

Ozone-induced toxicity in experimental animals and isolated cell systems

by Lars E Gustafsson & Ian Cotgreave

The scope of this section is extensively covered by the document Air Quality Criteria for Ozone and Related Photochemical Oxidants published by the Environmental Protection Agency (EPA) in the United States (1). The literature on animal experiments on exposure to ozone is vast, and it was felt not within the scope of this report to make a full review. Instead, emphasis has been put on literature covering principles and on more recent literature, especially that dealing with the mechanisms underlying various effects of ozone, such as proinflammatory effects, effects of low concentrations in vivo, and effects on primates.

Effects on respiratory system morphology

Changes in lung morphology constitute a very early sign of the effects of ozone, but the dose required to elicit such changes varies between species, and also within species. An important issue is how animal data can be related to the effects of ozone on humans. Many studies have been performed on rodents. The modeling of data obtained from studies on rodents suggests that existing anatomical differences within primates would cause the rodents to be more sensitive to damaging effects of the gas in the distal airways and alveoli (2). Therefore, studies on nonhuman primates are of importance. Earlier studies have used rather high concentrations of ozone and have put considerable emphasis on effects on alveolar cells. Recent data indicate, however, that bronchiolar cells might be the most sensitive cell group. An important advancement in striving to reduce variability in results is the systematic microdissection of identical airway segments between different animals and the use of double-blind and automated techniques for morphological evaluations (2).

Affected cells and sites

The most striking effects of ozone occur in the proximal alveolar region and in the most distal parts of the airways, the respiratory bronchioles.

Effects on lung morphology in primates

When exposed to an ozone concentration of $300 \mu\text{g} \cdot \text{m}^{-3}$, 8 h a day for 6 or 90 d, the Macaque monkey exhibits

significant nasal epithelial lesions with ciliated cell necrosis, diminished cilia number, and hyperplasia of Clara cells (3).

When the Macaque monkey was exposed to 300 or $600 \mu\text{g} \cdot \text{m}^{-3}$ for 8 h a day for 6 or 90 d, hyperplasia and hypertrophy of nonciliated cuboidal epithelial cells were observed in terminal and respiratory bronchioles (4). The effect was fully developed already at the lowest dose rate, since there was no further increase with increased ozone concentration or exposure time. Intraluminal accumulation of macrophages was also seen in the respiratory bronchioles. The validity of the results was underlined by the use of systematic microdissection and the identification of matching bronchiolar segments in control and exposed animals.

Effects on morphology in other species

A study employing identical concentration-versus-time exposures to ozone and to the combination of ozone and nitrogen dioxide has been reported for rats (5). The cell proliferation in larger, as well as peripheral, airways was monitored after bromo-deoxy-uridine had been incorporated into airway cells, which were continuously infused subcutaneously by miniosmotic pumps. Alveolar cell labeling was unaltered by 400 and $1600 \mu\text{g} \cdot \text{m}^{-3}$ of ozone, whereas a marked effect was seen in the bronchiolar cells. The large airway cells showed increased labeling only at the highest dose rates. There was a clear synergy with nitrogen dioxide for the effect on airway epithelium. Hypertrophy and hyperplasia were verified by electron microscopy.

In both juvenile and adult rats, epithelial alterations were observed after exposure to an ozone concentration of $500 \mu\text{g} \cdot \text{m}^{-3}$ for 12 h a day for six weeks. The lesions were localized to the proximal parts of the most peripheral parts of the lungs, namely, in the proximal alveolar region (6). A decrease in the surface area of type I epithelial cells of alveoli was noted; the cells also doubled in number and increased in thickness. In addition, the number of type II cells and alveolar macrophages increased. No age-related difference in sensitivity was seen. In addition, adult animals showed an increased number of tissue macrophages, a clear sign of inflammation. Adult animals were also exposed to $240 \mu\text{g} \cdot \text{m}^{-3}$ and showed significant changes in the number and size of alveolar type I cells already at this exposure level. The

findings are consistent with a dose dependently increased turnover rate of alveolar cells and show that the effect occurs already at $240 \mu\text{g} \cdot \text{m}^{-3}$.

Rats (Jcl:Wistar, male, 8 weeks old) exposed to an ozone concentration of $800 \mu\text{g} \cdot \text{m}^{-3}$ continuously for 14 d showed a 214% increase in lung cytochrome P₄₅₀ 2B1, the major pulmonary P₄₅₀ enzyme in rats (7). A hypertrophy of Clara cells, the unciliated cuboidal cells of terminal bronchioles, was seen in histological sections. The Clara stained immunologically positive for P₄₅₀ 2B1. The appearance of a cell type intermediate in morphology between types I and II alveolar cells was also seen in proximal alveolar regions, and these cells also expressed P₄₅₀ 2B1. The data suggest that ozone exposure may activate P₄₅₀-mediated xenobiotic metabolism in pulmonary epithelial cells and that ozone exposure leads to alveolar cell metaplasia.

At higher ozone concentrations (1400 to $6000 \mu\text{g} \cdot \text{m}^{-3}$) several studies have shown effects on alveolar cells, with a decrease in the number of the flat type I cells and hyperplasia of type II (lamellar bodies containing) cells clearly evident. The lamellar bodies were shown to be uneven in density, as well as undergoing hypersecretion into the alveoli, suggesting increased secretion of surfactant.

In the rat, bronchiolar epithelium exhibits changes when exposed to inhaled ozone at $500 \mu\text{g} \cdot \text{m}^{-3}$ (8). The effects included loss of surface area contributed by cilia, a decrease in the luminal surface area contributed by Clara cells, and a decrease in the number of brush cells per square millimeter of bronchiolar basement membrane.

When rats were exposed to $1900 \mu\text{g} \cdot \text{m}^{-3}$ of ozone for 3 d, ciliary damage and necrosis occurred in the trachea, with a concomitant increased density of intermediate cells and an increased thymidine labeling index (9). After 60 d of exposure the only remaining effect was shortened cilia of the ciliated cells. At 42 d postexposure no effect remained.

Another study (10) showed that cell renewal takes place at an increased rate, as indicated by thymidine incorporation, in rats exposed to 700 or $1000 \mu\text{g} \cdot \text{m}^{-3}$ of ozone (30-day-old male Sprague-Dawley rats exposed continuously up to 8 d). The increase in thymidine incorporation occurred during the first 2 d of exposure and returned to base line at day 4. This first "wave" of increase was due to thymidine incorporation in all types of alveolar cells. Animals which received an increase in ozone exposure from 700 to 1000 or $1400 \mu\text{g} \cdot \text{m}^{-3}$ from day 4 showed a second "wave" of increase in thymidine incorporation. The second increase was mainly due to thymidine incorporation into type II alveolar cells. This finding shows that, despite the initial adaptation, a new response can be evoked by a second "wave" of ozone if the concentration is higher.

The effects of long-term ozone exposure on pulmonary morphology has been studied in rats and mice in conjunction with the NTP study on the potential carcinogenesis of ozone (11). Rats were exposed for two years to ozone at 240 , 1000 or $2000 \mu\text{g} \cdot \text{m}^{-3}$ for 6 h a day, 5 d a week for 104 weeks. No effects on alveolar cell morphology were seen in control animals. The animals exposed to $240 \mu\text{g} \cdot \text{m}^{-3}$, both males and females, exhibited minimal, but significant, alveolar cell metaplasia. The changes were further enhanced at higher concentrations of ozone. Mild, but significant infiltration of histiocytes (macrophages) into alveoli and interstitial fibrosis were noted at 1000 and $2000 \mu\text{g} \cdot \text{m}^{-3}$.

Comparison between ozone and oxygen toxicity

Detrimental effects of ozone on alveolar cells occur at $200 \mu\text{g} \cdot \text{m}^{-3}$, which is only slightly above current ambient background levels (12). Another gas which is toxic at concentrations only slightly above background levels is oxygen. It is well known that prolonged exposure to twice the normal concentration of oxygen in inspired air causes pulmonary injury with functional impairment in humans (13). Exposure of primates (baboons) to 60% oxygen (ie, three times the normal concentration) for two weeks with subsequent eight weeks of recovery led to significant morphological changes (14). Changes noted directly after exposure included increases in type II alveolar cells and macrophages and signs of interstitial fibrosis. The fibrosis progressed further during the eight-week recovery period. These alveolar changes are similar to those observed in Macaque monkeys exposed to ozone, as has already been described. If it is assumed that the defense mechanisms against oxygen and ozone are similar, the effects of low concentrations of ozone on morphology in animals are, therefore, likely to have a high degree of relevance for humans, for whom similar studies cannot be performed for ethical reasons.

Summary

Alveolar macrophages proliferate in rats exposed to an ozone concentration of $200 \text{ mg} \cdot \text{m}^{-3}$ of ozone. Effects on bronchiolar cells also occur at the lowest ozone level tested, $200 \text{ mg} \cdot \text{m}^{-3}$, and these effects indicate both a growth of and an increased number of epithelial cells. This response was still seen 4 d after the cessation of a 3-d exposure, and it was thus not a transient response. Macrophages proliferate and changes occur in bronchiolar cells, with consequent overgrowth into alveoli, also in primates, and these effects might have implications both as to an understanding of the mechanisms behind direct ozone lesions in the lung and an understanding of indirect effects such as increased sensitization to allergens and hypersensitivity to bronchoconstrictor agents. Thus an increased number of macrophages, which is seen both in the alveoli and in the

interstitial tissue, increases the possibilities for these cells to adsorb allergens and present them to cells of the immune system. This phenomenon facilitates an increase in the development of antibodies.

The morphological changes and biochemical measurements performed provide evidence of increased rates of cell division, and this increase may be important in that it increases the possibilities for a malignant transformation of lung cells. Oxygen, another gaseous oxidant, is toxic to animal and human lung already at twice the normal ambient level. Considering the similarity between the defense against the mechanism of action and ozone and oxygen effects, the toxicity of ozone in animals at levels only slightly above ambient levels might be relevant to the estimation of toxicity in humans. Indeed, morphological changes are seen in monkeys exposed for more than 60 d at ozone levels near the present ambient concentrations. Likewise, in mice subjected to life-long ozone exposure, morphological changes and fibrosis are present in increased frequency in the exposed groups. The fibrosis also remains despite the cessation of ozone exposure. Therefore, the morphological changes must be regarded as a nontransient, long-term effect.

