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The Effects of Ozone on Immune Function

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A review of the literature reveals that ozone (O_3) exposure can either suppress or enhance immune responsiveness. These disparate effects elicited by O_3 exposure depend, in large part, on the experimental design used, the immune parameters examined as well as the animal species studied. Despite the apparent contradictions, a general pattern of response to O_3 exposure can be recognized. Most studies indicate that continuous O_3 exposure leads to an early (days 0–3) impairment of immune responsiveness followed, with continued exposures, by a form of adaptation to O_3 that results in a re-establishment of the immune response. The effects of O_3 exposure on the response to antigenic stimulation also depend on the time at which O_3 exposure occurred. Whereas O_3 exposure prior to immunization is without effect on the response to antigen, O_3 exposure subsequent to immunization suppresses the response to antigen. Although most studies have focused on immune responses in the lung, numerous investigators have provided functional and anatomical evidence to support the hypothesis that O_3 exposure can have profound effects on systemic immunity.
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Introduction

Broadly defined, the function of the immune system is to protect the body from damage by infectious microorganisms and neoplastic cells. The immune response is mediated by a variety of soluble factors and cells grouped according to their capacity to mediate innate (natural) or adaptive (acquired) immune responses. Innate immunity is primarily dependent on the phagocytic effector mechanisms (including humoral factors such as complement, lysozyme, etc.), which are neither specific for particular infectious agents nor improved by repeated encounters with the same agent. Acquired immunity is specific for the inducing agent and is marked by an enhanced response upon repeated encounters with that agent, thus displaying memory. These systems do not function independently since innate and acquired immune mechanisms can interact: specific antibodies enhance the phagocytic ingestion process and cell-mediated immune responses increase the intracellular microbicidal and tumoricidal capabilities of the phagocytes. Both innate and acquired immune responses and the cells that carry out these processes are regulated by soluble factors such as cytokines and various autocoids.

The multiple interlocking mechanisms involved in the complex sequence of events that lead to the expression of the immune response can present a plethora of targets for air pollutants and xenobiotic compounds. Recognition that environmental agents can impair immune function has lead to the emerging field of immunotoxicology (*1,2*). As with any new area of scientific endeavor, uncertainties abound as the field is defining itself and gaining recognition. This is especially true when many questions remain unanswered in the parent discipline immunology. Against this background the literature on the effects of ozone on immune function is reviewed.

Ozone and the Immune Response

A survey of the literature on the effects of O_3 exposure on innate and acquired immunity reveals that the responses range from no effect to impaired and/or enhanced effects. Several factors related to the experimental systems used are responsible for this ambiguous situation. First, many studies have examined a diverse range of immunologic parameters with varying sensitivities to O_3. Second, there are differences in responses to O_3 among animal species. Third, it is becoming increasingly apparent that the O_3 concentrations employed and the exposure protocols utilized (i.e., continuous versus intermittent) heavily influence the responses measured. Finally, the time at which O_3 exposure occurs, relative to the time at which responsiveness is measured, appears to be critical.

To unravel the variables and to determine the trends that emerge, it is helpful to group the approaches that have been used to investigate the effect of O_3 on the immune system into several categories. These include: *a*) measurement of lymphoid organ weights and cellular composition; *b*) determination of the functional capacity of lymphocytes in the absence of antigenic stimulation; *c*) measurement of the immune response following antigenic stimulation; *d*) assessment of the phagocytic capacity of alveolar macrophages; and *e*) measurement of the host response to infectious agents. Because multiple variables determine the response of the immune apparatus to experimental O_3 exposure, attention is given herein to the exposure conditions utilized. Thereafter, the studies performed in our laboratories in an attempt to validate and gain insights into the mechanisms of the divergent effects of O_3 on the immune system will be presented.

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Effect of Ozone on Lymphoid Organ Weight and Cell Numbers

The effect of O_3 exposure on lymphoid organ weights and/or cellularity have been examined in several studies. Although there is no clearcut conclusion, a general pattern of response to O_3 is reflected in the changes in lymphoid tissues that can be correlated with changes in immune parameters.

Continuous 0.3 to 0.8 ppm O_3 exposure for 20 to 24 hr/day elicits decreases in murine spleen, thymus, and mediastinal lymph node (MLN) weights after 1 to 3 days of exposure (*3,4*). The changes in MLN weight were found to be dose dependent over a range of 0.3 to 0.7 ppm O_3 (*4*). With prolonged exposures (7–14 days), spleen, thymus, and MLN weights returned to control levels (*3,4*). Concomitant with the return of lymphoid organ weights to control levels is an increase in the number of T-cells in these organs. Thus, after an initial decrease, T-cells increase in lymphoid organs after continued O_3 exposure (*5–8*). The increases in MLN T-cell numbers were abolished by pretreatment with a monoclonal antibody for T-cells (*8*).

The increase in T-cell numbers is not paralleled by a change in B-cell numbers. IgG- and IgM-secreting cells in murine bronchus-associated lymphoid tissue (BALT) (*9*) and B-cell numbers in the lung as a whole did not change with prolonged O_3 exposures (*7*). Likewise, exposure of rats to 0.13 ppm O_3 for 1 week increased T/B cell ratios in the MLN, with the increases lasting for at least 5 days after cessation of exposure (*10*).

Based on the studies cited above, it is apparent that a pattern in the response to O_3 can be recognized. Subsequent to an initial decrease in lymphoid organ weights and cellularity after 1 to 7 days of exposure, continued exposure of 1 to 3 weeks reveals that the organ weights not only stop decreasing, but actually return to control levels and, in some cases, exceed control levels (*4*). Most of these changes in lymphoid organ weights can be correlated to changes in T-cell numbers and proliferation. This apparent adaptation to O_3 exposure is also mirrored in the functional studies of the immune response.

The general pattern of response to O_3 exposures does not always hold, however. For example, it was observed that thymus weights continued to decrease as continuous exposures to 0.8 ppm O_3 were extended to periods as long as 56 days (*11*). On the other hand, when mice were continuously exposed to 0.31 ppm O_3 (103 hr/week for 6 months) and allowed to breathe filtered air for 5 months following exposure, an increase in the spleen weights was observed (*12*). Furthermore, an analysis of BALT tissue in rats showed that exposures of 2 to 3 days actually enhanced DNA synthesis and the mitotic index, both of which gradually returned to control levels with continued exposures. These changes in proliferation in BALT tissue were not reflected by changes in BALT size, which was unaffected by O_3 exposure (*13*).

