechanism underlying interaction between acid sulfates and O_3 is not known. Last et al. (1986) noted that similar sites of deposition for O_3 and acid aerosols favored synergism. A synergistic response of biochemical indices in rat lung with exposure to 1,000 μg/m^3 H_2SO_4 + 0.6 ppm O_3 was found when the acid droplet diameter was 0.5 μm, whereas no increase compared to the O_3-only response was noted when the droplet diameter was 0.02 μm. Apparently, the larger particles that deposited to a greater extent within the bronchoalveolar junction, the major target site for O_3, were most interactive.

Observed synergism between O_3 and acid sulfates in rats also was suggested to be due to a shift in the local microenvironmental Ph of the lung following deposition of acid, enhancing effects of O_3 by producing a change in the reactivity or residence time of reactants, such as free radicals, involved in O_3-induced tissue injury (Last et al., 1984a). If this were the only explanation, then the effects of O_3 should be enhanced consistently by the presence of acid in an exposure atmosphere. However, in the study of Schlesinger et al. (1992b) in which rabbits were exposed for 3 h to combinations of O_3 at 0.1, 0.3, and 0.6 ppm + H_2SO_4 (0.3 μm) at 50, 75, and 125 μg/m^3, antagonism was noted in the evaluation of stimulated production of superoxide anion by AMs harvested by lavage immediately after exposure to 0.1 or 0.3 ppm O_3 in combination with 75 or 125 μg/m^3 H_2SO_4 and also for AM phagocytic activity at all of the O_3/acid combinations. Mixtures of O_3 (0.6 ppm) and another acid, HNO_3 vapor (1,000 μg/m^3), also produced antagonism for certain aspects of the function of Ams harvested from acutely exposed rats (Nadziejko et al., 1992). Although the deposition sites of both acid and O_3 should be comparable in these two studies, perhaps the particular cellular endpoints examined are subject to this type of interaction.

Last (1989) observed an apparent all-or-none response in rats exposed to the acid sulfate/O_3 mixtures. That is, there was no concentration-response relationship between the concentration of acid in the mixture and the extent of change in various endpoints, compared to effects observed with O_3 alone. In the study of Schlesinger et al. (1992b), a similar phenomenon was noted, but, in this case, the concentration of O_3 in the mixture did not always influence the response compared to that seen with acid alone. Thus, exposure-concentration-response relationships noted with individual pollutants may not necessarily hold following exposure to their mixtures. This is consistent with the results of Gelzleichter et al. (1992a) for mixtures of O_3 and NO_2.

The above studies involved simultaneous exposures to O_3 and acidic pollutants, but some studies involving sequential exposures to O_3 and acid sulfate aerosols were described in the previous O_3 criteria document. For example, Gardner et al. (1977) found an additive increase in infectivity when mice were exposed to 0.1 ppm O_3 for 3 h prior to a 2-h exposure to 900 μg/m^3 H_2SO_4, whereas no difference from air control was noted when the acid was administered prior to O_3. Grose et al. (1980) noted a reduction in ciliary activity in isolated tracheal sections obtained from hamsters exposed to 0.1 ppm O_3 for 3 h, followed by exposure to 1,090 μg/m^3 H_2SO_4 for 2 h, that was less in magnitude than that found with exposure to acid alone; O_3 alone had no effect.

Silbaugh and Mauderly (1986) examined the ability of O_3 to increase susceptibility to a subsequent exposure to H_2SO_4 in terms of producing airway constriction. Guinea pigs were exposed to 0.8 ppm O_3 for 2 h followed by H_2SO_4 (12 mg/m^3 for 1 h). An increased volume of trapped gas in the lungs (the metric of constriction) was seen with both O_3 alone and with the mixture, but the response to the latter did not differ from that due to the former, and

acid alone had no effect. Thus, in this case, preexposure to O_3 did not affect response to a subsequent exposure to acid.

Chen et al. (1991) examined the reverse exposure scenario, whether exposure to H_2SO_4 affected subsequent response to O_3. Guinea pigs were exposed to H_2SO_4 or ultrafine zinc oxide (ZnO) particles coated with H_2SO_4. A 1-h exposure to 0.15 ppm O_3 following a 1-h exposure to acid (300 $\mu g/m^3$, 0.09 μm) did not alter the response seen with acid alone, a decline in DL_{CO}. However, when single (1-h) or multiple (3-h/day, 7-day) exposures to acid-coated ZnO (24 or 84 $\mu g/m^3$ equivalent H_2SO_4) were followed by a 1-h exposure to 0.15 ppm O_3, the effect on DL_{CO} appeared to be greater than additive, although no specific statistical test for interaction was performed. This study suggested that prior exposure to acid increased the susceptibility of the guinea pig to subsequent exposure to O_3, but it also showed that the manner in which the acid was delivered affected whether or not any interaction occurred. It is likely that the number of particles was greater in the ZnO-H_2SO_4 aerosol than in the H_2SO_4 aerosol, and the interaction may reflect this greater particle number.

6.4.2.3 Other Copollutants

Although the bulk of the database for binary mixtures of O_3 involves NO_2 or acids, a few studies examined responses to combinations of O_3 with other pollutants.

Reuzel et al. (1990) exposed rats to mixtures of O_3 (0.2, 0.4, or 0.8 ppm) + HCHO (0.3 to 3.0 ppm). Although exposure to the mixtures did not alter the nature or extent of histological lesions, (cilia loss and epithelial hyperplasia) compared to exposure to each pollutant alone, a site-specific synergistic increase in turnover of nasal epithelial cells was found with all concentrations of HCHO together with 0.4 ppm O_3. A lack of such response with 0.8 ppm O_3 was ascribed to an O_3-induced alteration in breathing pattern, which reduced the delivered dose. It was, however, noted that interaction occurred only when one constituent of the mixture was administered at cytotoxic concentrations, an exposure scenario that rarely occurs in ambient air. In any case, the authors concluded that because cell proliferation likely plays a role in carcinogenesis, and that if mixtures potentiate cell proliferation, then exposure to pollutant mixtures may increase cancer risk.

Mautz et al. (1988) exposed rats for 3 h, both at rest and with exercise, to a mixture of 0.6 ppm O_3 + 10 ppm HCHO. A synergistic increase in nasal epithelial cell turnover followed exposure with exercise, whereas exposure at rest resulted in no difference from that seen with HCHO alone. Likewise, exposure to the mixture with exercise resulted in an increase in the number of focal lesions in lung parenchyma compared to either O_3 or HCHO alone, but exposure at rest resulted in a lower incidence of lesions than seen with O_3 alone. This latter observation was ascribed to an effect of HCHO on breathing pattern, producing a change in inhaled dose of O_3 that did not occur with exercise.

Nishikawa et al. (1992) examined the effect of sequential exposure to cigarette smoke and O_3 in altering airway responsivity to inhaled bronchoconstrictor challenge and tracheal vascular permeability in guinea pigs. Animals were exposed to 1 ppm O_3 for 0.5 h, followed by 5 puffs of cigarette smoke, or to 1 ppm O_3 for 1.5 h, followed by 10 puffs. Exposure to O_3 and five puffs increased responsivity and vascular permeability immediately after exposure, whereas no effect on either endpoint was noted with either pollutant given alone. Exposure to O_3 and 10 puffs also increased responsivity and permeability, but to the same extent as did the lower concentration mixture or exposure to O_3 alone, whereas exposure to 10 puffs of smoke only increased responsivity. Thus, sequential exposure to O_3 and

cigarette smoke enhanced the magnitude of response compared to either pollutant alone, but the duration of response was not altered.

The potential role of O_3 in enhancing fibrotic lung disease by interaction with silica was examined by Shiotsuka et al. (1986), who exposed rats with developing silica-induced fibrosis to O_3 at 0.8 ppm for 6 h/day, 5 day/week for 37 exposure days. Silica had been instilled (2, 12, or 50 mg) on Day 1 of the study and exposure to O_3 began on Day 3 or 4 postinstillation. There was found to be no interaction between silica and O_3 in development of fibrosis, as assessed biochemically (lung content of hydroxyproline) or histopathologically. Although an increase was found in the ratio of hydroxyproline to total protein in the group exposed to the mixture and instilled with the highest amount of silica, this was not considered by the authors to be biologically significant.