Host defense mechanisms

Host defense mechanisms of the lung are directed either at transporting foreign material out of the lungs, or at inactivating the offending material at the place of intrusion. The elimination from alveoli is mainly accomplished through the action of alveolar macrophages, or through diffusion or penetration of matter into the lung parenchyma, where recruited macrophages and other inflammatory cells engulf it. Above the alveolus the bronchial mucociliary transport mechanisms are of major importance, and the concerted actions of cilia and the secreted mucous lining fluid are necessary for transport. These mechanisms are nonspecific, whereas specific immunological mechanisms can be activated both in the parenchyma and in bronchi with the emergence of inflammatory cells and mediator and cytokine release. The response may involve both the action of preformed antibodies and a long-term activation of antibody formation.

Mucociliary clearance

For normal mucociliary clearance it is necessary that normal mucous production occurs, that ciliary cells function normally, and that mucus membrane function and lung morphology are normal (15, 16). Ozone inhalation can result in increased mucous secretion, but, if other pulmonary functions are disturbed, for example, by smooth muscle contraction or inflammation with

consequent invasion of the mucosum by white blood cells, the net effect may be negative for a person with, for example, an accumulation of mucus in the airways (13). The numbers of ciliated cells in the terminal bronchioli of rats are decreased by the inhalation of ozone at a concentration of $500 \mu\text{g} \cdot \text{m}^{-3}$ for 12 h a day for six weeks (7). The surface area contributed by ciliated cells in the bronchioli is also diminished. These effects are equivalent in adult and newborn rats. Such effects on morphology might impair ciliary transport mechanisms in a long-term perspective, despite having a short-term stimulatory influence.

In hamsters, *in vivo* exposure to the gas for 3 h at $200 \mu\text{g} \cdot \text{m}^{-3}$ does not alter ciliary beating frequency when assessed postexposure *in vitro* (17). However, the inhalation of combinations of ozone and sulfuric acid, or ozone and nitrogen dioxide, have been found to inhibit ciliary beating and mucociliary transport (15). In rabbits, exposure to $1200 \mu\text{g} \cdot \text{m}^{-3}$ for 2 h causes retarded particle clearance (18). In rats, combined exposure to $120 \mu\text{g} \cdot \text{m}^{-3}$ for 13 h a day, in combination with spikes of $500 \mu\text{g} \cdot \text{m}^{-3}$ for 9 h a day, for 5 d a week with a total duration of six weeks causes the pulmonary retention of asbestos fibers to increase (19). Increased and decreased clearance have been found in rabbits, the former in exposures to 200 and $1200 \mu\text{g} \cdot \text{m}^{-3}$ and the latter at $2400 \mu\text{g} \cdot \text{m}^{-3}$ (20). It is possible that the variation in response type is due to the presence or absence of effects on glycoprotein secretion (1). Glycoprotein is a component of mucus, and its increase is a sign of increased mucous secretion. The increased secretion would be expected to carry foreign material out of the airways more rapidly.

Thus the most sensitive effects of ozone on host defense are the stimulation of clearance at $200 \mu\text{g} \cdot \text{m}^{-3}$ in rabbits and the inhibition of clearance at $500 \mu\text{g} \cdot \text{m}^{-3}$ in rats.

Alveolar macrophages

Alveolar macrophages increase in number, decrease in their ability for phagocytosis, decrease bactericidal activity, and diminish superoxide production upon exposure to ozone. The concentration dependency of these effects varies (1). In general effects can occur at $200 \mu\text{g} \cdot \text{m}^{-3}$ and seem well established at $500 \mu\text{g} \cdot \text{m}^{-3}$. A recent *in vivo* study in rats showed that a one-week exposure to $200 \mu\text{g} \cdot \text{m}^{-3}$ increased alveolar macrophage numbers, as well as macrophage enzyme activities in rats (21). Of importance for other effects of the gas might be that the mitotic index of macrophages is increased at $540 \mu\text{g} \cdot \text{m}^{-3}$ (12) and at $1600 \mu\text{g} \cdot \text{m}^{-3}$ (18, 22). The number of lavageable macrophages increases at concentrations from 200 to $2000 \mu\text{g} \cdot \text{m}^{-3}$, but can be unaffected or even decreased at concentrations ranging from $1600 \mu\text{g} \cdot \text{m}^{-3}$ up, perhaps as a sign of more drastic cytotoxicity at the higher con-

centrations. This is of importance for choosing and evaluating exposure concentrations.

Immunologic mechanisms

The effect of short-term ozone exposure on antibody response and on the thymus was investigated in mice (23). Mice were continuously exposed to an ozone concentration of $800 \mu\text{g} \cdot \text{m}^{-3}$ and $1600 \mu\text{g} \cdot \text{m}^{-3}$ for 1, 3, 7 or 14 d. Already from day 1 and throughout the exposure, both concentrations caused an increase in relative lung weight per body weight, and from day 3 and on there was a significant decrease in relative thymus weight. In addition, spleen weights were depressed at all time points by the higher concentration of ozone, whereas at $784 \mu\text{g} \cdot \text{m}^{-3}$ there was a drop only on day 14. The ability to develop an antibody response to sheep red blood cells was also reduced by the higher concentration of ozone, and this effect was seen already after the first day of exposure. Thus an immunodepressant effect of ozone exposure was found in parallel with the induction of an inflammatory response (edema and weight increase) in the lungs. The authors concluded that the impairment was due to a suppressed T-cell response, since the response to other antigens, independent of T-cells, was normal.

The action of ozone on the thymus and on pulmonary lymph nodes has been investigated in mice during acute and subchronic inhalation (24). Female mice (CD-1) were exposed to $1400 \mu\text{g} \cdot \text{m}^{-3}$ for 20 h a day for 1 to 28 d. The thymus showed an atrophic response, whereas the mediastinal lymph nodes showed a hyperplastic response, with the presence of blastic cell forms. The atrophy in the thymus was especially seen in the cortical regions, and it was markedly reversed if the adrenal glands had been removed before ozone exposure. This finding might suggest that steroid hormones, or some other factor derived from the adrenals, are involved in the ozone-induced depressant effect on the thymus. The removal of the thymus reduced the hyperplastic response caused in the mediastinal lymph nodes in response to ozone exposure. The lungs showed signs of oxidant injury, with terminal bronchiolitis and alveolitis evident and with the infiltration of white blood cells, septal thickening, type I cell damage, type II cell proliferation, and macrophage accumulation. It seems likely that mediators from the lungs caused the changes in lymph node and thymus morphology. These results substantiate that exposure to relatively high concentrations of ozone can have an immunomodulatory effect.

In a follow-up study, athymic mice were exposed to an ozone concentration of $1400 \mu\text{g} \cdot \text{m}^{-3}$ for 20 h a day for 7 or 14 d (25). The athymic mice showed more marked lung damage than the control mice did, with more pronounced increases in lung weight and more marked peripheral damage to alveolar ducts and alveoli,

which exhibited a more-pronounced inflammatory reaction. The results support the hypothesis that the mediastinal lymph node reaction seen in normal animals is part of a response aimed at minimizing lung damage.

In female CD-1 mice exposed to an ozone concentration of $200 \mu\text{g} \cdot \text{m}^{-3}$ for 5 h a day, 5 d a week for 103 d, splenic T-lymphocyte function, measured as the response to mitogens and alloantigens, was reduced (26). In addition the *in vitro* bacteriostatic action of peritoneal macrophages was enhanced. However, the mortality of mice infected with group C streptococci was enhanced, despite an increased bactericidal effect on pulmonary alveolar macrophages. Co-exposure with sulfur dioxide or ammonium sulfate aerosol did not increase the effect above that of ozone, but it reversed the effect on splenic lymphocytes.

By use of immunofluorescence against T- and B-lymphocyte markers it was possible to demonstrate an increased T-cell infiltration in lungs from mice exposed to ozone at $1400 \mu\text{g} \cdot \text{m}^{-3}$ for 20 h daily for 4 or 14 d (27). Furthermore, B lymphocytes were decreased in number in the lung parenchyma. The lungs of the mice were significantly increased in weight at both days 4 and 14, whereas spleen and thymus showed decreased weights at day 4, an effect normalized by day 14.

In the NTP study with long-term ozone inhalation (11) no changes in thymus morphology were seen after either two-year or life-long exposure to ozone in either rats or mice. However, in this study a four-week inhalation of $2000 \mu\text{g} \cdot \text{m}^{-3}$ caused a significant decline in thymus weight in female rats. This effect was absent in male rats or in mice of either sex.

Interactions between infectious agents

A potentiating interaction with infectious agents is one of the most sensitive effects of ozone on animals. Effects are seen from $200 \mu\text{g} \cdot \text{m}^{-3}$ in mice, female mice being more sensitive than males. Despite an ozone-induced increased bactericidal effect on alveolar macrophages at $200 \mu\text{g} \cdot \text{m}^{-3}$, exposed female animals infected with group C streptococci showed an increased mortality (23). A detailed account of the interaction of ozone with infectious agents can be found in a document of the EPA in the United States (1).

Effects on some cellular responses in vitro

Arachidonic acid was found to react with ozone *in vitro* to form aldehydic derivatives (28) when the arachidonic acid was exposed to an ozone concentration of 200 or $2000 \mu\text{g} \cdot \text{m}^{-3}$ for 30 min. When such incubates were given to natural killer (NK) cells, the cells exhibited decreased cytotoxicity against K562 target cells. In addition, a human bronchial epithelial cell line was found to release arachidonic acid-derived aldehydic substances when exposed to 200 or $2000 \mu\text{g} \cdot \text{m}^{-3}$. The results

indicate that a specific mechanism of arachidonic acid metabolism involving aldehyde generation, rather than general lipid peroxidation, might function in bronchial epithelial cells and that this phenomenon may exert inhibitory effects on NK cell cytotoxicity and T-cell proliferation.

With the use of a culture technique in which the basolateral side of cells is exposed to medium while the top (apical part) of the cells is kept moist, human airway epithelial cells and alveolar macrophages were exposed to ozone in vitro (29). The primary epithelial cultures were exhibited after 1 h of exposure to an ozone level of $200 \mu\text{g} \cdot \text{m}^{-3}$ and macrophages already at 30 min. The human bronchial epithelial cell-line BEAS excreted increased amounts of interleukins 6 and 8 (IL-6, IL-8) and fibronectin upon ozone exposure, whereas the macrophages did not. The IL-6 excretion from the BEAS cells was directed only from the apical surface at 4 h of exposure, whereas by 24 h a secretion in both directions was at hand. The results suggest that epithelial cells may have a preferential role in the secretion of cytokines upon ozone exposure and that the secretion is stimulated at low ozone concentrations.