Effect of Ozone on Immune Function in the Absence of Antigenic Stimulation

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 populations, a critical step during the amplification of the immune response. Thus, MLN cells stimulated with concanavalin A (ConA) during the course of continuous 0.7 ppm O_3 exposure (20 hr/day, 7 days/week for 28 consecutive days) showed little change in responsiveness during the first week; however, by day 14, an enhanced reactivity was observed that continued to increase through day 28 (*4,8*). Short-term exposure studies have shown that the rat splenic cell responses to T-cell mitogens phytohemagglutinin (PHA) and ConA and a B-cell mitogen (*Escherichia coli* LPS) were significantly enhanced by exposure to 1 ppm O_3 (8 hr/day for 7 days) (*14*). In contrast, intermittent exposure to 2 ppm O_3 (8 hr/day for 4 days, 2–4 days in ambient air followed by another day of O_3 exposure) was without effect while long-term exposure of mice to 0.1 ppm O_3 (5 hr/day, 5 days/week for 103 days) suppressed the splenic cell responses to T-cell mitogens (PHA and ConA) but not to a B-cell mitogen [*Salmonella typhosa* LPS; (*15*)].

Natural killer (NK) cell activity has also been used as an index of O_3-induced effects on the functional integrity of the immune surveillance system. One such study demonstrated that continuous exposure of rats to 1 ppm O_3 for 10 consecutive days results in a significant decrease in pulmonary NK cell activity on days 1, 5, and 7 of exposure, with activity recovering by day 10 (*16*). In another study, continuous exposure of rats to O_3 (0.2 ppm, 0.4 ppm, and 0.8 ppm) enhanced lung NK cell activity on day 7 at the two lower concentrations, whereas exposure at 0.8 ppm O_3 had a suppressive effect (*17*).

The above-mentioned studies on the effects of O_3 exposure on mitogen responses and NK cell activity used exposures to O_3 in great excess of that found in ambient air. To investigate the effects of acute and chronic exposure to O_3 at near ambient concentrations, Selgrade et al. (*18*) 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 a period of 13 hr, a broad exposure spike rising from 0.06 ppm to 0.25 ppm and returning to 0.06 ppm over 9 hr, and a 2-hr downtime. The exposures to this profile were 5 days/week: weekend exposures were to the background. After 1, 3, 13, 52, or 78 weeks of exposure, blood was drawn and the spleens removed. 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). Peripheral blood leukocytes were also assessed for responses to T-cell mitogens. The data show that O_3 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-hr exposure to 1 ppm of O_3 on spleen cell responses to the mitogens immediately after exposure or at 24, 48, and 72 hr thereafter.

Several studies have also investigated the effect of O_3 exposure on human peripheral blood lymphocytes. Exposure of subjects to 0.4 ppm O_3 for 4 hr impaired the ability of B-cells to form rosettes with human erythrocytes, whereas the ability of T-cells to form rosettes with sheep erythrocytes was unaffected (*19*). Another study examined the effects of a single 2-hr exposure to 0.6 ppm O_3 on the mitogenic response of peripheral lymphocytes following stimulation with PHA, ConA, pokeweed mitogen (PWM), and *Candida albicans*. The mitogenic response to PHA was significantly reduced at 2 and 4 weeks following O_3 exposure, with normal responses observed at 2 months. In contrast, no significant changes in lymphocyte responses to ConA, PWM, and *C. albicans* were observed (*20*). Finally, subjects were exposed for 2 hr to 0.12 and 0.4 ppm O_3 during moderate exercise, and PHA-induced peripheral T-cell proliferation was assessed before exposure and 24 and 72 hr after exposure (*21*). In the absence of O_3, exercise enhanced proliferation as compared to resting subjects, whereas exposure to O_3 with exercise appeared to reduce the capacity of the subjects to react to exercise with an enhanced proliferative response. This reduced response appeared to be more pronounced in subjects exposed to the higher concentration of O_3 and appeared to be more pronounced at 24 hr than 72 hr post-exposure.

Effect of Ozone on Immune Function with Antigenic Stimulation

A handful of studies have examined the effect of O_3 exposure on the allergic response to antigenic (ovalbumin, OA) stimulation. In general, these studies have used a Pandora's box of O_3 exposure regimens and varied the time and site (aerosol, systemic, or a combination of both) of antigenic stimulation. A common thread through most of these studies is that when the antigenic stimulation was performed during O_3 exposure, enhanced allergic responses were observed (*22–26*). However, when O_3 exposure preceded OA stimulation, IgE antibody production was suppressed (*27*).

In a series of studies, Fujimaki and colleagues (*3,11,28*) evaluated the systemic effects of short-term O_3 exposure on the humoral and cell-mediated immune response. In the first study, mice were continuously exposed to 0.8 ppm O_3 for 1, 3, 7, and 14 days. After each day of exposure, the animals were tested for the primary splenic antibody response to either sheep erythrocytes (SRBCs; T-dependent antigen) or DNP-Ficoll (T-independent antigen). The antibody response to SRBCs was suppressed following all exposure periods. In contrast, no suppression was observed in the primary antibody response to DNP-Ficoll (*3*). When the continuous 0.8 ppm O_3 exposure was extended to 56 days, suppression of the primary splenic antibody response to SRBCs, but not DNP-Ficoll, was still evident (*11*). Using the same exposure protocol (continuous 0.8 ppm O_3 for 1, 3, 7, and 14 days), the delayed-type hypersensitivity (DTH) response to SRBCs was also examined (*28*). Ozone exposure gradually depressed the DTH reaction from 1 day to 7 days of exposure and returned to control levels by day 14. In the same study, the timing between O_3 exposure and SRBC immunization for the DTH response was also examined. Continuous exposure to 0.8 ppm O_3 for 3 days immediately before immunization (with no post-immunization exposure) had no effect on the DTH reaction, whereas continuous 0.8 ppm O_3 exposure for 3 days following immunization significantly suppressed the DTH response (*28*). *In toto*, it appears that O_3 exposure preferentially affects the immune repertoire of the T-cells rather than that of the B-cells.

The effect of O_3 exposure on DTH responses to *Listeria monocytogenes* antigen has also been investigated (*10*). For this, rats were continuously exposed to 0.75 ppm O_3 for 7 days immediately before or immediately after intratracheal infection with *L. monocytogenes*. The DTH response, tested at 7 and 14 days after infection (immunization), was not significantly influenced by exposure to O_3 for 1 week before infection. However, if O_3 exposure occurred during the *Listeria* infection, the DTH response was suppressed. Using the same exposure protocol, the proliferative responses of spleen cells to *Listeria* antigen was also examined. The effect of O_3 exposure on this expression of immunity closely paralleled the effect found with DTH to *Listeria* antigen [i.e., whereas exposure before immunization did not decrease blastogenesis, the proliferative response was significantly suppressed in animals that were exposed to O_3 immediately after immunization (*10*)]. In a related study, mice were continuously exposed to 0.59 ppm of O_3 for 36 days. On the fifth day of exposure, the animals were immunized with tetanus toxoid and challenged with tetanus toxin on day 27. Compared with unexposed controls, O_3-exposed mice exhibited greater mortality and morbidity following the toxin challenge (*29*).