6.4.3 Complex (Multicomponent) Mixtures Containing Ozone

Ambient pollution in most areas is a complex mix of more than two chemicals, and a number of studies have examined the effects of exposure to multicomponent atmospheres containing O_3. Some of these attempted to simulate photochemical reaction products occurring under actual atmospheric conditions. However, the results of these studies are often difficult to interpret due to chemical interactions between the components, as well as the resultant production of variable amounts of numerous secondary reaction products, and a lack of precise control over the ultimate composition of the exposure environment. In addition, the role of O_3 in the observed biological responses is often obscure.

One type of experimental multicomponent atmosphere that has been examined is ultraviolet-irradiated and nonirradiated automobile exhaust mixtures. Irradiation leads to the formation of photochemical reaction products that are biologically more active than those in nonirradiated mixtures. Such mixtures are characterized by total oxidant concentrations (expressed as O_3) in the range of 0.2 to 1.0 ppm. Although the effects described following exposure were not necessarily uniquely characteristic of O_3, and, although O_3 could have been responsible for some, or even most of them, in most cases, the biological effects have been difficult to associate with any one particular component. Effects of exhaust mixtures on different species have been discussed in the previous O_3 criteria document (U.S. Environmental Protection Agency, 1986). Pulmonary function changes were demonstrated in guinea pigs after short-term exposures to irradiated exhaust and in dogs after long-term exposure to both irradiated and nonirradiated exhaust mixtures.

Additional studies of complex mixtures have been performed since publication of the previous O_3 criteria document. Kleinman et al. (1985) exposed rats (Sprague-Dawley, male, 7 weeks old, nose-only) for 4 h to atmospheres designed to represent photochemical pollution and consisting of 0.6 ppm (1,180 $\mu g/m^3$) O_3 + 2.5 ppm (4,700 $\mu g/m^3$) NO_2 + 5.0 ppm (13,100 $\mu g/m^3$) sulfur dioxide (SO_2) + particles. The particulate phase consisted of 1,000 $\mu g/m^3$ of either H_2SO_4 or $(NH_4)_2SO_4$ laced with iron sulfate [$Fe_2(SO_4)_3$] and manganese sulfate ($MnSO_4$). The metallic salts act as catalysts for the conversion of sulfur IV into sulfur VI and for the incorporation of gases into the aerosol droplets. The respiratory region was examined for morphological effects. A confounding factor in these studies was the production of HNO_3 vapor in atmospheres that contained O_3 and NO_2, a phenomenon discussed previously, and nitrate in those that contained O_3 and $(NH_4)_2SO_4$, but not NO_2. Nevertheless, a significant enhancement of tissue damage was noted with exposure to atmospheres containing H_2SO_4 or secondarily produced HNO_3 compared to those containing $(NH_4)_2SO_4$, a less acidic

compound. In addition, there was some suggestion that the stronger acidic atmospheres resulted in a greater area of the parenchyma becoming involved in lesions, which were characterized by a thickening of alveolar walls, cellular infiltration in the interstitium, and an increase in free cells within alveolar spaces. An increased rate of nasal epithelial cell turnover was noted following exposure to atmospheres containing particulate acids compared with exposure to either O_3 alone or to a mixture of O_3 + NO_2. Furthermore, exercise seemed to potentiate the nasal and parenchymal responses to the complex mixtures containing strong acids (Kleinman et al., 1989), a finding similar to that with the previously discussed mixtures of O_3 and NO_2 or O_3 and HCHO.

Bhalla et al. (1987) examined the effects of a seven-component atmosphere (similar to that above) on epithelial permeability of rat lungs (Sprague-Dawley, male, 47 to 52 days old). The animals were exposed for 2 h (chambers, relative humidity [RH] = 85%) to the following: O_3 (0.6 ppm) + NO_2 (2.5 ppm) + SO_2 (5.0 ppm) + ferric oxide (Fe_2O_3) (241 $\mu g/m^3$) + $(NH_4)_2SO_4$ (308 to 364 $\mu g/m^3$) + $Fe_2(SO_4)_3$ (411 to 571 $\mu g/m^3$) + $MnSO_4$ (7 to 9 $\mu g/m^3$). The response to this mixture was compared to that following exposure to O_3 (0.6 ppm) + NO_2 (2.5 ppm), O_3 alone (0.6 or 0.8 ppm), or NO_2 alone (6 or 12 ppm). As above, the complex mixture was found to result in production of HNO_3, in this case at measured concentrations of 1,179 to 2,558 $\mu g/m^3$ (0.46 to 1.02 ppm); the O_3 + NO_2 atmosphere also resulted in some HNO_3 vapor formation. Epithelial permeability was found to increase immediately following exposure to O_3, O_3 + NO_2, or to the complex mixture. Although the magnitude of this change was similar following exposure to O_3 alone or in combination with other pollutants, there was increased persistence of effect after exposure to either the binary or complex mixture.

Prasad et al. (1988) used a similar multicomponent atmosphere and examined effects on AM surface receptors. Rats (Sprague-Dawley, male, 200 g) were exposed for 4 h/day, for 7 or 21 days to a mixture of O_3 (0.3 ppm) + NO_2 (1.2 ppm) + SO_2 (2.5 ppm) + $(NH_4)_2SO_4$ (270 $\mu g/m^3$) + $Fe_2(SO_4)_3$ (220 $\mu g/m^3$) + $MnSO_4$ (4 $\mu g/m^3$) + Fe_2O_3 (150 $\mu g/m^3$), or to O_3 alone. Both the mixture and O_3 alone resulted in a decrease in Fc receptor activity beginning immediately after the last exposure. Exposure to the complex atmosphere for 7 days resulted in a response similar to that seen with O_3 alone, but continued exposure to this mixture for up to 21 days resulted in an even greater reduction in receptor function compared to O_3 alone. However, as with most studies of complex mixtures, although the response to the mixture was different from that found with O_3, the role of other constituents was not clear. Phagocytic function of AMs was also examined following exposure to the mixture, but there were no O_3-only controls for comparison.

Mautz et al. (1985a) examined the effects of a complex mixture on pulmonary mechanics in exercising dogs. Exposures (nose-only) were for 200 min to a mixture of O_3 (0.45 to 0.7 ppm) + SO_2 (4.8 to 5.2 ppm) + H_2SO_4 (800 to 1,200 $\mu g/m^3$, 0.2 μm) + catalytic salts of $Fe_2(SO_4)_3$ and $MnSO_4$. A greater increase in resistance and decrease in compliance was found with the complex atmosphere than with O_3 alone, but the effect was ascribed to the presence of H_2SO_4. Although synergism was implied, it could not be concluded definitively because the mixture was not tested without O_3.

Mautz et al. (1991) further examined the ability of components of acidic fogs to alter the response to O_3. Rats (Sprague-Dawley, male, 7 weeks old, n = 12/group) were exposed for 4 h (nose-only; temperature = 22 to 23 $^\circ$C, RH = 82 to 83%) to 0.4 ppm O_3 or to a mixture of 0.4 ppm O_3 + 670 $\mu g/m^3$ HNO_3 vapor + 610 $\mu g/m^3$ H_2SO_4 particles (0.32

m). Exposure to either O_3 or the mixture resulted in comparable changes: development of a rapid, shallow breathing pattern; a decrease in fatty acid composition of pulmonary surfactant; and focal parenchymal lesions with thickened alveolar septa and cellular infiltration. The lack of any modulation of the O_3-induced effects by acids prompted the authors to raise the question of the sensitivity of rats to inhaled acids. Although responses to any pollutant are somewhat species dependent, there is some evidence that rats are not the most sensitive species to acidic aerosols (U.S. Environmental Protection Agency, 1989). As discussed previously, the extent of interaction within any one species of animal is endpoint dependent, and it is likely that the sensitivity of various endpoints is species dependent. Thus, rats do show biochemical changes (e.g., in collagen metabolism) with exposure to fairly low levels of acidic aerosols in combination with O_3 (see Table 6-10), although these involved longer duration exposures. In any case, the underlying reasons for the lack of interaction in the complex-mixture study above remain unclear.