Summary

Ozone exerts immunodepressant effects on mice in that the thymus may undergo hypoplastic changes. As another, possibly secondary, effect the formation of antibodies from lines derived from B cells may be deranged. The effects occur from $800 \mu\text{g} \cdot \text{m}^{-3}$ or with 3 d of continuous exposure. After exposure to $200 \mu\text{g} \cdot \text{m}^{-3}$ for 5 h a day for 130 d in vivo, immune-competent cells from mice show abnormal immunologic responses when tested ex vivo. The cytokine-releasing effects of ozone on airway epithelial cells may be an important step in the further activation of the airway response to ozone. If so, it might explain some of the local proliferative effects on the cells and the immigration of inflammatory cells into the airways. (See the later section on inflammation.) The effect occurs at $200 \mu\text{g} \cdot \text{m}^{-3}$ already after 1 h of exposure. It is not possible to convert the in vitro exposure level directly to an inhaled concentration of the gas in vivo. It is noteworthy that the in vitro effects are observed at ozone concentrations which seem highly relevant for those effective in in vivo studies. The in vitro findings are important confirmatory observations of results obtained in vivo and may offer important mechanistic explanations for effects observed in morphological studies.

Sensitization to allergens

An important aspect of ozone toxicity is whether exposure to the gas can lead to enhanced sensitization to

allergens, and especially whether sensitization occurs in more persons in an ozone-exposed group than in a group similarly exposed to an allergen alone.

In an early study on guinea pigs (30) exposure to an ozone concentration of $10\,000 \mu\text{g} \cdot \text{m}^{-3}$ or $20\,000 \mu\text{g} \cdot \text{m}^{-3}$ for 30 min a day caused increased mortality due to anaphylaxis in animals exposed to albumin by inhalation. Hemagglutination tests showed increased antibody formation against the allergen in the animals exposed to $10\,000 \mu\text{g} \cdot \text{m}^{-3}$. The increased sensitization occurred only for inhaled antigen. Thus no increased sensitization was seen upon skin testing with an allergen in animals sensitized to albumin by intraperitoneal injection when a comparison was made between control animals and animals exposed to $2000 \mu\text{g} \cdot \text{m}^{-3}$ by inhalation.

In a primate model (cynomolgus monkey; *Macaca fascicularis*), exposure to $2000 \mu\text{g} \cdot \text{m}^{-3}$ for 6 h a day, 5 d a week for 12 weeks was found to increase allergic sensitization to inhaled platinum salt dust (ammonium hexachloroplatinate, $200 \mu\text{g} \cdot \text{m}^{-3}$), which was given simultaneously with the ozone exposure (31). As a group, the dose of allergen required to evoke a similar airway response decreased to half of that in animals not exposed to ozone. The model was chosen as a simulation of platinum dust exposure in a platinum refinery. Exposed workers develop asthma and rhinitis, and also show positive skin tests and immunoglobulin E (IgE) antibodies to platinum salts, and this phenomenon was therefore investigated in the animals in parallel with lung function tests. Three groups of animals (7 to 8 animals in each group) were studied (ozone alone, platinum salt dust alone, and combined exposure to ozone and platinum salt dust). Exposure to the combination of ozone and platinum salt dust caused a statistically significant increase in positive skin tests, as compared with exposure to platinum salt dust alone, whereas the results of immunoglobulin E (IgE), IgG, or radioallergosorbent tests did not differ between the three groups of animals. In addition, the enhanced sensitization manifested itself as an increase in airway resistance, occurring at a significantly lower concentration of inhaled allergen in the group exposed to ozone and platinum salt dust, as compared with the group exposed only to platinum salt dust. The increased sensitization was not accompanied by any change in base-line airway resistance between the three different groups. That specific sensitization to the allergen occurred was validated by the increase in positive skin tests, and the positive reaction could be transferred by the injection of serum from sensitized animals into the skin of nonsensitized animals, as expected for an immunoglobulin-mediated response. The sensitivity to inhaled methacholine was significantly also increased in the group exposed to the combination of ozone and platinum salt dust. Sensitivity to methacholine was unaltered in

the two other groups. This finding suggests that nonspecific airway sensitivity occurred in the combined exposure group.

A study on relatively low ozone concentrations was performed with mice (32). It showed a sensitizing effect of ozone on the allergenic action of ovalbumin. The mice were exposed continuously for 4 d to either filtered air or to ozone at concentrations of 200, 260, 320 or 480 $\mu\text{g} \cdot \text{m}^{-3}$. On the fourth day the animals received an inhalation of the allergen, ovalbumin, and then remained in ambient air for a week, whereafter the ozone and allergen exposure was repeated to a total of four ozone exposure periods. The ozone-exposed animals exhibited a significantly increased frequency of anaphylactic shock when challenged intravenously with ovalbumin one week after the four exposure periods, as compared with controls exposed to filtered ambient air and allergen. The effect of ozone was significant from exposure levels of 260 $\mu\text{g} \cdot \text{m}^{-3}$ and up.

There are several possible mechanisms for the enhanced sensitization to allergen after ozone exposure. One possibility is that ozone causes decreased elimination of the antigen from the lungs. Evidence for this phenomenon is found in the increased retention of radio-labeled albumin instilled into the trachea of guinea pigs previously exposed to 16 000 $\mu\text{g} \cdot \text{m}^{-3}$ for 30 min (33). In the same study an increased uptake of allergen into blood was also found. A mechanism for the increased retention or uptake of allergen might involve inflammation of the respiratory tract in relation to ozone exposure. This possibility is supported by an experiment with dogs in which acute exposure to 4200 $\mu\text{g} \cdot \text{m}^{-3}$ for 2 h caused an increase in the number of neutrophil granulocytes in the airway mucosa (34). The ozone exposure caused increased airway responsiveness to acetylcholine, and the degree of increased responsiveness correlated with an increase in the mucosal neutrophil number. In an ensuing study it was found that neutrophil depletion in dogs inhibited the effect of ozone on airway hyperresponsiveness (35). Thus a contribution from inflammatory mechanisms seems likely.

Another interesting possibility is that ozone exposure might cause changes in the production and secretion of regulatory proteins, such as cytokines, and these changes might shift the immune response to an antigen towards the development of an allergic pattern. Evidence for such cytokine formation has been found in experiments with cultured human bronchial epithelial cells exposed to ozone. In these cells the formation of the cytokines IL 8 and tumor necrosis factor alpha was increased by ozone exposure (36). Other cytokines may be involved in the increased allergen-sensitizing effect of ozone (37).

Increased cytokine formation can be expected to lead to increased antibody formation. Indeed, evidence has been found for an increased antibody formation in lungs

as a result of ozone exposure. In mice exposed to 1000 or 1600 $\mu\text{g} \cdot \text{m}^{-3}$, ozone exposure continuously for 15 or 24 d caused an increase in pulmonary interstitial cells producing IgA (38), and an increase in the secretion of IgA into the respiratory tract also occurred, as indicated by increments of IgA in lung lavage fluid. Respiratory tract concentrations of IgG and IgM were also increased, but as a result of a passive leakage from plasma, as indicated by a parallel increase in albumin leakage.

Measurement of IgE-containing cells in mice exposed to inhaled ovalbumin or to ovalbumin after ozone inhalation revealed an increase in IgE-positive cells near the mucosa in the ozone-exposed animals (39). In response to only the inhalation of ovalbumin there was a ninefold increase in IgE-containing cells. Exposure to an ozone concentration of either 1000 or 1600 $\mu\text{g} \cdot \text{m}^{-3}$ prior to ovalbumin sensitization increased the number of IgE-positive cells by a factor of 34, and this increase was a significant enhancement of the effect of the allergen. Ozone-exposed animals exhibited positive passive cutaneous anaphylaxis, whereas this sign of antibody formation was not present in the animals exposed to ovalbumin alone.

The increased sensitization to allergen caused by ozone inhalation might be accompanied by the activation of immune regulatory mechanisms modifying the response. Thus mice were subjected to 1600 $\mu\text{g} \cdot \text{m}^{-3}$ of ozone for one, two or four weeks and then subjected to a single albumin inhalation followed by intraperitoneal immunization with albumin a week later. The IgE response to albumin was suppressed in the ozone-exposed mice (40). The suppression was due to a modifying effect of the inhaled albumin on the immune response to the intraperitoneal injection given one week later. Thus it seems clear that ozone can enhance antibody formation when the exposure to allergen is through inhalation and that it is important to use allergen exposure schemes that do not run the risk of obscuring a real effect of allergen sensitization.

In summary, ozone causes increased sensitization to inhaled allergens in animals. The increase in sensitization seems well documented since it has been found in several species and involves increased airway sensitivity, as demonstrated by both the decreased allergen dose necessary and the number of exposed persons that become sensitized. The increased sensitization is also displayed as increased anaphylactic reactions or enhanced passive cutaneous skin tests for allergen.

Effects on lung function

Breathing pattern, airway resistance and lung compliance

A sensitive parameter describing lung function is that of lung compliance. In guinea pigs exposed to an ozone

concentration of 400, 800, or 1600 $\mu\text{g} \cdot \text{m}^{-3}$ for 1 to 2 h, a significant decrease of 22% to 24% in compliance (increase in "lung stiffness") occurred at 800 $\mu\text{g} \cdot \text{m}^{-3}$ and above (41). In guinea pigs exposed to 200 or 1600 $\mu\text{g} \cdot \text{m}^{-3}$ for 1 h, followed by histamine challenge, a significant augmentation of the decrease in compliance caused by histamine occurred already at 200 $\mu\text{g} \cdot \text{m}^{-3}$. The effect was the most marked 2 h after the ozone exposure (42). At 400 to 600 $\mu\text{g} \cdot \text{m}^{-3}$ the airway resistance to gas flow was affected and sensitivity to bronchoconstrictor agents increased. An increase in respiratory frequency occurred with a concomitant decrease in breath volume (tidal volume) at ozone concentrations of 600 $\mu\text{g} \cdot \text{m}^{-3}$ and above. Overt airway constriction was found to occur in guinea pigs and cats, respectively, at 800 and 1000 $\mu\text{g} \cdot \text{m}^{-3}$. The changes seem to parallel the effect of a 1500 $\mu\text{g} \cdot \text{m}^{-3}$ exposure of humans (43).