Effect of Ozone on Alveolar Macrophage Phagocytosis

Studies on the effects of O_3 exposure on innate immunity have focused on the phagocytic activity of alveolar macrophages (AMs). Acute exposures (2.5 ppm for 5 hr) decreased the *in situ* phagocytosis of inhaled *Staphlylococcus aureus* in rat lungs (*30*). In a similar manner, exposure to 0.4 and 0.8 ppm O_3 ppm for 3 hr decreased phagocytosis of intratracheally administered *Streptococcus zooepidemicus* in murine lungs in a dose-dependent manner (*31*).

Ex vivo studies on AM phagocytosis have found divergent effects of O_3 exposure. For example, when rats were continuously exposed to 0.8 ppm O_3 (for 3, 7, and 20 days), an enhancement in the ingestion of carbon-coated latex particles was observed, with the greatest increase following 3 days of exposure (*32*). On the other hand, rat AM phagocytosis of opsonized SRBCs was progressively suppressed from day 1 to day 3 of continuous 0.5 ppm O_3 exposure, with recovery of phagocytic activity by day 6 of exposure (*33*). When rabbits were given a single 2-hr exposure of 0.1 ppm O_3, AM phagocytosis was maximally decreased immediately after exposure and remained low throughout the ensuing day, albeit to a lesser extent; complete recovery of phagocytic activity occurred by day 7 post-exposure (*34*). Increasing the single 2-hr exposure dose of rabbits to 1.2 ppm O_3 resulted in a continued suppression of AM phagocytosis through post-exposure day 7. Intermittent exposure of rabbits (0.1 ppm O_3 for 2 hr/day for 14 days) resulted in a significant reduction of AM phagocytosis on day 3 and 7 with recovery of phagocytic potential by day 14.

To assess species differences in O_3-induced changes in AM phagocytosis, rats and mice were intermittently exposed to 0.4 ppm O_3 (12 hr/day for 7 days). After 1 day of exposure, AM phagocytosis of opsonized SRBCs was suppressed in mice and enhanced in rats. By days 3 and 7 post-exposure, rat AM phagocytosis returned to control levels, whereas mouse AM phagocytosis continued to be suppressed (*35*).

The effect of O_3 exposure on human AM-mediated phagocytosis has also been examined. For this, subjects were exposed for 6.6 hr to either 0.08 ppm or 0.10 ppm O_3 during moderate exercise (*36*). Bronchoalveolar lavage was performed 18 hr after exposure (*36*). Ozone exposure significantly decreased the ability of AMs to ingest *Candida albicans* yeast particles coated with serum (complement receptor-mediated phagocytosis). However, no such effect was observed with IgG-coated yeasts (Fc receptor-mediated phagocytosis) or unopsonized yeasts (nonspecific receptor-mediated phagocytosis). Finally, a human study investigated the effect of a single 0.4 ppm O_3 exposure on peripheral blood leukocyte function. The capacity of polymorphonuclear neutrophils (PMNs) to phagocytize and kill *Staphylococcus epidermidis* was significantly suppressed at 72 hr after exposure and returned to normal levels 2 weeks after exposure (*37*).

The Effect of Ozone on Resistance to Infectious Agents

Recognition of the association between exposures to air pollutants and the development of acute respiratory illnesses has led to animal models that use microbiologic parameters to evaluate toxicity (*38,39*). Many studies on the effects of O_3 on host defenses have been performed with a rodent model that is often referred to as the "infectivity model" (*39*). In this model, rodents are challenged by aerosol inhalation with highly virulent *Klebsiella pneumoniae* or *Streptococcus pyogenes* following various O_3 exposure protocols and observed for deaths over the subsequent 14-day period. The data on the effects of O_3 exposure are expressed as increases in mortality. The earliest of these studies (*40*) found significant increases in mortality following *K. pneumoniae* challenge when mice were exposed to 1 ppm and 4 ppm O_3 for 3 hr, 1 ppm O_3 for 100 hr, and 1 ppm O_3 for 4 hr/day, 5 days/week for 2 weeks. With the use of *Streptococcus pyogenes*, the sensitivity of the "infectivity model" increased in that significant differences in mortality were demonstrated when the infectious challenge was given simultaneously or 2 hr after exposure to 0.1 ppm O_3 for 3 hr (*41*). Mice exposed 5 hr/day, 5 days/week for 103 days to 0.1 ppm O_3 also exhibited increased susceptibility to death from streptococcus challenges (*15*). Other studies with the "infectivity model" involving O_3 exposure focused on the relationship between O_3 and NO_2 exposure on resistance to streptococcal pneumonia (*42–44*).

The "infectivity model" links interference with pulmonary antibacterial defenses to mortality following pulmonary challenges with virulent organisms. Another approach is to use minimally virulent organisms, such as *S. aureus*, that do not provoke injurious responses in the lungs (*45*). In this system, rodents are challenged by aerosol inhalation with staphylococci and intrapulmonary killing of the organism is assessed over a 4- or 5-hr period by standard microbiologic methods. Exposure to O_3 can occur before, after, or before and after bacterial challenge. Under normal conditions, approximately 90% of the staphylococci are killed over the initial 4-hr period of infection. The effects of O_3 exposure are assessed in terms of suppression of intrapulmonary bacterial killing.

Exposure of mice to 0.1 ppm, 0.5 ppm, and 1 ppm O_3 for 3 hr immediately before staphylococcal challenge significantly suppressed the intrapulmonary killing of *S. aureus* in a dose-related manner (*46,47*). Similarly, exposure to increasing concentrations of O_3 for 17 hr before or 4 hr after bacterial challenge also impaired the intrapulmonary killing of staphylococci in a dose-dependent manner (*48,49*).

More recent studies with the *Streptococcus zooepidemicus* infectivity model found that 0.4 ppm and 0.8 ppm O_3 exposure of 5- and 9-week-old CD-1 mice for 3 hr impaired intrapulmonary bacterial killing, with the most severe effect elicited by the higher dose of O_3 in the younger mice (*50*). When the study was performed in two different mouse strains, the results showed that C3H/HeJ mice were more sensitive to the effects of O_3 exposure on susceptibility to streptococcus infection than C57Bl/6 mice (*31*).

The effect of O_3 exposure on chronic respiratory infections has been assessed using *Mycobacterium tuberculosis* and *L. monocytogenes* as challenge organisms. Mice exposed to 1.5 ppm O_3 (4 hr/day, 5 days/week for 2 months) exhibited no increased mortality to intravenously administered *M. tuberculosis* (*51*). On the other hand, exposure of mice to 1 ppm O_3 (4 hr/day, 5 days/week for up to 8 weeks) beginning at 1 or 2 weeks after inhalation challenge with *M. tuberculosis* significantly enhanced the number of bacteria recovered from the lungs (*52*). With *L. monocytogenes*, rats were exposed continuously to 0.13 ppm, 0.25 ppm, 0.5 ppm, 0.75 ppm, 1.0 ppm, and 2.0 ppm O_3 for 1 week and thereafter intratracheally infected with *Listeria*. Exposure to 1 ppm O_3 significantly increased mortality through dysfunctions in the intrapulmonary elimination of the bacteria (*10*).