Kleinman et al. (1989) exposed rats (Sprague-Dawley, male, 7 weeks old, nose-only) to a mixture of O_3 (0.8 ppm) + SO_2 (5.0 ppm) + H_2SO_4 or $(NH_4)_2SO_4$ (1,000 $\mu g/m^3$) at high RH (85%) and noted a delay in early clearance of inert particles from the lungs, compared to air-exposed controls. However, it is difficult to relate any effects to the O_3 because responses to O_3 alone were not examined.

The ideal complex mixture is one that actually exists in the ambient environment. Saldiva et al. (1992) exposed rats (Wistar, male, 2 mo old) for 6 mo to actual atmospheres of São Paulo, Brazil, with controls maintained for the same period of time in a clean, rural environment. The mean pollution levels over the exposure period were as follows: 0.011 ppm O_3, 1.25 ppm CO, 29.05 $\mu g/m^3$ SO_2, and 35.18 $\mu g/m^3$ particles. The animals exposed to the urban air showed evidence of bronchial secretory cell hyperplasia, ciliary structural changes, increased viscosity of mucus, and impaired mucociliary clearance. Although chronic exposure to air pollution may result in pulmonary dysfunction, the specific components producing the response could not be determined.

Inhalation exposures to air pollutants are, of course, the ideal way to assess interaction, but in vitro exposures may provide indications of potential interactions. Shiraishi and Bandow (1985) exposed Chinese hamster V79 cells for 2 h to photochemical reaction products produced from the reaction of propylene and NO_2 in a smog chamber. The resultant exposure atmospheres consisted of various proportions of propylene (0.07 to 0.16 ppm), NO_2 (0.22 to 0.28 ppm), O_3 (0.09 to 0.38), PAN (0.04 to 0.41 ppm), and HCHO (0.23 to 1.50 ppm). Exposures to NO_2 and O_3 alone also were performed. All of the complex mixtures resulted in an increased frequency of sister chromatid exchange and growth inhibition. The effects of the mixture were greater than those due to either O_3 or NO_2 alone for sister chromatid exchange, but growth inhibition was similar to that induced solely by O_3. The authors concluded that the observed effects were not due to any single compound within the mixture, but rather to various compounds producing multiple effects.

6.4.4 Summary

It is difficult to summarize the role that O_3 plays in response to exposure to binary mixtures, and it is even harder to determine its role in response to multicomponent atmospheres. One of the problems in understanding interactions is that, although the specific mechanisms of action of the individual pollutants within a mixture may be known, the exact bases for toxic interactions have not been elucidated clearly. There are, however, certain

generic mechanisms that may underlie pollutant interactions. One is physical, involving adsorption of one pollutant onto another and subsequent transport to more or less sensitive sites or to sites where one of the components of the mixture normally would not deposit in concentrated amounts. This, however, probably does not play a major role in O_3-related interactions. A second mechanism involves production of secondary products that may be more toxicologically active than the primary materials. This has been demonstrated or suggested in a number of studies as a basis for interaction between O_3 and NO_2. A third mechanism involves biological or chemical alterations at target sites that affect response to O_3 or the copollutant. This has been suggested to underlie interactions with mixtures of O_3 and acid sulfates. A related mechanism is an O_3- or copollutant-induced physiological change, such as alteration in ventilation pattern, resulting in changes in the penetration or deposition of one pollutant when another is present. This has been implicated in enhanced responses to various O_3-containing mixtures with exercise.

Evaluation of interactions between O_3 and copollutants is a complex procedure. Responses are dependent on a number of host and environmental factors, such that different studies using the same copollutants may show different types or magnitudes of interactions. The occurrence and nature of any interaction is dependent on the endpoint being examined and is also highly related to the specific conditions of each study, such as animal species, health status, exposure method, dose, exposure sequence, and the physicochemical characteristics of the copollutants. Because of this, it is difficult to compare studies, even those examining similar endpoints, that were performed under different exposure conditions. Thus, any description of interactions is really valid only for the specific conditions of the study in question and cannot be generalized to all conditions of exposure to a particular chemical mixture. Furthermore, it is generally not possible to extrapolate the effect of pollutant mixtures from studies on the effects of each component when given separately. In any case, what can be concluded from the database is that interactions of O_3-containing mixtures are generally synergistic (antagonism has been noted in a few studies), depending on the various factors noted above, and that O_3 may produce more significant biological responses as a component of a mixture than when inhaled alone. Furthermore, although most studies have shown that interaction occurs only at higher than ambient concentrations with acute exposure, some have demonstrated interaction at more environmentally relevant levels (e.g., 0.05 to 0.1 ppm O_3 with NO_2) with repeated exposures.

6.5 Summary and Conclusions

6.5.1 Introduction

In the past 30 years, thousands of research studies on the effects of O_3 in laboratory animals have been reported in the literature. This body of evidence presents a clear picture of the types of alterations O_3 can cause on respiratory tract host defense mechanisms, biochemistry, structure, and lung function. Less is known about carcinogenic potential and effects on organs distant from the lungs. These types of effects are observed in many animal species from mice to nonhuman primates, lending credence to the qualitative extrapolation of these effects to humans. The major issue is what levels, durations, and patterns of exposure are capable of causing these effects in humans. Extrapolation is discussed in Chapter 8. Suffice it to say here that the animal toxicological studies assist in interpreting observations

made in O_3-exposed humans and extend the knowledge of potential human hazards that never can be studied adequately in humans.

This summary and conclusion section deals exclusively with the effects of O_3, alone and in mixture. Other photochemical oxidants either have been evaluated elsewhere (NO_2 and HCHO; U.S. Environmental Protection Agency, 1993; Grindstaff et al., 1991) or in an earlier O_3 criteria document (U.S. Environmental Protection Agency, 1986). This section is organized by molecular mechanisms of effects, respiratory tract effects, systemic effects, and effects of mixtures. Generally, it is an interpretative, factual summary of the array of effects observed in animals. Chapter 8 presents the current state of extrapolation of these effects to humans, and Chapter 9 integrates knowledge from animal toxicology, epidemiology, and human clinical studies.

Together, this chapter and the animal toxicological chapter in the 1986 document (U.S. Environmental Protection Agency, 1986), contain more than 1,000 references. Although all of them contribute to choosing and understanding the key issues to be summarized here, there obviously must be a highly selective choice made as to which references to include here. Generally, the papers discussed here were selected either because they represent the lowest effective concentration for an endpoint or they significantly influenced a particular conclusion.

6.5.2 Molecular Mechanisms of Effects

Molecular mechanisms (the manner in which chemical reactions of O_3 are translated into biological effects) are alluded to in different sections of this document. Studies that link O_3 chemistry with O_3 effect measurements would greatly strengthen the theoretical basis for understanding the biological effects of O_3. They also would allow examination of the similarity between animals and humans, thus strengthening interspecies extrapolations. Ozone has been shown to react directly with a variety of biomolecules that are present in both animals and humans. Most of the attention has been centered on polyunsaturated fatty acids and carbon-carbon double bonds, although reactions with sulfhydryl, amino, and some electron-rich compounds may be equally important. Free radicals may be involved, and antioxidant defenses appear to lessen the effect of these reactions. A "molecular target" for O_3 (the biomolecules most affected by reaction with O_3 or most crucial in mediating the observed responses) has not been identified for any of the endpoints studied. In fact, the target may be different for different endpoints.

An important concept in evaluating molecular targets was elucidated recently by Pryor (1992), who suggests (based on reaction and diffusion rate data) that the O_3 molecule does not penetrate through cell membranes or even the surfactant layer of the lung. Instead, a "reaction cascade" forms intermediates (organic or oxygen-free radicals, lipid hydroperoxides, aldehydes, hydrogen peroxide, etc.), which penetrate into the cells, causing the biological effects observed (Pryor et al., 1991). Confirmation of such O_3-induced free-radical autoxidation of lipids has been sought in vivo, but the indirect nature of the measurement methods produced equivocal results. More direct evidence has been obtained by Kennedy et al. (1992), who used electron spin-trapping methods to measure a concentration-related increase in radical adducts of the lipid fraction of lungs from O_3-exposed rats. Increased radical signals were detected after a 2-h exposure to 0.5 ppm O_3, but because the rats' respiration was stimulated by CO_2, the effective dose would be greater than it appears. Oxidized (oxygenated) biomolecules that result from reaction with O_3 also may mediate the

effects of O_3. Studies by Hatch et al. (1994) show that crude fractions of the lung lining layer become labeled with oxygen-18 after exposure to oxygen-18-labeled O_3. The label is concentrated in the airway lining layers, and the amount of oxygen-18 incorporation in this layer appears to be correlated with effects of O_3 (permeability and inflammation) in both rats and humans. These findings are consistent with the hypothesis that O_3 reacts with the lining of the lung, that the same types of interactions occur in both animals and humans, and that these reactions lead to similar effects.