Airway responsiveness

The hyperresponsiveness to the immunizing effects of allergens as a result of ozone exposure has already been discussed. Another phenomenon of relevance is that ozone can cause an increased responsiveness to inhaled bronchoconstrictor agents. This phenomenon can be called hyperresponsiveness. This effect also involves an increased response to allergen, but it is an effect which is not due to increased immunization and is likely to be a sign of increased effects of released mediators.

Hyperresponsiveness to bronchoconstrictors occurs in several species. In the dog and sheep the hyperresponsiveness caused by a 2-h exposure to an ozone concentration of 1400 and 1000 $\mu\text{g} \cdot \text{m}^{-3}$ is the most marked 1 d after the exposure (44, 45). The effect is likely to be due to lung inflammation. The inflammation sensitizes the lung to allergens and agonists. The hyperresponsiveness decreases but does not disappear during a 7-d period after exposure.

In guinea pigs ozone exposure to concentrations of 2000 or 6000 $\mu\text{g} \cdot \text{m}^{-3}$ for 18 h prior to challenge with inhalation histamine or allergen (ovalbumin) caused similar enhancements of bronchial obstruction (46).

Summary

It is clear that inhaled ozone causes changes in breathing pattern, bronchial obstruction, and hyperresponsiveness to bronchoconstrictive agents in animals. The effects occur at concentrations of the gas that are slightly higher than those necessary to cause changes in morphology, changes in mucociliary transport, and aberrant host defense. Inflammation is likely to be involved in these effects.

Proinflammatory effects of ozone in animal models

The inhalation of ozone has been shown to result in detrimental effects in the lungs of all the animal species tested, ranging from rodents up to nonhuman primates. Among the major observed effects, the induction of pulmonary inflammation and the resultant increase in airway permeability seem to be a recurrent phenomenon, particularly after acute exposures (47). This section deals with the signs and symptoms of ozone-induced inflammation in animals and tries to relate them systematically to exposure factors, such as the concentration of the gas inhaled and the duration and pattern of exposure.

Inflammatory process, friend or foe?

Acute inflammation is a *normal* stress response to local injury or infection within a tissue. The overall process involves the integrated activity of specialized cell types, whose combined activity ensures the destruction of infectious pathogens, the involution of damaged cells within the tissue, and the processes of tissue repair or replacement (figure 1).

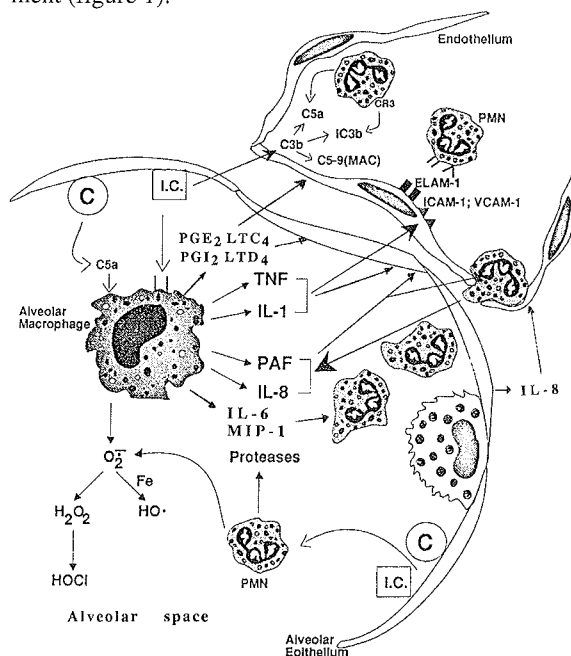


Figure 1. The cells and intracellular mediators involved in alveolar inflammation. (C = complement factor, C3b = complement factor 3b, C5—9 (MAC) = complement factor 5—9 membrane attack complex, C5a = complement factor 5a, CR3 = complement receptor 3 (CD11/CD18), ELAM-1 = endothelial-leukocyte adhesion molecule-1, Fe = iron, H_2O_2 = hydrogen peroxide, HOCl = hypochlorous acid, $\text{HO}\cdot$ = hydroxyl radical, I.C. = immune complex, IC3b = inhibitor fragment of complement factor 3b, ICAM-1 = intercellular adhesion molecule-1, IL-1 = interleukin-1, IL-6 = interleukin-6, IL-8 = interleukin-8, LTC_4 = leukotriene C_4 , LTD_4 = leukotriene D_4 , MIP-1 = macrophage inflammatory protein-1, O_2^- = superoxide radical, PAF = platelet activating factor, PGE_2 = prostaglandin E_2 , PGI_2 = prostaglandin I_2 , PMN = polymorphonuclear leukocyte, TNF = tumor necrosis factor, VCAM-1 = vascular cell adhesion molecule-1)

Macrophages, such as those present in the alveolar space or in the interstitial tissues of the airways, are generally thought to occupy a key role in instigating the inflammatory process. Macrophages respond to the phagocytosis of foreign (particles) or endogenous material (cellular debris), or to antigen presentation, by releasing many potent biological mediators that serve to amplify tissue response (figure 1).

Of these mediators, arachidonate metabolites (eicosanoids), such as the peptide-leukotrienes (LTC_4 and LTD_4), prostaglandins such as PGE_2 , PGI_2 and $\text{PGF}_{2\alpha}$ and peptide cytokines (various interleukins, eg, IL-1, IL-6 and IL-8), tumor necrosis factor alpha ($\text{TNF}\alpha$), the macrophage inflammatory proteins (MIP), and low molecular-weight agents such as nitric oxide (NO), play major roles in propagating the stress signal. Many of these mediators act by increasing the permeability of epithelial layers, such as the airway epithelium. In the lung this phenomenon is characterized by an increased permeability of the epithelium to interstitial proteins, and the increased permeability causes an accumulation of protein in the lung lumen. These changes also allow the permeability of airborne molecules (ie, radiolabeled tracers and particles) from the airways into the interstitium.

Most other epithelial cell types, particularly those within the airways, are also able to release such inflammatory mediators. Another important function of some of these inflammatory mediators is to activate the local vascular endothelium and therefore stimulate the expression of specific adhesion molecules directed against blood leukocytes and increase the endothelial layer permeability to proteins, solutes, and fluid. Finally, some of these mediators act as chemoattractants for specific blood leukocytes, such as mast cells, neutrophils, eosinophils, basophils, and lymphocytes. Thus, in a highly coordinated manner, these mediators stimulate the influx of cells from the circulation into the area of damage or infection, pave the way for their rapid infiltration to the initial area of the affected tissue or organ, and ensure that the cells are correctly activated to function optimally in tissue defense and repair. It should be remembered, however, that extremely vigorous acute inflammations can lead to considerable tissue damage.

In contrast to these acute events, the long-term maintenance of the inflammation response may lead to detrimental effects within tissues. For instance, many of the inflammatory cells recruited to the site of inflammation release agents, such as nitric oxide and reactive oxygen metabolites, proteolytic enzymes and cytotoxic proteins, and these cells can cause extensive tissue damage and loss of defined tissue function. Such damage may also lead to extensive cellular proliferation and lead to increased cellularity in the tissue, such as thickening of the airways, or to an imbalance in the cellular composition of the tissue, such as the replacement of type I

epithelial cells by proliferating type II cells in the alveolus of the airways. In extreme situations, stimulated proliferation of fibroblasts can occur and lead to the development of the fibrotic lesions characteristic of many chronic inflammations.

Ozone and pulmonary inflammation in animals

As the effects of exposure to ozone are dependent both on the concentration of the gas and the pattern and duration of exposure and as actual exposure to ozone within the human population is likely to be of a largely chronic nature, interspersed by acute periods of elevated exposure, for the purpose of this review, we have segregated the description of the proinflammatory effects of ozone into those after acute exposure (continuous exposure up to 24 h) and those after more chronic exposures of >1 d, either continuous or repeated intervals of exposure. Due to the multiplicity of studies in these areas, we have limited our description to selected examples which best illustrate the major findings in each case.

Acute inflammatory responses to ozone

The objective of the many studies on the acute proinflammatory effects of ozone have been to define characteristic symptoms of pulmonary response and relate these symptoms to the factors governing exposure, such as the minimum concentration required to induce a response in animals and the effect of the duration and pattern of exposure.

Early work by Alpert et al (47) demonstrated dose-dependent (200 to $5 \text{ mg} \cdot \text{m}^{-3}$) transient increases in lavage albumin and IgG concentrations in rats exposed acutely to ozone; these increases were then confirmed for other species (48, 49). These pioneering studies clearly show a threshold concentration of ozone in the range of 200 to $400 \text{ mg} \cdot \text{m}^{-3}$ for the induction of these types of changes in airway permeability, which are characteristic of inflammation. Since then, a plethora of information has become available describing the mechanism of the inflammatory response in more detail and defining suitable markers of animal species differences in response to the gas and the complex relationship between the concentration of inhaled ozone and the time of exposure. All of these parameters are important to the eventual assessment of the risk to human health.

One of the biggest problems in determining the lowest threshold for an acute inflammatory response to ozone is presented by the choice of markers. Thus, in contrast to the preceding permeability observations, it seems that the release of inflammatory mediators such as PGE_2 and $\text{PGF}_{2\alpha}$ into lavage fluid after acute exposures (2 h) of

rabbits only occurs at ozone concentrations of >1 ppm, whereas $200 \text{ mg} \cdot \text{m}^{-3}$ was totally without activity (50, 51). In another study, Fouke et al (52) exposed dogs and baboons to ozone levels of $1 \text{ mg} \cdot \text{m}^{-3}$ for 2 h and also failed to detect eicosanoid release into the lavage fluid.

In an extensive permeability study, in which several animal species were acutely exposed to ozone between 400 and $4 \text{ mg} \cdot \text{m}^{-3}$ for 4 h, species differences in response were clearly evident. Thus guinea pigs were shown to respond to $400 \text{ mg} \cdot \text{m}^{-3}$ with an increase in lavage protein 20 h after treatment. On the other hand, mice, rats, and hamsters were relatively insensitive, responding first to an ozone level of $2 \text{ mg} \cdot \text{m}^{-3}$, while rabbits were relatively resistant, requiring $4 \text{ mg} \cdot \text{m}^{-3}$ to initiate a response (53).