Only a few studies have investigated the effects of O_3 exposure on the outcome of viral respiratory infection. Fairchild (*53*) demonstrated that exposure to 0.6 ppm O_3 for 3 hr after aerosol infection of mice with mouse-adapted influenza A2/Japan 305/57 virus inhibited viral replication in the nose but did not alter influenza virus growth in the lungs of the same animals. Continuous 1.5 ppm O_3 exposure also had no effect on the proliferation of virus in the lungs of mice infected by aerosol inhalation with a sublethal dose of influenza A/PR8/34 virus (*54*). In another study (*55*), mice were exposed to 1 ppm O_3, 3 hr/day for 5 days and intranasally infected with mouse-adapted influenza virus A/Hong Kong/68 immediately after the first, second, third, fourth, and fifth of the five daily exposures. A 2-fold increase in the incidence of mortality was observed in mice infected after the second exposure, with no effects on percentage mortality in mice infected on the first, third, fourth, or fifth exposure. When the exposure concentration was lowered to 0.5 ppm, there were no effects on mortality in mice after the second exposure. Five daily 3-hr exposures to 1 ppm O_3 had no effect on virus titers in the lungs of mice infected after either the second or fifth day of exposure.

Wolcott et al. (*56*) observed a protective effect of O_3 exposure to influenza virus infection. Mice were continuously exposed to 0.5 ppm O_3 for 2 weeks before and after aerosol infection with influenza A virus (WSN strain). Four groups were included in the study. The control mice were exposed to air for 2 weeks, virus infected and reexposed to air for 2 weeks. The experimental groups were exposed to O_3 either before, after, or before and after virus infection. It was found that all animal groups exposed to O_3 had a significant decrease in mortality, which correlated with a less widespread infection of the lung parenchyma. The reduced mortality was independent of peak pulmonary virus titers, pulmonary interferon titers, and pulmonary and serum-neutralizing antibodies.

Other Studies

Ozone exposure may also induce aberrant immune responses through modification of "self" determinants in the lungs by ways such as cross-linking of antigen (*57*), denaturation of protein (*58*), or by other means (*59*). Indeed, an autoimmune response has been attributed to O_3 exposure (*60*).

The effect of *in vitro* O_3 exposure on the immune responsiveness of isolated cells has also been investigated. These experiments have demonstrated that acute exposure to O_3 reduces AM phagocytosis (*61*) in a dose-dependent manner (*62*). In addition, PGE_2 production by AMs was increased by O_3 exposure, and superoxide release from stimulated cells decreased (*62*). Exposure of peripheral mononuclear cells from healthy individuals to various concentrations of O_3 revealed a general decrease in immune parameters such as NK activity (*63*), responsiveness to mitogens, expression of activation markers on monocytes and lymphocytes (*64,65*), and antibody production (*66*). These are important observations because they demonstrate similar responses to O_3 between human cells exposed *in vitro* and animals cells exposed *in vivo*. Confirming the validity of the animal models, these studies demonstrate that the response to O_3 of human cells exposed *in vitro* is similar to that of animal cells exposed *in vivo*.

Studies Performed

The studies performed in our laboratories were designed to clarify some of the issues regarding the effects of O_3 exposure on the innate and acquired immune system. The first goal was to investigate the modulating effect of O_3 exposure on AM phagocytosis and to determine whether an adaptive phase followed prolonged exposure. The second goal was to explore whether changes in the local (lung) immune responses were mirrored by changes in the systemic immune responses. The third goal was to gain some insight into the mechanisms of the systemic effects and, finally, we attempted to elucidate the mechanisms by which O_3 exposure reduced the pathogenesis of experimental influenza virus infection.

Suppression and Recovery of the Alveolar Macrophage Phagocytic System

To investigate the effect of O_3 exposure on AM phagocytosis and to determine whether prolonged O_3 exposure results in an adaptive response, mice were exposed to 0.5 ppm O_3 for 23 hr/day for 14 consecutive days. At 1, 3, 7, and 14 days of exposure, AM phagocytosis of opsonized SRBCs was assessed *in vitro* (*67*). Figure 1*A* shows that 1 day of O_3 exposure suppressed AM phagocytosis. This impairment of phagocytic activity was further exacerbated at day 3 but began to recover at 7 days and returned to control levels by 2 weeks.

Using another particle to probe the functional capacity of the AM phagocytic system *in situ* (*45*), mice were challenged by aerosol inhalation with *S. aureus*, and intrapulmonary bacterial killing was assessed over a 4-hr period. The data (Figure 1*B*) reflect the results obtained with the *ex vivo* assay on AM phagocytosis of opsonized SRBCs (Figure 1*A*); that is, an initial suppression of intrapulmonary staphylococcal killing was followed by recovery of bactericidal activity by day 14 (*67*).

Modulation of the Immune Response

In nonimmunized mice, continuous 0.7 ppm O_3 exposure results in an initial reduction of the number of cells recovered from MLN, which was followed by an increase and maintenance above basal levels as the O_3 exposure continued (*4,8*). To determine whether the splenic events were comparable to the changes in the MLN, mice were exposed 23 hr/day for 14 consecutive days to 0.8 ppm O_3. After 1, 3, 7, and 14 days of exposure, the number of cells in the MLN and the spleen/body weight ratios were determined. Then, to test the functional activity of these two cell populations, the proliferative response of MLN and splenic lymphocytes to PHA stimulation was measured (*68*). The data (Figure 2*A*) demonstrate that continuous O_3 exposure causes a reversible decline in MLN cells followed by hypertrophy. Spleen weights were decreased with respect to body weight after 1 day of O_3 exposure (Figure 2*B*). This effect was still evident at day 3 but returned to control values following 7 days of exposure. With continuous exposure, the spleens were larger in size relative to body weight than in control animals.

Cells from both MLN and splenic sources obtained after 1 day of O_3 exposure had reduced responsiveness to PHA mitogen (Figure 3*A*). However, this effect was ablated following prolonged exposure. In a similar manner, the NK cell activity of splenic lymphocytes was significantly reduced following 1 and 3 days exposure but was restored upon continued exposure (Figure 3*B*).

Another set of experiments was designed to investigate the effect of O_3 exposure on antigen-stimulated immune responsiveness. For this, an immunization protocol was adopted that maximizes the local (pulmonary) immune response. Briefly, mice were primed with an ip injection of OA and 2 weeks later boosted by aerosol inhalation of OA. One week after the aerosol boost (i.e., 3 weeks after the intraperitoneal priming injection), the MLN and splenic lymphocytes were assayed for proliferative responses to OA antigen, and the bronchoalveolar lavage fluid was assayed for specific IgG and IgA antibody to OA. Ozone exposure was incorporated into this immunization protocol to the effect that groups of animals were exposed to 0.8 ppm of O_3 for 23 hr/day for 1, 3, 7, or 14 days prior to assay as shown schematically in Table 1.