6.5.3 Respiratory Tract Effects

6.5.3.1 Effects on Host Defenses

Several systems defend the respiratory tract of the host against infectious and neoplastic disease as well as nonviable inhaled particles; all of these systems can be affected by O_3. The mucociliary clearance system moves particles deposited on the mucous layer (either through deposition from the air stream or entry of cells or cellular debris from the alveoli) upwards and out of the lower respiratory tract. The nasal passages also have an effective clearance system. Concentrations as low as 0.15 ppm O_3 (8 h/day, 6 days) caused structural changes in the nasal respiratory epithelium (e.g., ciliated cell necrosis, shortened cilia) of monkeys (Harkema et al., 1987). Ciliated cells also are lost or damaged in the conducting airways of the lower respiratory tract after short exposures (e.g., 0.96 ppm O_3, 8 h, monkeys; Hyde et al., 1992). Mucous chemistry also is changed (McBride et al., 1991). Sufficient morphologic damage would be expected to have functional consequences. Acute exposures (0.6 ppm O_3, 2 h) slow mucociliary particle clearance in rabbits, but repeated exposures (up to 14 days) caused no effects. Alveolar clearance is slower and involves clearance of particles through interstitial pathways to the lymphatic system or movement of particle-laden AMs up to the bottom of the mucociliary escalator. Effects on alveolar clearance are concentration-dependent. A single 2-h exposure of rabbits to 0.1 ppm O_3 accelerated clearance up to 14 days postexposure, exposure to 0.6 ppm caused no effect, and a higher concentration (1.2 ppm) slowed alveolar clearance (Driscoll et al., 1986). Alveolar clearance of asbestos particles was slowed by a 6-week exposure to an urban pattern of O_3 (Pinkerton et al., 1989).

Alveolar macrophages are the first line of defense against microbes and nonviable particles that reach the pulmonary region of the lung. They phagocytize particles, kill microbes, and interact with lymphocytes in the development of an immune response. Thus, their proper functioning is critical. Alveolar macrophages from several species of animals exposed acutely to O_3 can exhibit decreased phagocytosis; decreased lysosomal enzyme activities and superoxide anion radical production, both of which function in killing bacteria; alterations in membrane morphology; chromosomal damage; decreased cytotoxicity to tumor cells; increased release of PGE_2 and $PGF_{2\alpha}$; and alterations in the number of AMs. Phagocytic changes are the most investigated. Exposure of rabbits to a level as low as 0.1 ppm O_3 (2 h/day) decreased nonspecific phagocytosis (of latex microspheres) after 2 or 6 but not 13 days of exposure (Driscoll et al., 1987). Recovery from the single 2-h exposure to 0.1 ppm O_3 was complete by 7 days postexposure. This pattern of response was confirmed in mice for Fc-receptor mediated phagocytosis (Gilmour et al., 1991; Canning et al., 1991).

The humoral- and cell-mediated immune system of the lung also is affected by O_3. Generally, T-cell-dependent immunity is more susceptible than B-cell-dependent immunity, but most immune functions examined have exhibited effects. However, because relatively few studies have been conducted, it currently is not possible to adequately interpret the impacts of

O_3-induced alterations on the immune system (e.g., decreases in mitogenic responses of T cells, alterations in T:B-cell ratios in the MLN). Only a few studies have attempted to correlate immunological changes and infectious disease outcome. Van Loveren et al. (1988) infected rats with Listeria, exposed them to O_3 for 1 week (0.26 to 1.02 ppm) and measured several endpoints. Ozone concentrations of 1.02 and 0.77 ppm, respectively, increased Listeria-induced mortality and severity of pathologic lesions in the lung and liver. They interpreted these findings as due to O_3-induced impaired clearance of the bacteria caused by decreased AM function and decreased cellular immunity (e.g., decreased delayed-type hypersensitivity and decreased T:B-cell ratios in MLN).

A reasonably large body of evidence indicates that the impact of O_3 on one or several host defense mechanisms leads to the inability of animals to fight bacterial infection and alters the course of viral infection. Antibacterial models are more commonly used. Mice exposed for 3 h to 0.4 ppm O_3 have decreased intrapulmonary killing of *S. zooepidemicus* (Gilmour et al., 1993a; Gilmour and Selgrade, 1993). Similar results have been obtained for *S. aureus* at a slightly higher concentration (Goldstein et al., 1971b). Correlations have been made between O_3 exposure and decreases in AM phagocytosis, decreases in bactericidal activity, growth of bacteria in the lungs, presence of bacteria in the blood, and mortality in mice (Coffin and Gardner, 1972; Gilmour and Selgrade, 1993). The lowest O_3 exposure causing increased streptococcal-induced mortality is 0.08 ppm for 3 h in mice (Coffin et al., 1967; Coffin and Gardner, 1972; Miller et al., 1978). However, prolonged intermittent exposure to 0.1 ppm O_3 for 15 weeks only slightly increased the mortality (Aranyi et al., 1983), and continuous exposure for 15 days to 0.1 ppm with two daily 1-h peaks (5 days/week) to either 0.3 or 0.5 ppm did not enhance mortality in the same model system (Graham et al., 1987). Prolonged exposure (1 to 2 weeks) also did not affect bactericidal activity to *S. aureus* (Gilmour et al., 1991).

Generally, short-term exposure to O_3 does not affect viral titers in the lungs of mice infected with influenza virus; however, reduced numbers of lung tissue T and B cells will reduce antibody titers to the virus, and mortality, lung pathology, and increased lung wet weight do occur (Selgrade et al., 1988; Jakab and Hmieleski, 1988). Ozone also enhances postinfluenzal alveolitis and structural changes that begin at 30 days postinfection (Jakab and Bassett, 1990). The complexity of the interaction of viral infection and O_3 exposure is further illustrated by Selgrade et al. (1988), who found that the effects of O_3 on influenza virus infection were dependent on the temporal relationship of O_3 exposure and day of infectious challenge. Also, interferon, which can be induced by viral infection, mitigates the O_3-induced lung lessons in mice, raising the possibility that certain stages of viral infection may have interactions with the lung that are different from other stages (Dziedzic and White, 1987b).

6.5.3.2 Effects on Inflammation and Permeability

The barrier function of the respiratory tract is disrupted by O_3, allowing cellular and fluid components from the blood to enter the lung and allowing certain types of substances in the lung to enter the blood. Markers of inflammation generally included increased proteins and PMNs in BAL. Concurrent with these events, but not necessarily interdependently, AMs liberate more arachidonic acid, which results in the production of biologically active LTs and PGs. Similar responses are observed in mice, rats, rabbits, guinea pigs, hamsters, and nonhuman primates. After acute exposure, the lowest effective concentration that increases BAL protein and number of PMNs is 0.12 ppm O_3 (mice, 24 h of exposure, BAL immediately

after exposure) (Kleeberger et al., 1993a). However, the increase in BAL protein typically is maximal roughly 16 to 24 h postexposure. In rats exposed to 0.8 ppm O_3 for 6 h and examined by lavage and morphometry several times postexposure, the increase in nasal PMNs occurs sooner and wanes about the time that these cells are increasing in number in the lungs (Hotchkiss et al., 1989a,b). It should be recognized that BAL can enable measurement of the protein and cells accessible by lavage, including the resident material (i.e., may include protein from O_3-induced cellular destruction) and the material entering from the tissue or circulation. Thus, interstitial inflammation, which has been observed in several species microscopically, is not detectable by BAL.