In addition to detecting permeability changes and the release of eicosanoids in response to relatively short-term acute exposure to ozone, several studies on rats exposed to ozone between 3 and $240 \text{ mg} \cdot \text{m}^{-3}$ for 6 h have shown transient increases in lavageable macrophages and polymorphonuclear leukocytes (PMN) (54, 22).

Interestingly, although the threshold for an increase in PMN in the total lavage was $1.6 \text{ mg} \cdot \text{m}^{-3}$ in these studies, when the airways were differentially lavaged, $240 \text{ mg} \cdot \text{m}^{-3}$ was shown to increase significantly the number of PMN lavaged from the nasal portion of the airways (22). It should be stressed that lavageable PMN are only one component of the inflammatory response to consider and that changes in the interstitial populations of macrophages may also be relevant. This point is considered later in detail.

Three studies have been published that probe the interrelationship of concentration (C) and time (T) in controlling the response of animals to acute, continuous exposure to ozone. Rombout and his co-workers exposed rats for 1 to 8 h to 760 or $4.08 \text{ mg} \cdot \text{m}^{-3}$ during daytime conditions and for 4 to 12 h to 260 to $760 \text{ mg} \cdot \text{m}^{-3}$ under nighttime conditions. The minimal C x T product required to increase the lavageable protein in the lung lumen was $260 \text{ mg} \cdot \text{m}^{-3}$ for 4 h. Animals exposed under nighttime conditions were more sensitive to ozone. Multivariate analysis revealed that the influence of time increased with increasing concentration and that the influence of time was still important at the lowest concentration of the gas tested ($260 \text{ mg} \cdot \text{m}^{-3}$) (55). Similar results were obtained for guinea pigs, but there was some disparity in the relationship between concentration and time at low C x T products (56). The diurnal sensitivity of animals to the acute effects of ozone has been confirmed in a study by Van Bree et al (57), who demonstrated greater increases in lavage protein and PMN in rats exposed acutely to 12 h of $800 \text{ mg} \cdot \text{m}^{-3}$ at night versus a similar exposure under daytime conditions.

Many studies have been performed using single ozone exposures to $>1.6 \text{ mg} \cdot \text{m}^{-3}$. Using radiolabeled tracers, either injected into circulation or introduced into airways, Bhalla and his co-workers have exposed rats to $1.6 \text{ mg} \cdot \text{m}^{-3}$ for 2 h and demonstrated a transient permeabilization of the lung in both directions (58–60). The same authors showed that exercise enhanced the response of the animals to ozone under these conditions (61) and that the effects of ozone were attenuated in leukopenic rats, or rats pretreated with indomethacin, a cyclooxygenase inhibitor, and antiinflammatory agent (62). In addition, the PMN isolated from control rats treated with ozone under these conditions (58–60) showed morphological changes indicative of activation and increased cellular adhesiveness (63).

In more-detailed temporal studies of the influx of PMN into the lung and the relationship of this influx to increased epithelial permeability, Young & Bhalla (64) have shown that increases in tracheal permeability in response to $1.6 \text{ mg} \cdot \text{m}^{-3}$ for 3 h clearly preceded the influx of PMN into the lumen (64). In addition, it was shown that the influx of PMN into the lumen was clearly preceded by a transient increase in interstitial PMN, which began immediately after the inhalation was terminated, and that this later event correlated best of all with increases in airway permeability (65).

Higher concentrations of ozone ($>2 \text{ mg} \cdot \text{m}^{-3}$) have been generally shown to induce acute proinflammatory responses rapidly in the lung. In a unique study (66), in which dogs were exposed to $2 \text{ mg} \cdot \text{m}^{-3}$ for only 5 min, in the form of a bolus in the intubated lung, even this short exposure was sufficient to elevate the interstitial PMN and lavageable PMN transiently at 1 to 3 h and 24 h postexposure, respectively. In addition, unlike the lack of eicosanoid response to lower levels of ozone (52, 53), a 1- to 2-h exposure to $2 \text{ mg} \cdot \text{m}^{-3}$ has been clearly shown to elevate the levels of eicosanoids, such as PGE_1 , PGE_2 , LTB_4 and thromboxane A_2 , in lavage fluid from a variety of species (67, 68). Interestingly, Gunnison et al (67) clearly demonstrated age-related differences in the released eicosanoids, younger animals clearly favoring the release of prostaglandin metabolites, while the release of leukotrienes, the increase in epithelial permeability, and the influx of PMN into the lung lumen were not altered by age. It should be mentioned that some of the stimulated release of eicosanoids may not have any functional bearing on the development of the inflammatory reaction. Recent experiments by Stevens et al (69) have demonstrated that the administration of MK 0591, a phospholipase-activator protein antagonist, did not diminish the response of dogs exposed acutely to ozone. Thus these authors postulate that leukotrienes do not play a role in the response of dogs to ozone.

After 6 h of ozone exposure of rats to $2 \text{ mg} \cdot \text{m}^{-3}$, Gunnison et al (70) noted frank cytotoxicity in the

lavage PMN population of young but not mature animals. In a recently published study Pino et al showed that the exposure of rats to $2 \text{ mg} \cdot \text{m}^{-3}$ for between 4 and 24 h resulted in time-dependent increases in PMN in the lavage and in biopsies of the centriacinar region of the lung, as well as in increased airway permeability. They noticed, however, that the number of lavageable macrophages decreased after exposure, while the interstitial macrophage numbers remained elevated (71). Using a neutrophil-deficient rat model, these authors also showed that the induction of epithelial cell permeability was independent of the presence of neutrophils in the lung (72).

Only a limited number of acute exposure studies have been performed with concentrations of ozone above $2 \text{ mg} \cdot \text{m}^{-3}$ (73, 74). Despite showing signs of inflammation in most cases, a clear decrease in the number of lavageable macrophages was noted in rats exposed to $3.6 \text{ mg} \cdot \text{m}^{-3}$ for 2 to 4 h (73). In a recent study utilizing some interesting inbred mice strains, ozone exposure to concentrations of up to $4 \text{ mg} \cdot \text{m}^{-3}$ for 3 h was shown to attenuate the transepithelial electric potential of the tracheal airways of C57-BL/6J mice prone to inflammation after ozone exposure. In contrast, the ozone-resistant C3H/HeJ strain showed no such acute alteration in transepithelial potential (75). In a similar series of experiments, Tankersley et al (76) also studied the proinflammatory effects of ozone in the C57BL/6J mice, which were again shown to respond to both high-level exposure ($4 \text{ mg} \cdot \text{m}^{-3}$, 3 h) and relatively low-level exposure ($600 \text{ mg} \cdot \text{m}^{-3}$, 7 h), whereas the resistant C3H/HeJ strain showed little response. Comparative genetic studies performed on these mice strains indicate the involvement of two critical, and as yet unidentified, genes in the susceptibility to ozone-induced lung inflammation (76). In another series of experiments with susceptible and resistant mice strains, Kleeberger et al (77) showed that WBB6F1-W/Wv mice with a mast cell deficiency were resistant to the influx of PMN into the lung lumen and the increase in lavage protein caused by $3.5 \text{ mg} \cdot \text{m}^{-3}$ for 3 h. On the other hand, the C57-BL/6J mice and mature WBB6F1-W/Wv, which had received a bone marrow transplant, showed a considerable inflammatory response under these conditions. These results indicate a potentially critical role for interstitial mast cells in mediating the acute inflammatory response to ozone, at least in mice, at these relatively high concentrations.

In a recent study on the differential cellular response to the inhalation of very high ozone levels ($4 \text{ mg} \cdot \text{m}^{-3}$) for 4 h, Schultheis & Bassett (78) have shown that lavaged cells obtained from guinea pigs over a period of 2 to 14 d postexposure had transient changes in the numbers of mast cells, macrophages, and eosinophils in the lung after exposure. Using conventional lavage and lavage following the collagen digestion of pulmonary connective tissue, these studies also confirmed (64, 65) that

the progression of the pulmonary inflammation was best reflected in the interstitial cell response, rather than in the responses detected by conventional lavage of the lung lumen (78).

Only very few studies have been performed on the effect of the acute co-exposure of animals to ozone and other agents. In a recent study, Bhalla (79) co-exposed rats to ozone levels of 1.2 or $1.6 \text{ mg} \cdot \text{m}^{-3}$ and an enzyme system generating hydrogen peroxide in the trachea for 2 h. The study clearly showed an additive effect between the two agents in the induction of tracheal permeability.

In summary, it appears that a single acute exposure to ozone between 200 and $4 \text{ mg} \cdot \text{m}^{-3}$ results in several changes in animal pulmonary physiology which are characteristic of the induction of acute inflammation. One primary characteristic of the acute response is that the effects are often delayed after exposure to about $1 \text{ mg} \cdot \text{m}^{-3}$ or below, whereas concentrations above $1.6 \text{ mg} \cdot \text{m}^{-3}$ can result in immediate proinflammatory changes after the termination of exposure. It also appears that the longer the exposure time, the more exaggerated the response, both in terms of the recruitment of proinflammatory mechanisms and the threshold of the response. Nevertheless, the molecular mechanism underlying acute ozone-induced inflammation remains questionable. Several studies have also revealed interspecies variations in the sensitivity of animals to ozone, and these variations complicate extrapolation to human exposure. The vast majority of the studies indicate, however, that single exposures to ozone for up to 24 h result in transient inflammatory changes in the lung, but that the changes eventually appear to subside. It must be stressed, however, that appropriate studies have not been performed which critically probe for long-term effects of acute exposure to the gas. In addition, careful comparison of the studies reveal intraspecies variation in the response of the lungs to ozone inhalation. Further work is required to determine the molecular basis of this variation in order to clarify eventual extrapolations from animal studies to situations of human exposure.

Chronic inflammatory responses to ozone

In contrast to the plethora of studies on the acute, proinflammatory effects of ozone in the lungs of animals, there have only been a few controlled studies on the effects of repeated interval or continuous exposures to the gas over extended periods. Such experimental exposure patterns more realistically represent the conditions under which humans are exposed to ozone in the environment.