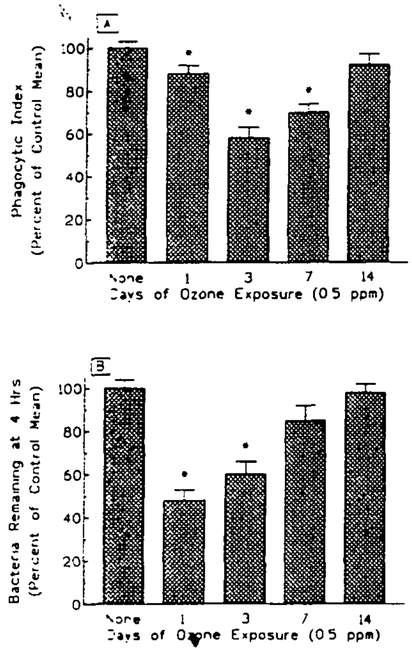


Figure 1. Alveolar macrophage Fc-receptor-mediated phagocytosis (*A*) and intrapulmonary bacterial killing of *Staphylococcus aureus* (*B*) from mice exposed continuously to 0.5 ppm ozone. * = $p < 0.05$. From Gilmour et al. (*67*).

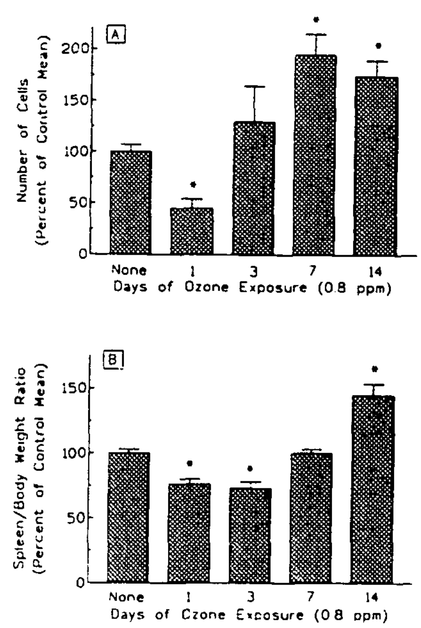


Figure 2. Number of cells recovered from the mediastinal lymph nodes (*A*) and spleen/body weight ratios (*B*) from mice exposed continuously to 0.8 ppm ozone. * = $p < 0.05$. From Gilmour and Jakab (*68*).

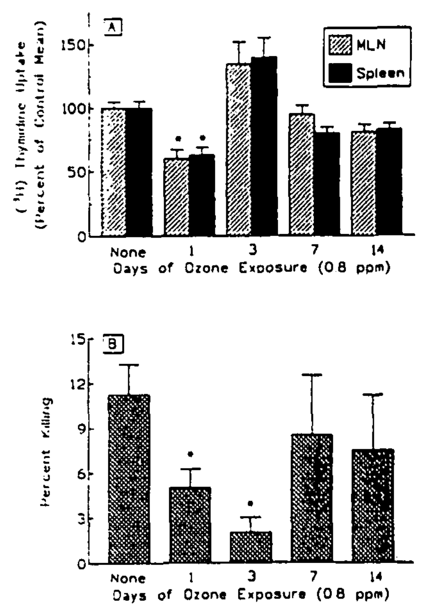


Figure 3. Proliferative responses of mediastinal lymph node and splenic lymphocytes to phytohemagglutinin stimulation (*A*) and natural killer cell activity of splenocytes (*B*) from mice exposed continuously to 0.8 ppm ozone. * = $p < 0.05$. From Gilmour and Jakab (*68*).

Table 1. Ozone exposure protocol

	Day					
	0 Prime	7	14 Boost	17	20	21
1 day exposure					Ozone ———	>Assay
3 day exposure				Ozone ———	———	>Assay
7 day exposure			Ozone ———	———	———	>Assay
14 day exposure		Ozone ———	———	———	———	>Assay

Figure 4*A* shows that the MLN cell responses to OA antigen were not significantly different from controls for 1, 3, and 7 days of O_3 exposure. However, following 14 days of exposure to O_3 the MLN lymphocyte responses to OA antigen were significantly enhanced. Splenocyte responses to OA antigen were similarly unaffected by 1 and 3 days of exposure, but were significantly lower than control values at the 7- and 14-day time points.

Serum antibody titers to OA antigen were high in all animals and were unaffected by any period of O_3 exposure (*68*). Pulmonary IgG and IgA antibody titers to OA antigen (Figure 4*B*) were lower in bronchoalveolar lavage fluid from animals exposed to O_3 for 7 and 14 days. At the earlier time points, IgA titers remained unchanged; IgG levels were at first suppressed (day 1) and then returned to control values (day 3) before decreasing again at the later time points.

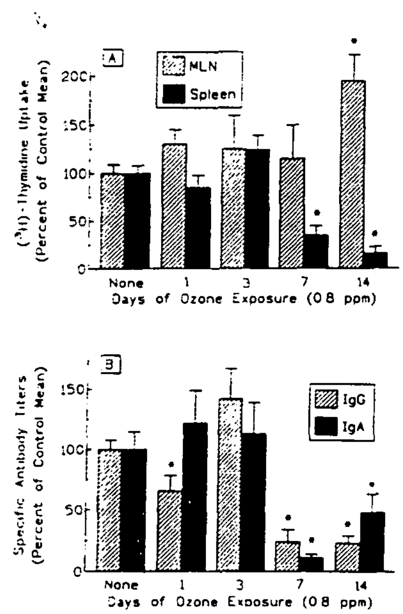


Figure 4. Proliferative responses to ovalbumin antigen of mediastinal lymph node and splenic lymphocytes (*A*) and specific IgG and IgA antibody to ovalbumin in bronchoalveolar lavage fluid (*B*) from immunized mice exposed to 0.8 ppm ozone for various days before assay. * = $p < 0.05$. From Gilmour and Jakab (*68*)

Finally, all the experiments detailed above with exposure to 0.8 ppm O_3 were also performed with continuous exposure to 0.5 ppm O_3. The results of these studies showed similar trends in responses, however, the magnitude of these responses was not as large as that observed with 0.8 ppm O_3 exposure (MI Gilmour and GJ Jakab, unpublished observations).