Several C × T studies have been conducted in mice using BAL protein as an endpoint. In two studies, there was combination of various Cs (0.1 to 2.04 ppm O_3) and Ts (1 to 12 h), resulting in a number of different C × T products (Rombout et al., 1989; Highfill et al., 1992). Both of these studies showed that the influence of T increased as C increased (i.e., there was no simple relationship of $C^a \times T^a$ = constant product; however, at the lowest C × T products, there was a more equivalent influence of C and T). Gelzleichter et al. (1992b) used a single C × T product composed of a variety of Cs and Ts for up to 3 days of exposure. The 24 h/day exposure group had less response than the other groups that responded equivalently. Effects of longer term exposure on permeability and inflammation are more complex to interpret (also see subsequent discussion on lung structure). Histological examination of rat lungs exposed to 0.5 ppm O_3 (2.25 h/day) showed more inflammatory cells in the alveoli after 5 days of exposure than after 1 day of exposure (Tepper et al., 1989). In contrast, the increase in BAL PMNs that occurred after Day 1 of exposure of rats had resolved by Day 4 (7 h/day) (Donaldson et al., 1993).

Some studies suggest that, although protein and PMN increases are observed concurrently, this may be more a function of experimental design than the actual biological sequence of events. For example, in rats depleted of PMNs with anti-PMN serum, O_3 did not increase BAL PMNs, but BAL protein still was increased (Pino et al., 1992b). Also Young and Bhalla (1992) observed an increase in tracheal protein earlier than increased tracheal PMNs. They interpreted this and other related results to suggest that the recruited PMNs may serve to sustain an increase in permeability.

6.5.3.3 Effects on Structure, Function, and Biochemistry

Theoretically, and in some cases empirically, lung structure, function, and biochemistry are linked. Correlations are not exact because of differences in available measurement methods (e.g., most lung function tests used do not measure sensitively the function of the smallest airways, where the "classical" O_3 lesion is observed) and some independence of effects (e.g., a transient change in breathing frequency would not be morphologically detectable). Also, most biochemical measurements are made of whole lung, rather than focal areas of damage, and only some enzyme activities measured would be expected to be correlated to structure or function (e.g., collagen metabolism, antioxidant metabolism).

After acute exposure to O_3, the most commonly observed effect in several species is tachypnea (increased f and decreased V_T) with little (if any) change in $\dot{V}_E$; the lowest exposure causing tachypnea was 0.2 ppm O_3 for 3 h in rats (Mautz and Bufalino, 1989). Other effects reported after acute exposure to ≥1 ppm include increased R_L and decreased Cdyn, TLC, VC, FRC, RV, FVC, DL_{CO}, and the multibreath N_2 slope (e.g., Fouke et al., 1991; Mautz et al.,

1985b; Miller et al., 1988). However, these changes are not observed in all studies, probably due to differences in animal species, measurement method, and exposure protocols. With rare exception, concentrations well in excess of 1 ppm O_3 are required to increase airway reactivity.

Two C × T studies of pulmonary function using acute exposure periods have been performed. Costa et al. (1989) found that FVC, DL_{CO}, and the multibreath N_2 slope decreased with increasing C × T products in rats and that the influence of T is greater at higher Cs. In guinea pigs, Nishikawa et al. (1990) observed that airway responsiveness to methacholine increased at higher C × T products (e.g., at 90 but not at 30 ppm · min); the authors concluded that T was an important factor in the O_3 response.

When rats were exposed for 5 days (2.25 h/day, with CO_2 to stimulate ventilation equivalent to light exercise in humans) to 0.35, 0.5, and 1.0 ppm O_3, the change in shape of the flow-volume curve occurred and tachypnea peaked on Days 1 and 2, but by Day 5, there was no difference from control (except at 1 ppm) (Tepper et al., 1989). This attenuation is similar to that observed in humans. However, in other, similar groups of animals, histological changes in the lung progressed, and BAL protein remained elevated. Other similarities between laboratory animals and humans in their pulmonary function responses to short-term O_3 exposure are explored in Chapter 8.

Ozone causes similar types of alterations in lung morphology in all laboratory animal species studied. The most affected cells are the ciliated epithelial cells of the airways and Type 1 cells in the gas exchange region. Within the nasal cavity, anterior portions of the respiratory and transitional epithelium are affected. Cilia are lost or damaged; some ciliated cells become necrotic, are lost, and are replaced with nonciliated cells. Mucus-secreting cells are affected.

The CAR (the junction of the conducting airways and the gas exchange region) is a primary target, possibly because it receives the greatest dose of O_3 delivered to the lower respiratory tract (see Chapter 8) and has Type 1 epithelial cells covering a large surface area. Even though there are significant interspecies differences in the structure of the CAR (e.g., primates, including humans, have RBs, which are rudimentary or absent in laboratory animals such as rats or mice), it is the target in all species studied. Exposure to O_3 causes loss of cilia or necrosis of the ciliated cells, leaving a bare basement membrane that is replaced by nonciliated bronchiolar cells, which may become hyperplastic after longer exposures. Mucous secreting cells can be affected, but not as significantly as ciliated cells. Type 1 cells also are damaged and can be sloughed from the surface; Type 2 cells, which are thicker, replace them. Sometimes, Type 2 cells differentiate into Type 1 cells. This epithelial remodeling is accompanied by an inflammatory response in the CAR, primarily consisting of an increase in number of PMNs in the earlier stages and an increase in number of AMs in later stages; interstitial edema occurs. With increased duration of exposure, alveolar septa in the CAR thicken due to increased matrix, basement membrane, collagen, and fibroblasts and a thickened alveolar epithelium.

These patterns of change have different relationships to duration of exposure, as illustrated by Dungworth (1989) (see Figure 6-3; Section 6.2.4.5). Inflammatory changes peak after a few days of O_3 exposure; are still observable, but to a much lesser degree, in tissue during months of exposure; and begin to return to control values after exposure ceases. In contrast, epithelial hyperplasia rapidly increases during about the first week of exposure, plateaus as exposure continues, and begins to decrease slowly when exposure stops. Interstitial

fibrosis requires months of exposure to be observed microscopically and increases slowly, but when exposure ceases, interstitial fibrosis still can persist or continue to increase. Numerous studies using several different species and experimental approaches support these findings. Only a few of the studies (primarily those using more sensitive morphometric measurements) are used here to illustrate key points and to show correlations with pulmonary function and lung biochemistry. Only rat and nonhuman primate studies are discussed because most investigations were conducted on them. At equivalent exposures, nonhuman primates appear to be more responsive than rats (Section 6.2.4).

Generally, short-term exposures to concentrations 0.2 ppm O_3 do not cause changes detectable by LM in the nasal cavities of rats or nonhuman primates, except for inflammation and an occasional delayed postexposure finding of mild hyperplasia. For example, Hotchkiss et al. (1989a) reported inflammation in the nasal epithelium of rats up to 66 h after a 6-h exposure to levels as low as 0.12 ppm O_3; there was no necrosis, loss of cilia, or hyperplasia even at 1.5 ppm. After 3 days (22 h/day) of exposure, 0.4 ppm caused loss of cilia and hyperplasia and metaplasia of the nasal epithelium of rats (Reuzel et al., 1990). Nonhuman primates appeared to be more responsive. Harkema et al. (1987) observed that exposure to 0.15 or 0.3 ppm O_3 for 6 or 90 days (8 h/day) caused necrosis of ciliated cells, shortened cilia, and increased mucous granule cells in the respiratory epithelium; alterations in cell numbers also were found in the transitional epithelium.

Within the CAR, a number of alterations occur. In rats and monkeys, ciliated and Type 1 cells become necrotic and are sloughed from the epithelium as early as the first 2 to 4 h of an exposure to about 0.5 ppm O_3 (Stephens et al., 1974a,b). Repair, as shown by increased DNA synthesis by nonciliated bronchiolar and Type 2 cells, begins by about 18 to 24 h of exposure (Evans et al., 1976a,b; Stephens et al., 1974a; Castleman et al., 1980), although cell damage continues (Castleman et al., 1980). The lesion is fully developed by about 3 days of continuous exposure, after which the rate of repair exceeds the rate of damage. The increase in antioxidant enzyme activities (e.g., succinate oxidase, G6PD, and 6PGD) parallels the increase in Type 2 cells, which are rich in these enzymes; the increase in the Type 2 cell population is probably responsible for these biochemical changes (Bassett et al., 1988a; U.S. Environmental Protection Agency, 1986).