Early work by Castleman et al with primates (80) and by Moore & Schwartz with rodents (81) clearly demonstrate proinflammatory effects of chronically administered ozone in a concentration range similar to that in-

ducing acute effects (ie, 100 to 400 mg · m⁻³). In addition to demonstrating effects on PMN infiltration into the lung, these studies closely detailed long-term ultrastructural changes in tissue composition (ie, thickening of the epithelial-interstitial layers) which are generally not studied after acute exposure. However, these and more recent studies clearly show that repeated exposures of animals to ozone often result in an attenuation of the response with time and indicate the induction of an adaptive response (82).

Several subchronic studies have been performed which highlight adaptive responses to ozone inhalation in animals. In a study in which rabbits were exposed to ozone concentrations between 200 and 2.4 mg · m⁻³ for 2 h a day over a two-week period, Driscoll et al (83) demonstrated an increase in alveolar macrophages 7 d after a single exposure to as little as 200 mg · m⁻³. However, even upon repeated exposures for up to two weeks, significant elevations in lavageable macrophages and PMN were only evident after more than 6 d of repeated exposure. However, the infiltration response occurred more rapidly. In another study in which rats were exposed to 400 or 1.6 mg · m⁻³ for 7 h a day over 4 d, no significant increase in total lavage cells was noted over control values, but PMN were increased at concentrations above 1.2 mg · m⁻³ after 1 d of exposure; the levels then decreased to control values thereafter (84). Tepper et al (85) demonstrated an adaptive change (attenuation of the response with time) in pulmonary function after exposure to 1 mg · m⁻³ in rats exposed for 2.25 h a day for 5 d, but not in inflammatory cell infiltration into the lung lumen. Indeed, this study revealed significantly more PMN in the lung and a persistence of elevated protein levels in the lavage after repeated ozone administration over this period. More recently, van der Val and his colleagues (86) demonstrated that preexposing rats to daily intervals of near ambient levels of ozone for 7 d, significantly diminished the subsequent inflammatory cell response to an acute challenge with high concentrations of the gas.

Several long-term studies have been published utilizing interval exposures. Last and his co-workers (87) exposed rats to between 60 and 2 mg · m⁻³, 6 h a day, 5 d a week, for 20 months. In rats exposed to concentrations above 1 mg · m⁻³, increases in collagen deposition were noted in the centriacinar region of the lung; this change is characteristic of the induction of mild, persistent fibrosis. In a recently published study on primates, exposure to 300 mg · m⁻³, 8 h a day for up to 90 d was clearly shown to cause morphological changes in the lung. The changes included increases in epithelial thickness and the number of cuboidal epithelial cells and increased cellular proliferation in the interstitium (3). These results are disturbing in terms of the risks to human health as the levels of ozone employed were

close to ambient values, and the clearly hyperplastic response can have serious implications for situations of co-exposure with other airborne pollutants, particularly carcinogens.

In addition to the concentration of ozone and duration of exposure, the pattern of exposure clearly affects the response of animals to the gas. Several studies have been performed using continuous exposure for extended periods. Bassett and his co-workers (88) have exposed rats to 1.4 mg · m⁻³ for 3 days continuously. Increases in alveolar macrophages, PMN, and albumin were noted as early as 12 h into the exposure. In contrast, while PMN numbers rapidly subsided after exposure, the elevated protein and macrophage number persisted in the lavage fluid over several days. Similar effects were obtained by Canning et al (89) after the continuous exposure of mice to 1 mg · m⁻³ for up to two weeks. Thus PGE₁ and protein peaked in lavage fluid by the third day of the exposure, declining thereafter. These authors noted declining numbers of cells with time in the lavage following 1—3 d of exposure.

With the induction of lung fibrosis in mind as a consequence of chronic exposure to ozone, it is interesting to note that the continuous exposure of rats to 1 ppm of the gas for up to two weeks caused considerable induction of the expression of both fibronectin and type 1 collagen mRNA. An induction of the growth-related protooncogene *c-myc* was also noted during the treatment (90). However, it should be emphasized that these effects were seen with relatively high concentrations of ozone.

Extending the exposure of rats to between 8 and 200 mg · m⁻³ continuously for between 1 and 12 weeks has been shown to induce persistent infiltration of cells into the lung lumen. However, in view of the C x T products used, relatively little proinflammatory action was noted after extended periods of inhalation. Thus the lowest effect limit was 400 mg · m⁻³, which caused a significantly higher accumulation of macrophages, but not PML, only after 11 weeks of exposure (12). In a very interesting study by Chang and his co-workers (91) rats were continuously exposed to ozone over a long period using a cyclic pattern matched to environmental variations of the gas. Thus animals received ambient concentrations of the gas (120 mg · m⁻³) for 13 h, followed by a short peak of 500 mg · m⁻³, returning to ambient levels over 9 h. These authors clearly demonstrated an inflammatory response in the lungs by one week of exposure, interstitial macrophages being increased in the proximal airways and the terminal bronchioles and interstitial edema being present. Once again, adaptive responses were noted in these experiments, as the levels of interstitial macrophages returned to control values after three weeks of exposure to the cycle. However, importantly, some evidence of interstitial fibrosis in the proximal airways was noted after the exposure was stopped.

Finally, as the human population is simultaneously exposed to many airborne pollutants, it is of interest to determine any additive or synergistic effects of other agents on ozone-induced inflammation. One agent which has received considerable attention is nitrogen dioxide, and this possibility has been the subject of many reviews (83). Apart from this oxidant gas, the literature is beginning to reveal interactions between ozone and other agents. For instance, the concomitant chronic inhalation of carbon-black particles with high concentrations of ozone ($3 \text{ mg} \cdot \text{m}^{-3}$) clearly potentiated the inflammatory response of mouse lung (92). Such interactions, although a possibility, may not, however, be plainly evident, and revealing them requires a careful study design. In a very recent study by Cassee & Feron (93), in which rats were intermittently exposed for 3 d to formaldehyde (3.6 ppm), a known nasal or respiratory tract irritant, ozone ($800 \text{ mg} \cdot \text{m}^{-3}$) and combinations thereof, little or no synergism was demonstrated despite each agent causing its own spectrum of inflammatory and cell proliferatory responses. Further interaction studies involving chronic exposure of animals to ozone and other airborne agents are needed if we are to define the risks to human health from the inhalation of ozone better, particularly in the urban environmental setting.

Summary

Perusal of the available studies clearly shows that the initial inflammatory response of animals to the inhalation of continuous or repeated periodic bouts of ozone

resembles the response of the animal to acute exposures. However, with increasing lengths of exposure, it is clear that adaptive responses in lung function and biochemistry occur which instigate tolerance to the gas. The relevance of such adaptive responses in animals to the situation of human exposure to ozone is clear. However, the demonstration of such a response in humans may be difficult as the threshold for response in the animal models tested appears to be between 240 and $300 \text{ mg} \cdot \text{m}^{-3}$, which is readily achieved in the urban environment at present. In addition, the pattern of exposure ($C \times T$ product), more than the absolute exposure concentration, seems to dominate the response. Thus continuous exposure to ambient levels of ozone, interspaced by short peak exposures to the gas, seems to elicit as strong a proinflammatory response as does continuous exposure to the peak level used. The detrimental inflammatory and cell proliferatory responses of animal lungs to chronic exposures to these near-ambient concentrations of ozone are disturbing in terms of the risks to human health.

Summary and concluding remarks

Exposure of animals to ozone causes effects on lung morphology and function, as well as inflammation and decreased resistance to infectious agents. Significant effects are seen at $200 \text{ } \mu\text{g} \cdot \text{m}^{-3}$ and above, and dose-dependency occurs from this concentration and above, mak-

Table 1. Summary of the low-level effects of ozone and effects of ozone on primates (selected for low effective concentrations, 0.1 ppm approximated to $200 \text{ } \mu\text{g} \cdot \text{m}^{-3}$).

Reference	Effect	Ozone exposure ($\mu\text{g} \cdot \text{m}^{-3}$)	Species
	Morphology		
Mochitate et al 1992 (21)	Alveolar macrophages, increase in number and enzyme activity	200	Rat
Barry et al 1985 (6)	Increase in alveolar type I cell turnover	240	Rat
Harkema et al 1993 (3)	Bronchiolar cell proliferation	300	Monkey
Harkema et al 1987 (4)	Nasal epithelial lesions	300	Monkey
Raijini et al 1993 (5)	Bronchiolar cell proliferation	300	Rat
Rithideh et al 1989 (12)	Increase in alveolar macrophage mitotic index	540	Rat
	Mucociliary clearance		
Driscoll et al 1986 (20)	Increase	200	Rabbit
Pinkerton et al 1989 (19)	Decrease	500	Rat
	Infection defense		
Aranyi et al 1983 (26)	Decreased resistance	200	Mouse
	Sensitivity to allergen or bronchoconstrictors		
Osebold et al 1988 (32)	Increased sensitization to allergen; inhaled ovalbumin, i.v. anaphylaxis	260	Mouse
Biagini et al 1986 (31)	Inhaled platinum salt dust, inhaled and skin tests	2000	Monkey
Gordon & Amdur 1980 (42)	Increased sensitivity to bronchoconstrictive effect; increased sensitivity to histamine	200	Guinea pig

ing the observed effects highly reliable. A summary of some important effects at low levels or in primates is given in table 1. Apart from effects on lungs, the effects of ozone can be seen in thymus and lymphoid tissues after acute exposure. Very disturbing, from a health perspective, are recent, well-controlled studies on monkeys showing that, at $300 \mu\text{g} \cdot \text{m}^{-3}$, an increased proliferation of both epithelial and inflammatory cells occurs in the airways. Thus effects on animals are apparent at concentrations which are at, or near, background levels of ozone. In mice subjected to life-long ozone exposure, morphological changes and fibrosis are present in increased frequencies. The fibrosis also remains despite the cessation of ozone exposure. Therefore, the morphological changes must be regarded as nontransient, long-term effects.

The lowest effective concentration of ozone evoking morphological changes and decreased defense against infectious agents in rodents and changes in mucociliary clearance in rabbits is $200 \mu\text{g} \cdot \text{m}^{-3}$. The morphological effects are seen both after acute exposure and after 90-d (monkeys) or life-long (mice, rats) exposure. In primates morphological changes are seen with exposure to $300 \mu\text{g} \cdot \text{m}^{-3}$, involving changes in alveolar morphology in the lung and alterations in nasal epithelium morphology. Regardless of differences in sensitivity between species, it is clear that ozone can cause effects on animals from $200 \mu\text{g} \cdot \text{m}^{-3}$ up and that ozone causes increased sensitization to allergens from $260 \mu\text{g} \cdot \text{m}^{-3}$ on.