Impairment of Peritoneal Macrophage Phagocytosis: The Role of Prostanoids

During the course of studies on the effect of continuous 0.5 ppm O_3 exposure on AM Fc-receptor-mediated phagocytosis, it was also observed that the exposure impaired peritoneal macrophage (PM) phagocytosis. Due to its high reactivity, it seemed unlikely that O_3 could directly mediate systemic changes in phagocyte function. Rather, as has been suggested by others, the systemic effects of oxidant gases are likely indirect, due to mediators produced upon oxidant exposure (*69*). One such class of mediators may be the prostaglandins, potent immunomodulatory autacoids found in high concentrations in the airways (*70,71*) and serum of animals (*72*) and man (*73*) following acute O_3 exposure. Furthermore, exogenously added prostaglandin E_2 (PGE) inhibits macrophage phagocytosis (*74*). To test the hypothesis that PGE plays a role in mediating the O_3-induced suppression of macrophage phagocytosis, mice were continuously exposed to 0.5 ppm O_3 and the effect of the cyclooxygenase inhibitor indomethacin on AM and PM Fc-receptor-mediated phagocytosis and PGE levels in the bronchoalveolar lavage fluid (BAL) were assessed.

Figure 5*A* shows that the time course for O_3-induced impairment of PM phagocytosis paralleled that of the AMs in that maximal suppression occurred at 3 days of exposure, which progressively abated as exposure continued. PGE levels in BAL fluid during the course of O_3 exposure are presented in Figure 5*B*. The data show that the time course of PGE content in BAL fluid is strikingly similar to the time course of O_3 induced suppression of AM and PM phagocytosis. Treatment of mice with

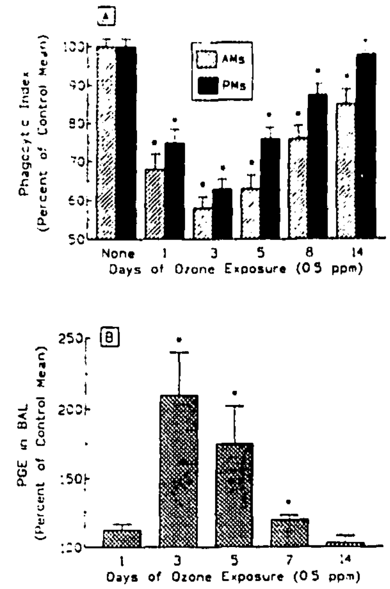


Figure 5. Alveolar and peritoneal macrophage Fc-receptor mediated phagocytosis (*A*) and PGE levels in bronchoalveolar lavage fluid (*B*) from mice continuously exposed to 0.5 ppm ozone. * = $p < 0.05$. From Canning et al. (*74*)

indomethacin for 3 days before and during O_3 exposure ablated the O_3-induced increases in bronchoalveolar lavage PGE levels (Figure 6) and suppression of PM phagocytosis (Figure 7). Indomethacin also significantly reduced the effect on AM phagocytosis on the third day of exposure (Figure 7). The effect of indomethacin on O_3-induced suppression of PM phagocytosis were mimicked by pretreatment of mice with the active enantiomer of the cyclooxygenase inhibitor naproxen (*74*). Thus, prostanoids seem to play a key role in the

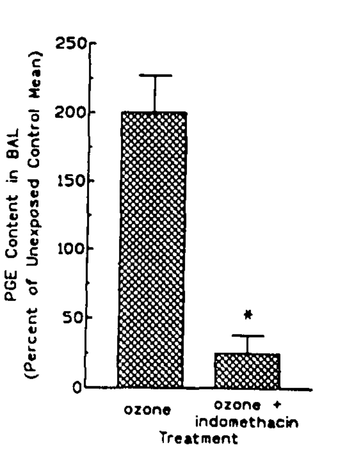


Figure 6. Effect of indomethacin treatment of mice on ozone-induced increases in PGE levels in bronchoalveolar lavage fluid on the third day of continuous 0.5 ppm ozone exposure. * = $p < 0.05$. From Canning et al. (*74*)

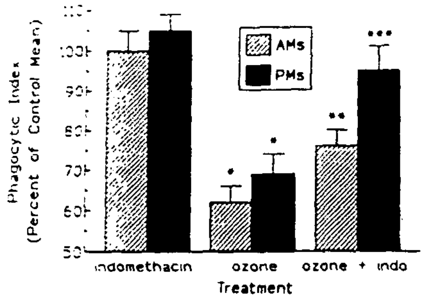


Figure 7 Effect of indomethacin treatment on ozone induced suppression of alveolar and peritoneal macrophage Fc-receptor mediated phagocytosis on day 3 of continuous exposure to 0.5 ppm ozone. * = $p < 0.05$ from control; ** = $p < 0.05$ from controls and O_3 exposed; *** = $p < 0.05$ from 3 exposed O_3. From Canning et al. (74).

local and systemic decreases in phagocytic activity induced by O_3.

The observations that prostanoids play a significant role in the suppression of AM phagocytic function has been recently confirmed with *S. zooepidemicus* as shown by the reduced susceptibility of mice to O_3-enhanced infection by indomethacin treatment (*50*).

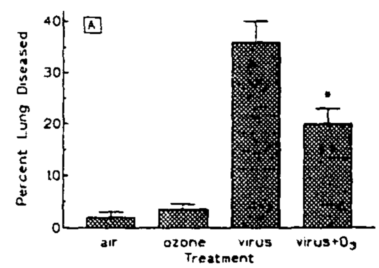
Reduction of Influenza Virus Pathogenesis

Although O_3 exposure has been shown to increase susceptibility to infectious bacterial challenges, the opposite has been observed with viral infections: ozone exposure actually diminishes the severity of influenza virus infection, as evidenced by decreased mortality and increased survival time (*56*).

Mortality from experimental influenza pneumonitis is due to extensive damage of alveolar tissue resulting in impaired gas exchange (*75*). The antiviral immune response is known to participate in influenza-induced lung injury since immunodeficient (*76–80*) and immunosuppressed (*81–83*) mice develop much less cellular infiltration and histopathology than their immunocompetent counterparts. Also, adoptive transfer of specific immunity to immunosuppressed animals during the course of influenza virus infection reestablishes lung injury and increases pneumonic deaths (*84*). Because the integrity of the antiviral immune response is required for the tissue destructive phase of viral pneumonitis, we tested the hypothesis that O_3 exposure reduced the pathogenesis of influenza pneumonitis through suppression of the antiviral immune response.

Mice were infected with a nonlethal dose of influenza A (PR8/34 strain) and continuously exposed to 0.5 ppm O_3 (23 hr/day for 14 consecutive days). On the ninth day of infection, at the height of the virus-induced pathologic changes (*85,86*), morphometric analysis of lung tissue, lung wet/dry weight ratios, and lavage albumin concentrations showed that O_3 exposure was associated with a significant reduction in these parameters of lung injury (Figure 8). Because viral-induced pathologic changes are infectious dose related (*87,88*), the effect of O_3 exposure on viral proliferation and antigen accumulation in the lungs was quantitated. Figure 9 shows that neither virus proliferation nor the accumulation of viral antigen was affected by O_3 exposure.