Lesions in the CAR are one of the hallmarks of O_3 toxicity, having been well established. The study by Chang et al. (1992) provides examples of some of the patterns of cellular alterations. Chang et al. (1992) exposed rats to an urban pattern of O_3 (0.06 ppm background, 7 days/week on which were superimposed 9-h peaks [5 days/week] slowly rising to 0.25 ppm) for 78 weeks and made periodic examinations of the CAR TB and proximal alveoli by TEM morphometry during and after exposure. Type 1 cells had a larger volume at Week 13 and increased numbers at Weeks 13 and 78; there were no such changes at 17 weeks after exposure ceased. Type 2 cell volume per area of basement membrane increased immediately after Week 78 and was still increased 17 weeks after exposure ceased. Interstitial cells and matrix were increased after Weeks 1, 13, and 78, but returned to control by 17 weeks after exposure ceased. However, epithelial and endothelial basement membrane were thickened and accompanied by increased collagen fibers at the later examination times and 17 weeks after the 78-week exposure ended. In TBs, surface areas of ciliated and nonciliated cells decreased during exposure. Pulmonary function studies conducted in identically exposed groups of rats were consistent with the morphometric findings (Tepper et al., 1991). Generally, expiratory resistance was increased (suggesting central airway narrowing), but it

was only significantly different from control at 78 weeks. Tidal volume was reduced at all evaluation times. Overall, breathing frequency was reduced, but no single evaluation time was significant. Monkeys exposed to a higher concentration of O_3 (0.64 ppm, 1 year) also showed increased resistance and decreased flows, which were interpreted as central and peripheral airway narrowing; during a 3-mo postexposure period, decreases in static lung compliance persisted (Wegner, 1982).

Several studies have demonstrated distal airway remodeling. This bronchiolization of CAR alveoli is so named because bronchial epithelium replaces the Type 1 and 2 cells typical of ADs, resulting in the appearance of RBs in rats and increased volume fraction and volume of RBs in monkeys. This has been observed at exposures as low as 0.5 ppm O_3 (50 days) in rats (Moore and Schwartz, 1981) and as low as 0.25 ppm (8 h/day, 18 mo) in monkeys (Tyler et al., 1988). Inflammation occurs concurrently, perhaps indicating an influence on remodeling. In monkeys, such bronchiolization can persist 6 mo after the end of a 1-year (8 h/day) exposure to 0.64 ppm (Tyler et al., 1991b).

Exposure regimens can have unexpected impacts on experimental outcomes. Several investigations of combinations of O_3 "episodes" or O_3 "seasons" with clean-air periods have been examined. In the first of these, Last et al. (1984b) compared air control rats to two groups of rats exposed to 0.96 ppm. One group received a 90-day (8 h/day) exposure ("daily"); the other group had intermittent units of 5 days of O_3 (8 h/day) and 9 days of air, such that there were 35 O_3 exposure days over the 90-day period (episodic). Both groups had equivalent increases in lung collagen. Using a similar exposure regimen, Barr et al. (1990) found equivalent CAR remodeling and volumes of CAR lesions in both groups. In contrast, RB thickness increased in the daily group only, and the CAR interstitium increased in thickness only in the episodic group. Monkeys were studied more extensively after a daily (8 h/day) exposure to 0.25 ppm for 18 mo and a seasonal exposure only during the odd months of the 18-mo period (Tyler et al., 1988). Most morphometric measurements were similar between the two groups (e.g., both had respiratory bronchiolitis). However, only the daily group had an increased number of AMs in the lumen and interstitium. Only the seasonal group had increased lung collagen content; increased chest wall compliance, suggesting delayed lung maturation; and increased inspiratory capacity. This body of work indicates that under these types of exposure circumstances, the simple product of $C \times T$ does not predict the outcome. Indeed, half the O_3 (on a $C \times T$ basis) caused equivalent or more effects than a "full" O_3 exposure.

The complexity of understanding $C \times T$ relationships is further illustrated by Chang et al. (1991), who compared two different exposure regimens (one a square wave and the other an urban pattern) on the basis of $C \times T$ products. There was a linear relationship between $C \times T$ products and the increase in Type 1 cell volume in the CAR; a similar observation on Type 2 cell volumes was less robust. There was no such relationship for other morphometric endpoints in the same animals. Cell proliferation in the nasal epithelium does not increase linearly with increasing $C \times T$ but does increase linearly with increasing C (Henderson et al., 1993).

Long-term exposure also thickens CAR alveolar septa, due to an increase in inflammatory cells, fibroblasts, and amorphous extracellular matrix (Fujinaka et al., 1985; Barry et al., 1985; Zitnik et al., 1978). There is some morphological evidence of mild fibrosis (i.e., local increase in collagen) in CAR interalveolar septa (Last et al., 1979; Boorman et al., 1980; Chang et al., 1992; Pickrell et al., 1987b; Freeman et al., 1974; Moore and Schwartz,

1981). Biochemical evidence supports these findings, even though biochemical approaches would be expected to be less sensitive because the whole lung (rather than focal lesions) is examined. Last et al. (1979) directly demonstrated the correlation by observing increased collagen histologically and biochemically (collagen synthesis rate) in rats similarly exposed to 0.5 to 2.0 ppm O_3 for 7 to 21 days. The increase became greater with increasing concentration and duration of exposure. Similar correlations were observed at a higher concentration by Pickrell et al. (1987b). The increased collagen content can persist after exposure ceases (Chang et al., 1992; Hussain et al., 1976a,b; Last et al., 1984b), but some studies suggest that higher concentrations (>0.5 ppm) may be required for such persistence (Last and Greenberg, 1980; Pickrell et al., 1987b). Collagen cross-links were studied in monkeys exposed to 0.61 ppm O_3 for 1 year (8 h/day) (Reiser et al., 1987). Earlier examination of these same monkeys revealed that collagen content was increased (Last et al., 1984b). When specific collagen cross-links were measured, the increase in "abnormal" cross-links observed immediately after exposure remained in the lungs at 6 mo postexposure.

These morphologic/morphometric and biochemical findings of fibrotic changes are supported by some pulmonary function studies. For example, rats exposed for up to 78 weeks, using the same urban exposure protocol as Chang et al. (1992), exhibited reduced lung volume and hastened N_2 washout patterns, consistent with a "stiffer" lung (i.e., restrictive lung disease) (Costa et al., 1994).

The chronic O_3 study by the NTP and the Health Effects Institute (HEI) (Last et al., 1994; Szarek, 1994; Radharkrishnarmurthy, 1994; Parks and Roby, 1994; Harkema and Mauderly, 1994; Harkema et al., 1994; Chang et al., 1995; Pinkerton et al., 1995; Catalano et al., 1995a,b) further illustrates some of the complex interrelationships between lung structure, function, and biochemistry. All of these endpoints were evaluated in a collaborative project using rats exposed 6 h/day, 5 days/week for 20 mo to 0.12, 0.50, or 1.00 ppm O_3. Although lung biochemistry and structure were affected at the higher O_3 concentrations ($\geq$0.50 ppm), there were no observed effects on pulmonary function. This is consistent with the relative sensitivity of the tests used and suggests that the observed effects were not sufficient to overcome the reserve function of the lung.

Combined analyses of the NTP/HEI collaborative studies showed that 0.50 and 1.00 ppm O_3 caused a variety of structural and biochemical effects; 0.12 ppm O_3 did not cause any major effects, although a few specific endpoints were altered. Hallmarks of chronic rhinitis (e.g., inflammation, mucous cell hyperplasia, decreased mucous flow) were observed in focal regions of the nasal cavity. Structural and biochemical changes included some, but not many hallmarks of airway disease. Typical O_3-induced changes (e.g., bronchiolarization, increased interstitial matrix) observed in the tracheobronchial region and in the CAR were characteristic of centriacinar fibrosis; however, diffuse pulmonary fibrosis was not observed.

An integrative, multiple endpoint analysis (Catalano et al., 1995a) utilizing median polish techniques produced composite variables for disease surrogates that were tested for trends across all three O_3 concentrations. Trends for centriacinar fibrosis, airway disease, and chronic rhinitis were examined for 10, 18, and 3 endpoints, respectively, from the individual NTP/HEI studies. A statistically significant trend was noted for the association between chronic rhinitis and increasing O_3 concentration. The differences between control and exposed rats were statistically significant at 0.50 and 1.00 ppm O_3. Marginally significant and significant trends were found for the association between centriacinar fibrosis or airway disease

and increasing O_3 concentration; however, no statistically significant differences were found between control and O_3-exposed rats.