References

1. US Environmental Protection Agency (EPA). Air quality criteria for ozone and related photochemical oxidants. Washington (DC): US Environmental Protection Agency.
2. Hyde DM, Bolender RP, Harkema JR, Plopper CG. Morphometric approaches for evaluating pulmonary toxicity in mammals: implications for risk assessment. *Risk Anal* 1994;14:293—302.
3. Harkema JR, Plopper CG, Hyde DM, St George JA, Wilson DW, Dungworth DL. Response of the macaque nasal epithelium to ambient levels of ozone: a morphologic and morphometric study of the transitional and respiratory epithelium. *Am J Pathol* 1987;128:29—44.
4. Harkema JR, Plopper CG, Hyde DM, St George JA, Wilson DW, Dungworth DL. Response of Macaque bronchiolar epithelium to ambient concentration of ozone. *Am J Pathol* 1993;143:857—66.
5. Rajini P, Gelzleichter TR, Last JA, Witschi H. Alveolar and airway cell kinetics in the lungs of rats exposed to nitrogen dioxide, ozone, and a combination of the two gases. *Toxicol Appl Pharmacol* 1993;121:186—92.
6. Barry BE, Miller FJ, Crapo JD. Effects of inhalation of 0.12 and 0.25 parts per million ozone on the proximal alveolar region of juvenile and adult rats. *Lab Invest* 1985;53:692—704.
7. Takahashi Y, Aida S, Suzuki E, Ito Y, Miura T, Kimura Y. Cytochrome P450 2B1 immunoreactivity in bronchiolar and alveolar epithelial cells after exposure of rats to ozone. *Toxicol Appl Pharmacol* 1994;128:207—15.
8. Barry BE, Mercer RR, Miller FJ, Crapo JD. Effects of inhalation of 0.25 ppm ozone on the terminal bronchioles of juvenile and adult rats. *Exp Lung Res* 1988;14:225—45.
9. Nikula KJ, Wilson DW, Giri SN, Plopper CG, Dungworth DL. The response of the rat tracheal epithelium to ozone exposure. *Am J Pathol* 1988;131:373—84.
10. Evans MJ, Johnson LV, Stephens RJ, Freeman G. Renewal of the terminal bronchiolar epithelium in the rat following exposure to NO_2 or O_3 . *Lab Invest* 1976;35:246—57.
11. National Toxicology Program (NTP). NTP technical report on the toxicology and carcinogenesis studies of ozone and ozone/NNK in F344/N rats and B6C3F1 mice (inhalation studies). NTP, 1993. NTP TR 440:NIH publication, no 94—3371.
12. Rithideh K, Hotchkiss JA, Griffith WC, Henderson RF, Brooks AL. Chromosome damage in rat pulmonary alveolar macrophages following ozone inhalation. *Mutat Res* 1989;241:67—73.
13. Jenkinson SG. Oxygen toxicity. *N Horiz* 1993;1:504—11.
14. Crapo JD, Hayatdavoudi G, Knapp MJ, Fracica PJ, Wolfe WG, Piantadosi CA. Progressive alveolar septal injury in primates exposed to 60% oxygen for 14 days. *Am J Physiol* 1994;267:L797—L806.
15. Gardner DE. Oxidant-induced enhanced sensitivity to infection in animal models and their extrapolations to man. *J Toxicol Environ Health* 1984;13:423—39.
16. Bergofsky EH. The lung mucosa: a critical environmental battleground. *Am J Med* 1991;91 suppl 4A:4S-10S.
17. Grose EC, Richards JH, Illing JW, Miller FJ, Davies DW, Graham JA, et al. Pulmonary host defence response to inhalation of sulfuric acid and ozone. *J Toxicol Environ Health* 1982;10:351—62.
18. Schlesinger RB, Driscoll KE. Mucociliary clearance from the lungs of rabbits following single and intermittent exposure to ozone. *J Toxicol Environ Health* 1987;20:125—34.
19. Pinkerton KE, Brody AR, Miller FJ, Crapo JD. Exposure to low levels of ozone results in enhanced pulmonary retention of inhaled asbestos fibres. *Am Rev Respir Dis* 1989;140:1075—81.
20. Driscoll KE, Vollmuth TA, Schlesinger RB. Early alveolar clearance of particles in rabbits undergoing acute and subchronic exposure to ozone. *Fundam Appl Toxicol* 1986;7:264—71.
21. Mochitate K, Ishida K, Ohsumi T, Miura T. Long-term effects of ozone and nitrogen dioxide on the metabolism and population of alveolar macrophages. *J Toxicol Environ Health* 1992;35:247—60.
22. Hotchkiss JA, Harkema JR, Kirkpatrick DT, Henderson RF. Response of rat alveolar macrophages to ozone: quantitative assessment of population size, morphology, and proliferation following acute exposure. *Exp Lung Res* 1989;15:1—16.
23. Fujimaki H, Ozawa M, Imai T, Shimizu F. Effect of short-term exposure to O_3 on antibody response in mice. *Environ Res* 1984;35:490—6.
24. Dziedzic D, White JH. Thymus and pulmonary lymph node response to acute and subchronic ozone inhalation in the mouse. *Environ Res* 1986;41:598—609.
25. Dziedzic D, White HJ. Response of T-cell-deficient mice to ozone exposure. *J Toxicol Environ Health* 1987;21:57—71.
26. Aranyi C, Vana SC, Thomas PT, Bradof JN, Fenters JD, Graham JA, et al. Effects of subchronic exposure to a mixture of O_3 , SO_2 , and $(\text{NH}_4)_2\text{SO}_4$ on host defenses of mice. *J Toxicol Environ Health* 1983;12:55—71.

27. Bleavins MR, Dziedzic D. An immunofluorescence study of T and B lymphocytes in ozone-induced pulmonary lesions in the mouse. *Toxicol Appl Pharmacol* 1990;105:93—103.
28. Madden MC, Friedman M, Hanley N, Siegler E, Quay J, Becker S, et al. Chemical nature and immunotoxicological properties of arachidonic acid degradation products formed by exposure to ozone. *Environ Health Perspect* 1993;101:154—64.
29. Devlin RB, McKinnon KP, Noah T, Becker S, Koren HS. Ozone-induced release of cytokines and fibronectin by alveolar macrophages and airway epithelial cells. *Am J Physiol* 1994;266:L612-L619.
30. Matsumura Y. The effects of ozone, nitrogen dioxide, and sulfur dioxide on the experimentally induced allergic respiratory disorder in guinea pigs, I: the effect on sensitization with albumin through the airway. *Am Rev Respir Dis* 1970;102:430—7.
31. Biagini RE, Moorman WJ, Lewis TR, Bernstein IL. Ozone enhancement of platinum asthma in a primate model. *Am Rev Respir Dis* 1986;134:719—25.
32. Osebold JW, Zee YC, Gershwin LJ. Enhancement of allergic lung sensitization in mice by ozone inhalation. *Proc Soc Exp Biol Med* 1988;188:259—64.
33. Matsumura Y. The effects of ozone, nitrogen dioxide, and sulfur dioxide on the experimentally induced allergic respiratory disorder in guinea pigs, II: the effects of ozone on the absorption and the retention of antigen in the lung. *Am Rev Respir Dis* 1970b;102:438—43.
34. Holtzman MJ, Fabbri LM, O'Byrne PM, Gold BD, Aizawa H, Walters EH, et al. Importance of airway inflammation for hyperresponsiveness induced by ozone. *Am Rev Respir Dis* 1983;127:686—90.
35. O'Byrne PM, Walters EH, Gold BD, Aizawa HA, Fabbri LM, Alpert SE, et al. Neutrophil depletion inhibits airway hyperresponsiveness induced by ozone exposure. *Am Rev Respir Dis* 1984;130:214—9.
36. Rusznak C, Devalia JL, Davies RJ. The impact of pollution on allergic disease. *Allergy* 1994;49:21—7.
37. Leikauf GD, Simpson LG, Santrock J, Zhao Q, Abbinante-Nissen J, Zhou S, et al. Airway epithelial cell responses to ozone injury. *Environ Health Perspect* 1995;103(2):91—5.
38. Osebold JW, Owens SL, Zee YC, Dotson WM, LaBarre DD. Immunological alterations in the lungs of mice following ozone exposure: changes in immunoglobulin levels and antibody-containing cells. *Arch Environ Health* 1979;34:258—65.
39. Gershwin LJ, Osebold JW, Zee YC. Immunoglobulin E-containing cells in mouse lung following allergen inhalation and ozone exposure. *Int Arch Allergy Appl Immunol* 1981;65:266—77.
40. Ozawa M, Fukimaki H, Imai T, Honda Y, Watanabe N. Suppression of IgE antibody production after ozone in mice. *Int Arch Allergy Appl Immunol* 1985;76:16—9.
41. Amdur MO, Ugro V, Underhill DW. Respiratory response of guinea pigs to ozone alone and with sulfur dioxide. *Am Ind Hyg Assoc J* 1978;39:958—61.
42. Gordon T, Amdur MO. Effect of ozone on respiratory response of guinea pigs to histamine. *J Toxicol Environ Health* 1980;6:185—95.
43. Mauderly JL. Respiratory function responses of animals and man to oxidant gases and to pulmonary emphysema. *J Toxicol Environ Health* 1984;13:345—61.
44. Lee L-Y, Bleecker ER, Nadel JA. Effect of ozone on bronchomotor response to inhaled histamine aerosol in dogs. *J Appl Physiol* 1977;43:626—31.
45. Abraham WM, Jnauszkiewicz AJ, Mingle M, Welker M, Wanner A, Sackner MA. Sensitivity of bronchoprovocation and tracheal mucous velocity in detecting airway responses to O₃. *J Appl Physiol* 1980;48:789—93.
46. Vargas MH, Segura P, Campos MG, Hong E, Montano LM. Effect of ozone exposure on antigen-induced airway hyperresponsiveness in guinea pigs. *J Toxicol Environ Health* 1994;42:435—42.
47. Alpert SM, Gardner DE, Hurst DJ, Lewis TR, Coffin DL. Effect of exposure to ozone on defensive mechanisms in the lung. *J Appl Physiol* 1971;31:247—52.
48. Reasor MJ, Adams GK, Brooks GK, Rubin R. Enrichment of albumin and IgG in the airways secretion of dogs breathing ozone. *J Environ Health Part C* 1979;13:335—46.
49. Hu PC, Miller FJ, Daniels MJ, Hatch GE, Graham JA, Gardner DE, et al. Protein accumulation in lung lavage fluid following ozone exposure. *Environ Res* 1982;29:377—88.
50. Driscoll KE, Leikauf GD, Schlesinger RB. The effects of in vitro and in vivo exposure on eicosanoid production from rabbit alveolar macrophages. *Inhalation Toxicol* 1988;1:109—22.
51. Schlesinger RB, Driscoll KE, Gunnison AF, Zelikoff JT. Pulmonary arachidonic acid metabolism following acute exposures to ozone and nitrogen dioxide. *J Toxicol Environ Health* 1990;31:275—90.
52. Fouke JM, Delmos RA, McFadden ER. Airway response to ultra short-term exposure to ozone. *Am Rev Respir Dis* 1988;137:326—330.
53. Hatch GE, Slade R, Stead AG, Graham JA. Species comparison of acute inhalation toxicity of ozone and phosgene. *J Toxicol Environ Health* 1986;19:43—53.
54. Hotchkis, JA Harkema JR, Sun JD, Henderson RF. Comparison of acute, ozone-induced nasal and pulmonary inflammation responses in rats. *Toxicol Appl Pharmacol* 1989;98:289—302.
55. Rombout PJA, Van Bree L, Heisterkamp SH, Marra M. The need for an 8 hour ozone standard. In: Schneider T, Lee SD, Wolters GJR, Grant LD, editors. *Atmospheric ozone research and its policy implications: proceedings of the 3rd US-Dutch international symposium, May 1988. Nijmegen (The Netherlands): Elsevier Scientific Publications, 1989:701—10. Studies in environmental sciences, 35.*
56. Highfill JW, Hatch GE, Slade R, Crissman KM, Norwood J, Devlin RB, et al. Concentration-time models for the effects of ozone on bronchoalveolar lavage fluid protein from rats and guinea pigs. *Inhalation Toxicol* 1992;4:1—16.
57. Van Bree L, Marra M, Rombout PJM. Differences in pulmonary and biochemical and inflammatory responses in rats and guinea pigs resulting from daytime or nighttime, single or repeated exposure to ozone. *Toxicol Appl Pharmacol* 1992;116:209—16.
58. Bhalla DK, Mannix RC, Kleinman MT, Crocker TT. Relative permeability of nasal tracheal and bronchoalveolar mucosa to macromolecules in rats exposed to ozone. *J Toxicol Environ Health* 1986;17:269—83.
59. Bhalla DK, Crocker TT. Tracheal permeability in rats exposed to ozone: an electron microscopic and autoradiographic analysis of the transport pathway. *Am Rev Respir Dis* 1986;134:572—9.
60. Bhalla DK, Crocker TT. Pulmonary epithelial permeability in rats exposed to ozone. *J Toxicol Environ Health* 1987;21:73—87.
61. Bhalla DK, Mannix RC, Lavan SM, Phalen RF, Kleinman