Since immunosuppression during the course of viral infection reduces the number of lymphocytes retrieved from the lungs, which, in turn, is related to reduced immunopathologic changes (*86,89*), the effect of O_3 exposure on the recovery of the two major classes of lymphocytes from lung tissue was phenotypically quantitated. In the presence of viral infection, both the T- and B-cell populations are significantly reduced by exposure to O_3 (Figure 10) as were the antiviral serum antibody responses (*85*). These data support the notion of the immunosuppressive nature of O_3 in that immunopathologic mechanisms are involved in the virus-induced lung injury, which are mitigated by O_3 exposure.



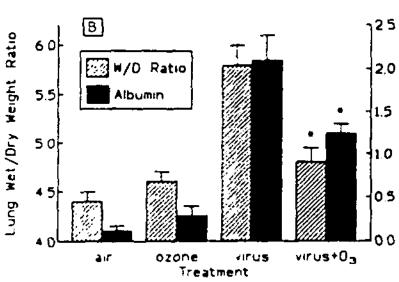


Figure 8. Morphometric analysis of lung tissue (*A*), lung wet/dry weight ratios and lung lavage albumin content (*B*) among virus-infected and -uninfected mice that were continuously exposed to 0.5 ppm ozone or allowed to breathe ambient air for 9 days. * = $p < 0.05$, virus-infected versus virus-infected and ozone-exposed. From Jakab et al. (*85*)

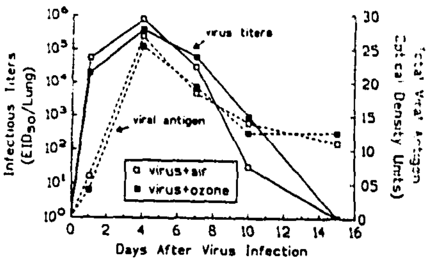


Figure 9. Comparison of infectious virus titers and total viral antigen in lung homogenates during the course of influenza virus infection in mice exposed continuously to 0.5 ppm ozone or ambient air. From Jakab et al. (*85*).

With lethal doses of virus, immunosuppressed mice still succumb to influenza virus infection. However, the deaths are not the early pneumonic deaths but occur later and are due to the extrapulmonary spread of the virus (*90,91*). The effects of continuous 0.5 ppm O_3 exposure on a severe influenza A/PR/34 infection were investigated. Twenty-five percent of the unexposed control mice died between day 8 and day 12 of infection, with no deaths occurring thereafter. In contrast, in O_3-exposed mice, 5% died between day 8 and day 12, with another 35% succumbing between day 12 and day 20 of the infection (GJ Jakab, unpublished observations).

Discussion

Before reaching any conclusions on the effects of *in vivo* exposure to O_3 on the immune system, several related issues warrant discussion. These issues broadly encompass inhalation toxicology, immunotoxicology, host factors, and experimental design.

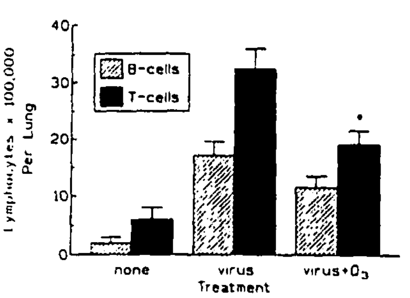


Figure 10. Comparison of phenotypically identified T- and B-lymphocytes recovered from lung tissue of virus-infected mice with or without continuous 0.5 ppm ozone exposure for 8 days. * = $p < 0.05$, virus-infected versus virus-infected and ozone-exposed. From Jakab et al. (*85*).

Considerations on the Inhalation Toxicology of Ozone

Concepts of inhalation toxicology dictate that the animals exposed to O_3 and unexposed animals are treated in an identical manner (i.e., the control animals are exposed to filtered air in an identical exposure chamber so that the only difference between the experimental and control group is the O_3 exposure). Assuming that this practice is followed, several issues arise with the longer term continuous O_3 exposure studies that may influence the outcome of an immunologic parameter under investigation. For example, continuous O_3 exposure leads to a loss of appetite in rodents as reflected by a slower weight gain in young animals (*92*) and, depending on the O_3 concentrations, weight loss in older mice (GJ Jakab, unpublished observations). Malnutrition is known to affect the integrity of the immune system (*93*) and, to a lesser extent, undernutrition also blunts the immune response (*94*). In addition to O_3 exposure retarding growth due to loss of appetite, the food consumed by control and exposed animals is not the same. Ozone exposure oxidizes the food thereby altering its nutritional value (*95*). Variations in diet composition are known to affect the immune response (*93,94,96*). Because of this, animals exposed to O_3 for longer periods of time have a nutritional disadvantage that may act in concert with O_3 exposure to suppress immunity. During long-term continuous O_3 exposure, little can be done about the loss of appetite except to match the food intake of the control animals to that of the exposed animals. To minimize the effects of ingesting O_3-oxidized food, it may be prudent to provide O_3-treated food for the control animals (*85,86*).

In addition to the nutritional aspect, the health status of the experimental animals also merits consideration. Animal husbandry has greatly improved over the past few decades to the point that reputable suppliers are capable of providing animals for research purposes that are free of intercurrent diseases. However, endogenous infections of rodents are pervasive (*97*), and assessment of the health status of the animals requires constant vigilance by suppliers and in the research setting since intercurrent infections are known to have profound effects on toxicity and research testing (*98,99*). For example, one of the most common infections of rodent colonies is Sendai (parainfluenza 1) virus (*100*). Sendai virus infection suppresses AM phagocytosis (*101,102*) and the primary antibody response of splenocytes to SRBCs (*103*) reduces T-cell mitogenesis (*104,105*), increases splenic NK cell activity (*106,107*), alters the *in vitro* response to heterologous erythrocytes (*108*), and alters immune cell function in aging mice (*109*). Other endogenous rodent infectious agents of concern to toxicologists include pneumonia virus of mice, mouse hepatitis virus, rat coronavirus, minute virus of mice, Kilham rat virus, Tooland's virus, and *Mycoplasma pulmonis* (*98,110*). Because of the possibility that intercurrent infections confound studies, the validity of observed results may be at risk if the health status of animals is not defined.

Considerations of the Immunotoxicology of Ozone

Undoubtedly, one of the overriding factors involved in the divergent responses to O_3 exposure on innate and acquired immune systems are the types of experimental methods used among the various studies. For example, a close examination of the methods used to determine the effects of O_3 exposure on AM phagocytosis reveals the following techniques: *a*) visual counting of the ingestion of 3.5 µm diameter carbon-coated latex microspheres by adherent AMs (*32*); *b*) visual counting of the ingestion of latex particles of unknown size by rabbit AMs in suspension (*34*); *c*) scintillation counting of Fc-receptor-mediated phagocytosis of ^{51}Cr-labelled SRBCs by mouse and rat AMs seeded in 96-well microtiter plates (*35*); and *d*) visual counting of Fc-receptor-mediated ingestion by adherent rat AMs (33) and mouse AMs (*67,74*). With the use of the 3.5 µm carbon-coated latex particles, it was observed that continuous exposure to 0.8 ppm O_3 for 3 days enhanced Sprague-Dawley rat AM phagocytosis (*32*). In contrast, the opposite was observed when the challenge particles were sensitized SRBCs in that continuous 0.5 ppm O_3 exposure suppressed Wistar rat AM phagocytosis (*33*). These data point to vast differences in sensitivities to O_3 exposure between the two assay systems, the response between the two strains of rats, or the 0.3 ppm difference in the O_3 exposure concentration.