As discussed above, long-term O_3 exposure can cause lung fibrotic changes; however, there is no evidence that O_3 causes emphysema, using the currently accepted morphological definition of human emphysema (U.S. Environmental Protection Agency, 1986).

6.5.3.4 Genotoxicity and Carcinogenicity of Ozone

A significant amount of research has been conducted to determine whether O_3 is genotoxic or carcinogenic. Many of the early experiments have flaws in experimental design or have used O_3 concentrations far above levels that could occur in ambient air. In evaluating the data, a number of conclusions can be made. In vitro exposure of naked plasmid DNA to very high O_3 concentrations results in single and double-strand breaks in the DNA, as confirmed by gel electrophoresis and electron microscopy studies (Hamelin, 1985). Testing of O_3 in various mutagenesis assays has led to marginal or small results in a number of assays and negative results in other assay systems. Ozone is not mutagenic in *Salmonella* strains TA98, TA100, TA104, and TA1535 and causes, at most, weak effects in strain TA102 that are not strictly concentration dependent (Dillon et al., 1992; Victorin and Stahlberg, 1988a,b). Extremely high concentrations of O_3 (50 ppm) caused mutation to streptomycin resistance in *E. coli* and caused various types of mutations in the yeast *S. cerevisiae*, but O_3 was a weak mutagen compared to known strong mutagens in the yeast system (L' Herault and Chung, 1984; Dubeau and Chung, 1982). Ozone was not mutagenic in the *N. tabacam* or *Tradescantia* mutation assay systems (Gichner et al., 1992). Hence, overall, the data on the mutagenicity of O_3 are mixed: negative in six assays, marginally positive in one assay, and weakly positive in two assays. The present data indicate that O_3 is, at most, a weak mutagen, but further data are needed in mammalian cell systems to draw definitive conclusions regarding this point. There are some data indicating that O_3 may cause chromosome breakage in cultured cells, but in vivo animal studies are conflicting (Zelac et al., 1971a,b; Tice et al., 1978). A human study with an appropriate experimental design was negative (McKenzie et al., 1977; McKenzie, 1982).

Regarding carcinogenicity, O_3 has been shown to induce morphological transformation in cultured C3H/10T1/2 mouse embryo cells and in SHE cells and to cause a synergistic morphological transformation in cells treated also with gamma radiation (Borek et al., 1986, 1989b). However, these results could be due to interactions of O_3 with the culture medium that generate chemical species different from those produced in vivo. Whole animal carcinogenesis assays performed in strain A mice have demonstrated marginal increases in tumor yield that were not statistically significant or concentration dependent (Hassett et al., 1985; Last et al., 1987). The NTP study demonstrated that O_3 was not a tumor promoter or a co-carcinogen when NNK-treated male F344/N rats were exposed for 2 years to 0.5 ppm O_3 (National Toxicology Program, 1994). In the NTP study, rats and mice were exposed to 0.12, 0.5, or 1.0 ppm O_3 for 6 h/day, 5 days/week for two years or a lifetime. This NTP study showed no evidence of carcinogenic activity in male or female F344/N rats, equivocal evidence of carcinogenic activity in male $B6C3F_1$ mice, and some evidence of carcinogenic activity in female $B6C3F_1$ mice at a high concentration (1.0 ppm). Hence, O_3 has been shown to be a weak pulmonary carcinogen only in female $B6C3F_1$ mice at toxic concentrations in one experiment.

At present, O_3 is shown to be nonmutagenic in some assay systems; at most, weakly mutagenic in a few assay systems; and clastogenic in vitro but not in vivo. Ozone can transform cells in vitro. Ozone does not cause concentration-dependent tumor induction that is statistically significant in hamsters, Wister male rats, F344/N male or female rats, male or female A/J mice, or Swiss-Webster male mice. There are ambiguous data for pulmonary carcinogenesis in male $B6C3F_1$ mice and weak carcinogenesis data in female $B6C3F_1$ mice from chronic exposure to 1.0 ppm O_3. Therefore, O_3 has been shown to be a carcinogen only in female $B6C3F_1$ mice in one experiment. Because a chronic exposure to 1 ppm O_3 was required to induce pulmonary tumors in female mice, it is possible that pulmonary toxicity, which occurs only at high O_3 concentrations (1.0 ppm) and does not occur at lower levels, contributed to the tumor development. Hence, the potential for animal carcinogenicity is uncertain at the present time.

6.5.3.5 Factors That Influence Ozone Exposure

Factors that increase the delivered dose of O_3, decrease biochemical defense mechanisms, or increase cellular sensitivity can increase the impact of a given O_3 exposure. The most commonly studied factors include exercise, age, and nutrition.

As discussed in Chapter 8, exercise increases the dose of O_3 delivered to the respiratory tract and alters the distribution of O_3. As would be expected, exercise during exposure enhances the effect of O_3. This has been demonstrated by Mautz et al. (1985b), who showed that exercising rats had more extensive lung lesions than rats exposed at rest. Similarly, Tepper et al. (1990, 1994) found that rats were more responsive to O_3 when coexposed to CO_2 to increase ventilation, simulating exercise.

A number of studies have been conducted to compare the effects of O_3 on various ages of mice and rats, from 1 day old to older adults. Interpretation of these studies is difficult because, prior to weaning, the huddling behavior of the neonates with their dams as well as the bedding material (present in some studies) may have affected the concentration of O_3 in the breathing zone and hence the subsequent delivered dose. Generally, in short-term exposure biochemical studies of antioxidant metabolism, there was a decrease or no change in enzyme activity in neonates. As age increased after weaning, the typical increase in antioxidant metabolism became greater with age (Elsayed et al., 1982; Tyson et al., 1982; Lunan et al., 1977; Mustafa et al., 1985). Stephens et al. (1978) found that morphological effects did not occur in animals exposed prior to weaning at 21 days of age. This may explain the results of Barry et al. (1985, 1988), who found no morphometric differences in the CAR and TB in rats that started a 42-day exposure at ages of 1 day and 42 days. In identically exposed rats, however, Raub et al. (1983) found more, though admittedly subtle, pulmonary function changes in the youngest group of animals. Yokoyama et al. (1984) did not detect any age-related differences in lung function of rats at 4, 7, and 10 weeks of age. Although O_3-induced increases in BAL protein and PMNs do not show age dependence, BAL prostaglandins increased sooner and more leukocytes were dead in younger (13-day-old) rats, compared to adults (e.g., 16 weeks old) (Gunnison et al., 1990, 1992a). Age (5 weeks versus 9 weeks) did not influence the O_3-induced decrease in lung bactericidal activity (Gilmour et al., 1993a).

The literature on O_3-exposed pregnant animals is extremely sparse. Exposure of rats (1 ppm O_3 6 h) on Day 17 of pregnancy or Days 3, 13, and 20 of lactation caused a greater increase in lung permeability and inflammation than that observed in nonpregnant rats (Gunnison et al., 1992b).

Numerous reports document that animals made vitamin E deficient are more susceptible to the biochemically detected effects of O_3 (e.g., lipid changes, antioxidant metabolism changes) (U.S. Environmental Protection Agency, 1986; Pryor, 1991). Generally, the research shows that, although vitamin E deficiency enhances susceptibility to lung biochemical changes, there is not a proportionate relationship between vitamin E supplementation (above normal levels) and protection from O_3. Also, vitamin E deficiency did not alter the impact of O_3 on lung structure (Chow et al., 1981). Vitamin C deficiency also has an influence. Guinea pigs deficient in vitamin C had a greater increase in BAL protein (compared to vitamin C-normal animals) when exposed acutely to 0.5 but not 1.0 ppm O_3 (Slade et al., 1989).