- MT, Crocker TT. Tracheal and bronchoalveolar permeability changes in rats inhaling oxidant atmospheres during rest or exercise. *J Toxicol Environ Health* 1987;22:417—37.
62. Bhalla DK, Daniels DS, Liu NK. Attenuation of ozone-induced airways permeability in rats by pretreatment with cyclophosphamide, FPL 55712 and indomethacin. *Am J Respir Cell Mol Biol* 1992;7:73—80.
 63. Bhalla DK, Rasmussen RE, Daniels DS. Adhesion and motility of polymorphonuclear leukocytes isolated from blood of rats exposed to ozone: potential biomarkers of exposure. *Toxicol Appl Pharmacol* 1993;123:177—86.
 64. Young C, Bhalla DK. Time course of permeability changes and PMN influx in the rat trachea following ozone exposure. *Fundam Appl Toxicol* 1992;18:175—80.
 65. Bhalla DK, Young C. Effects of acute exposure to O₃ on rats: sequence of epithelial and inflammatory changes in the distal airways. *Inhalation Toxicol* 1992;4:17—31.
 66. Kleeberger SR, Kolbe J, Turner C, Spanhake EW. Exposure to 1 ppm ozone attenuates the immediate antigenic response of canine peripheral airways. *J Toxicol Environ Health* 1989;28:349—62.
 67. Gunnison AF, Finkelstein I, Weideman P, Su WY, Sobo M, Schlesinger RB. Age-dependent effect of ozone on pulmonary eicosanoid metabolism in rabbits and rats. *Fundam Appl Toxicol* 1990;15:779—90.
 68. Miller PD, Ainsworth D, Lam, HF, Amador MO. Effect of ozone exposures on lung functions and plasma prostaglandin and thromboxane concentrations in guinea pigs. *Toxicol Appl Pharmacol* 1987;88:132—40.
 69. Stevens WH, Lane CG, Woolley MJ, Ellis R, Tagari R, Black C, et al. Effect of FLAP antagonist MK 0591 on leukotriene production and ozone-induced airway responsiveness in dogs. *J Appl Physiol* 1994;76:1583—8.
 70. Gunnison AF, Weideman P, Sobo M, Kznig KL, Lung CC. Age-dependence of response to acute ozone exposure in rats. *Fundam Appl Toxicol* 1992;18:360—9.
 71. Pino MV, McDonald RJ, Berry JD, Joad JP, Tarkington BK, Hyde DM. Functional and morphological changes caused by acute ozone exposure in the isolated and perfused rat lung. *Am Rev Respir Dis* 1992;145:882—9.
 72. Pino MV, Stoval MY, Levin JR, Devlin RB, Koren HS, Hyde DM. Acute ozone-induced lung injury in neutrophil-depleted rats. *Toxicol Appl Pharmacol* 1992;114:268—76.
 73. Bassett DJP, Brown-Kelly E, Brewster EL, Elbon CL, Reichenbaugh SS, Bunton T, et al. A reversible model of acute lung injury based on ozone exposure. *Lung* 1988;166:355—69.
 74. Schultheis AH, Bassett DJP. Inflammatory cell influx in ozone-exposed guinea pig lung interstitial and airways spaces. *Agents Actions* 1991;34:270—3.
 75. Takashi M, Kleeberger SR, Croxton TL. Effects of ozone on trans-epithelial potential in mouse trachea. *Am J Physiol* 1993;265:L33—L37.
 76. Tankersley CG, Kleeberger SR. Ozone-induced inflammation and altered ventilation in genetically-susceptible mice: a comparison of acute and sub-acute exposures. *Toxicol Lett* 1994;72:279—89.
 77. Kleeberger SR, Seiden J, Levitt RC, Zhang LY. Mast cells modulate acute ozone-induced inflammation of the murine lung. *Am Rev Respir Dis* 1993;348:1284—9.
 78. Schultheis AH, Bassett DJP. Guinea pig lung inflammatory cell changes following acute ozone exposure. *Lung* 1994;172:169—81.
 79. Bhalla DK. Alteration of ozone-induced airway permeability by oxygen metabolites and antioxidants. *Toxicol Lett* 1994;73:91—101.
 80. Castleman WL, Dungworth DL, Schwartz MW, Tyler WS. Acute respiratory bronchiolitis: an ultrastructural and autoradiographic study of epithelial cell injury and renewal in rhesus monkeys exposed to ozone. *Am J Pathol* 1980;98:811—40.
 81. Moore PF, Schwartz LW. Morphological effects of prolonged exposure to ozone and sulfuric acid aerosol in the rat lung. *Exp Mol Pathol* 1981;35:108—23.
 82. Berglund M, Boström C-E, Bylin G, Ewetz L, Gustafsson L, Moldéus P, et al. Health risk evaluation of nitrogen oxides. *Scand J Work Environ Health* 1993;19 suppl 2:1—72.
 83. Driscoll KE, Vollmuth TA, Schlesinger RB. Acute and sub-chronic ozone inhalation in the rabbit: response of alveolar macrophages. *J Toxicol Environ Health* 1987;21:27—43.
 84. Donaldson K, Brown GM, Brown DM, Slight J, MacLaren W, Davis JMG. Characteristics of bronchoalveolar leukocytes from the lungs of rats inhaling 0.2—0.8 ppm ozone. *Inhalation Toxicol* 1993;5:149—64.
 85. Tepper JS, Costa DL, Lehman JR, Weber MF, Hatch GE. Unattenuated structural and biochemical in the rat lung during functional adaptation to ozone. *Am Rev Respir Dis* 1989;140:493—501.
 86. van der Val WA, Van Bree L, Marra M, Robour PJ. Attenuation of acute lung injury by ozone inhalation. The effect of low level pre-exposure. *Toxicol Lett* 1994;72:291—8.
 87. Last JA, Gelzleichter T, Harkema J, Parks WC, Mellick P. Effects of 20 months ozone exposure on lung collagen in F344 rats. *Toxicology* 1993;84:83—102.
 88. Bassett DJP, Brown-Kelly E, Elbon CL, Reichenbaugh SS. Rat lung recovery from 3 days of continuous exposure to 0.75 ppm ozone. *J Toxicol Environ Health* 1988;25:329—47.
 89. Canning BJ, Hmielski RR, Spannhake EW, Jakab J. Ozone reduces murine alveolar and peritoneal macrophage phagocytosis: the role of prostanoids. *Am J Physiol* 1991;261:L277—L282.
 90. Choi AM, Elbon CL, Bruce SA, Bassett DJ. mRNA levels of lung extracellular matrix protein during ozone exposure. *Lung* 1994;172:15—30.
 91. Chang LY, Huang Y, Stocksill BL, Graham JA, Grose EC, Menache MG, et al. Epithelial injury and interstitial fibrosis in the proximal alveolar region of rats chronically exposed to a simulated pattern of urban ambient ozone. *Toxicol Appl Pharmacol* 1992;115:241—52.
 92. Jakab GJ, Hemenway DR. Concomitant exposure to carbon black particulates enhances ozone-induced lung inflammation and suppression of alveolar macrophage phagocytosis. *J Toxicol Environ Health* 1994;41:221—31.
 93. Cassee FR, Feron VJ. Biochemical and histopathological changes in nasal epithelium of rats after 3 days intermittent exposure to formaldehyde and ozone, alone or in combination. *Toxicol Lett* 1994;72:257—68.