6.5.4 Systemic Effects

Theoretical analyses (Pryor, 1992) indicate that the O_3 molecule does not penetrate to the blood, yet there are numerous reports of systemic effects (i.e., effects on lymphocytes, erythrocytes, serum, central nervous system, parathyroid gland, circulatory system, and liver). Possibly one or several of the reaction products of O_3 (see Section 6.2.1) penetrates the lung tissue, or perhaps some systemic responses are secondary to pulmonary effects. Although a variety of clinical chemistry changes occur after O_3 exposure, they cannot be interpreted and will not be discussed here (see U.S. Environmental Protection Agency, 1986, and Section 6.3). Effects on systemic immunity are discussed in Section 6.5.3.1.

6.5.4.1 Central Nervous System and Behavioral Effects

Acute exposure to O_3 caused transient changes in behavior. The lowest exposure causing effects was 0.12 ppm O_3 for 6 h in rats; wheel-running activity decreased (Tepper et al., 1985; Tepper and Weiss, 1986). Because exercising animals were exposed in these studies (i.e., they received a higher dose of O_3), it is not surprising that higher O_3 concentrations (0.5 ppm, 6 h) are required to affect sedentary behavior (e.g., operant behaviors such as lever pressing for food reinforcement) (Weiss et al., 1981). Mice show aversive responses to O_3 (0.5 ppm, 60 s) by terminating O_3 exposure (Tepper et al., 1985). The lowest exposures causing effects are impacted by the type of reward. For example, O_3 had less effect on behaviors to avoid electric shock (Ichikawa et al., 1988) than on behaviors to obtain food or access exercise (Tepper et al., 1982, 1985; Weiss et al., 1981).

6.5.4.2 Cardiovascular Effects

In rats, O_3 can cause bradyarrhythmia at exposures as low as 0.1 ppm for 3 days; bradycardia, at exposures as low as 0.2 ppm for 2 days; and decreased mean arterial blood pressure, at exposures as low as 0.5 ppm for 6 h (Arito et al., 1990, 1992; Uchiyama and Yokoyama, 1989; Watkinson et al., 1993; Yokoyama et al., 1989b; Uchiyama et al., 1986). There is an interaction between some of these responses and thermoregulation in the rat. For example, when heart rate decreased, the core temperature of the exposed rats also decreased, and when exposures were conducted at higher ambient temperatures, there was no change in core temperature or heart rate (Watkinson et al., 1993). Such interactions add to the complexity of extrapolating this type of response to humans, and therefore, without more information, qualitative extrapolation would be highly speculative.

6.5.4.3 Reproductive and Developmental Effects

No reports of "classical" (e.g., 2-generation studies) reproductive assays with O_3 were found. Kavlock et al. (1979, 1980) performed several developmental toxicity experiments in rats. Pregnant rats exposed intermittently (8 h/day) to 0.44 to 1.97 ppm O_3 during early, mid-, or late gestation or during the entire period of organgensis (Days 6 to 15) had no significant teratogenic effects. Continuous exposure during mid-gestation increased the resorption of embryos. Postnatal growth and behavioral development also were investigated. There was no effect on neonatal mortality (up to 1.5 ppm). Pups from dams exposed continuously to 1 ppm during mid- or late gestation weighed less 6 days after birth. Pups from pregnant rats exposed continuously to 1 ppm during late gestation had delays in behavioral development (e.g., righting, eye opening).

6.5.4.4 Other Systemic Effects

A number of investigations have shown the effects of O_3 on the pituitary-thyroid-adrenal axis, as evidenced by changes in circulating hormones and morphological changes in the thyroid and parathyroid glands (U.S. Environmental Protection Agency, 1986). No more recent studies could be found.

Several approaches have been used to study the effects of O_3 on the liver: increase in sleeping time following the injection of drugs (e.g., pentobarbital) metabolized by the liver, drug pharmacokinetics, and changes in liver enzymes. The lowest exposure causing increased sleeping time from pentobarbital was 0.1 ppm O_3 for at least 15 or 16 days (3 h/day) in female mice (Graham et al., 1981). In three species of animals, only females were affected (Graham et al., 1981). Pentobarbital pharmacokinetics was marginally ($p = 0.06$) slowed in mice exposed to 1 ppm O_3 for 3 h (Graham et al., 1985); theophylline clearance was slowed in older rabbits exposed to 0.3 ppm O_3 for 5 days (3.75 h/day) (Canada and Calabrese, 1985). Ozone has caused both increases, decreases, and no changes in liver xenobiotic metabolism, depending on the exposure and enzyme being measured (U.S. Environmental Protection Agency, 1986).

6.5.5 Effects of Mixtures

Humans in the real world are exposed to complex mixtures of gases and particles. Sufficient evidence exists to know that the health outcome is dependent on the mixture, but the relative role (or even the exact identity) of the "major" components is not known. Because of this, it is crucial to evaluate the health effects of O_3 in light of epidemiological, human clinical, and animal toxicological studies. For the purposes of this document, an interaction is considered to occur when the response to the mixture is statistically significantly higher (synergism) or lower (antagonism) than the sum of the individual pollutants. Most animal toxicological studies of O_3 interactions have been conducted with binary mixtures (predominantly NO_2 and H_2SO_4). The rarer reports on complex mixtures are interesting, but less helpful because often the studies did not include a group exposed only to O_3, and therefore knowledge of the role of O_3 is confounded. Thus, only the binary mixture studies will be summarized here. This research has demonstrated that exposure to O_3 in combination with another chemical can result in antagonism, additivity, or synergism, depending on the animal species, exposure regimen, and endpoint studied. Interpretation is further complicated by the fact that most studies used exposure regimens unlike the real world in terms of ratios of pollutant concentrations, "natural" sequencing of exposure patterns, and other factors. For example, when O_3 and NO_2 exposures were sequential (in any order), there was an additive

increase in BAL protein, as compared to a synergistic increase when the exposures were concurrent (Gelzleichter et al., 1992a).

A range of interactions has been shown with O_3 and NO_2 combinations. For example, a 2-week exposure to an O_3-NO_2 mixture (0.4 ppm of both) synergistically increased antioxidants in the lungs of rats but not guinea pigs, peroxide levels were synergistically increased in guinea pigs but not rats, and GST activity was decreased in guinea pigs and unchanged in rats (Ichinose and Sagai, 1989). Most of the interaction studies using lung biochemical endpoints display synergism. A rare exception was the antagonism to the increase in lung cytochrome P-450 content caused by 0.2 ppm O_3 (1 to 2 mo) when the rats were coexposed to 4 ppm NO_2 (Takahashi and Miura, 1989). Combinations of various acute exposure durations and of O_3 and NO_2 concentrations did not follow a C $\times$ T relationship for increased lung permeability, but were synergistic at higher C $\times$ T products (Gelzleichter et al., 1992b). For pulmonary host defenses against bacterial infection, the interaction is dependent on the exposure pattern. Graham et al. (1987) showed that a 15-day exposure of mice to mixtures of O_3 and NO_2, each having a baseline level with two daily 1-h peaks of the pollutant, resulted in synergism only when exposure to either gas alone caused an increase in bacterial-induced mortality.

Both synergistic and antagonistic interactions have been found with combinations of O_3 and acidic sulfates. Warren et al. (1986) reported that with 3 days of exposure to 0.2 ppm O_3 + 5 mg/m^3 $(NH_4)_2SO_4$, O_3 alone was responsible for increasing BAL protein, collagen synthesis rate, and other parameters, but, by 7 days of exposure, synergism occurred. When rabbits were exposed for 4 mo (2 h/day, 5 days/week) to 0.1 ppm O_3 + 125 μg/m^3 H_2SO_4, there was a synergistic increase in epithelial secretory cell number, whereas 8 mo of exposure resulted in antagonism (Schlesinger et al., 1992a). Antagonism also was observed for effects or certain AM functions after acute exposures to O_3-H_2SO_4 mixtures (Schlesinger et al., 1992b). Sequential exposures to O_3 and H_2SO_4 also have been examined. Exposure to O_3 did not influence the subsequent effects of H_2SO_4 on bronchoconstriction in guinea pigs (Silbaugh and Mauderly, 1986). Gardner et al. (1977) found an additive increase in bacterial infectivity when mice were exposed acutely to 0.1 ppm O_3 before (but not after) H_2SO_4.

In summary, the animal toxicological studies clearly demonstrate the major complexities and potential importance of interactions, but do not provide a scientific basis for predicting the results of interactions under untested ambient exposure scenarios.

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