In a manner similar to that exemplified with the effects of O_3 exposure on AM phagocytosis, questions arise about the relative sensitivities of the assays that measure lymphocyte function. Uptake of radiolabeled thymidine following stimulation with lectins or microbial products is a common method for assessing the ability of T- and B-cells to proliferate; a step required for the amplification of the immune response. These mitogens have specific binding sites which may not be present on all cell types (*111*). For example, PHA binds to the T cell receptor and CD2 antigens of most T-cells (*111*). Concanavlin A binds to all T-cells (CD3) and is more potent than PHA (*112*), while differences in the binding sites of PWM may exist between different species of animals. Although the binding of these mitogens to specific glycoproteins on the cell surface are integral to subsequent proliferation, these binding sites are many and varied on any given cell population. Thus, depending upon the mitogen employed and the species from which the cells are derived, immune defects in any population of cells may be exaggerated or underestimated during a proliferative event.

Interpretation of experimental results becomes more complex when examining specific immune responses toward antigens. Often the generation of antibody against T-cell-dependent antigens (e.g., SRBCs) are compared to antibody production against T-cell-independent antigens (e.g., DNP-Ficoll) as indicators of T- and B-cell competence respectively. Even though these regimens are optimized to provide maximal immune stimulation, the relative immunogenicity of the test antigens and the avidity and specificity of the antibodies generated may differ between assay systems. Thus, problems of antigen potency and antibody specificity may arise not only in the immunization stages *in vivo* but also in the detection of antibody during immunoassay.

Insofar as there are shortfalls in conventional testing of immune function and in comparing the relative sensitivity of xenobiotic agents on the different arms of the immune response, Luster et al. (*113,114*) have demonstrated concordance between a chemical effect on the ability of mice to form antibody to SRBCs and its potential to cause an immunotoxic effect in any of a number of other assays measuring immune function. Changes in lymphocyte surface markers and, to a lesser extent, cell proliferation to mitogens and NK activity also showed good correlation with immunotoxicity; however, B-cell proliferation to LPS and the mixed lymphocyte reaction (graft rejection) did not.

In studying the possible immunomodulatory effects of O_3 and other air pollutants, some attempts have been made to examine the effect of O_3 on pulmonary immune responses such as local antibody production (*22,23,68*), NK activity (*16*), and lymphocyte function in the draining lymph nodes (*68*). Although these experiments are technically feasible, their interpretation can be challenging because of the constant trafficking of serum factors and immune cells from the circulation to the lung and possible enhancement of these features during inflammation. For example, the observation of decreased lymphocyte sensitivity to antigen in the spleen following prolonged O_3 exposure and a contrasting increase in MLN lymphocyte activity (Figure 4A) could be explained by local inflammation and cellular recruitment to the site of oxidant damage (i.e., a simple matter of immune circulation). Until the cellular trafficking between the systemic immune compartment and that of the lung during immune stimulation are more clearly understood, these complicating factors will remain.

Considerations of Host Factors

A number of studies have investigated the age-dependent response to O_3 exposure on nonimmunologic bioparameters of the lung (*115–119*). *In toto*, these studies show that, depending on the end point, the age of the animals can play a significant role in the outcome of the effects of O_3 exposure. With respect to immunologic parameters, it should be kept in mind that the immune response is known to wane with age (*120*).

In addition to age, qualitative difference in nonimmunologic responses to O_3 among animal species have also been documented (*121–127*). Also, it is becoming increasingly clear that pulmonary responses to O_3 exposure is subject to genetic influence (*128–132*), and it is also known that the immune response is under genetic control (*133,134*). While the wide diversity of receptor sites on antibody and T-cell receptors occur by gene rearrangement of the immunoglobulin superfamily, a more innate and stable genetic influence exists over host susceptibility in mice to intracellular infections such as *Listeria*, *Mycobacteria*, and *Salmonellae* spp. (*135*). In many cases, the host response, which governs protection from these organisms, is the DTH reaction, involving both T-cells and subsequent activation of macrophages. Genetic differences have also been recently observed in the ability of two different strains of mice to resist streptococcal challenge (*50*). It is also becoming clear that some mouse strains produce high or low titers of circulating antibody following identical immunization procedures (*136*). Thus, in addition to species differences, strain differences may also play a role in the immunotoxicology of O_3; at this time, it is not known to what extent the combination of variables that include age, animal species, and genetic constitution may influence the effect of O_3 exposure on immune responsiveness.

Considerations of the Ozone Exposure Protocols

Investigations of the effects of O_3 on immunologic end-points that do not involve antigen stimulation have primarily used the following protocols: *a*) continuous (or intermittent) exposure on consecutive days with tests performed during the exposure protocol; *b*) a short (hourly) exposure with tests performed immediately after exposure or at intervals thereafter; and *c*) continuous (or intermittent) exposure for several days or weeks with tests performed immediately after exposure or at intervals thereafter.

Using continuous O_3 exposure with assays performed during a 2- to 4-week exposure period, four general response patterns emerge from the literature (Figure 11). In the first (Figure 11A), an initial suppression followed by recovery has been observed with spleen weights (*3*), spleen/body weight ratios (*68*), thymus weight (*4*), AM phagocytosis (*33,34,67,74*), PM phagocytosis (*74*), pulmonary NK and splenic NK cell activity (*16,68*), MLN proliferative response (*68*), and AM-dependent intrapulmonary bacterial killing (*67*). The second response pattern (Figure 11B), an initial suppression followed by an increased response, has been observed with MLN cell numbers (*4,68*). The third response pattern (Figure 11C), an initial absence of a response followed by increased activity, has been observed with MLN (*8*) and splenic (*14*) proliferative responses. Finally, in Figure 11D, a sustained response of thymic atrophy has also been observed (*11*).

The second exposure protocol consists of short (hourly) O_3 exposures with the tests performed immediately after exposure or at various times thereafter. The data show that immediately after exposure, AM phagocytosis is suppressed (*30,31,34*) and then followed by reestablishment of phagocytosis as the interval between exposure and assay was lengthened.

The third exposure protocol consists of continuous O_3 exposure for several days (or weeks) followed by tests immediately after exposure or at various intervals thereafter. Using this exposure protocol, T/B-lymphocyte ratios remained elevated for at least 5 days after exposure (*10*). Perhaps more studies were not performed with this O_3 exposure protocol because of a lack of an effect. A nonresponse situation may be predicted by the observations that continuous O_3 exposure, after a period of initial suppression, is followed by reestablishment

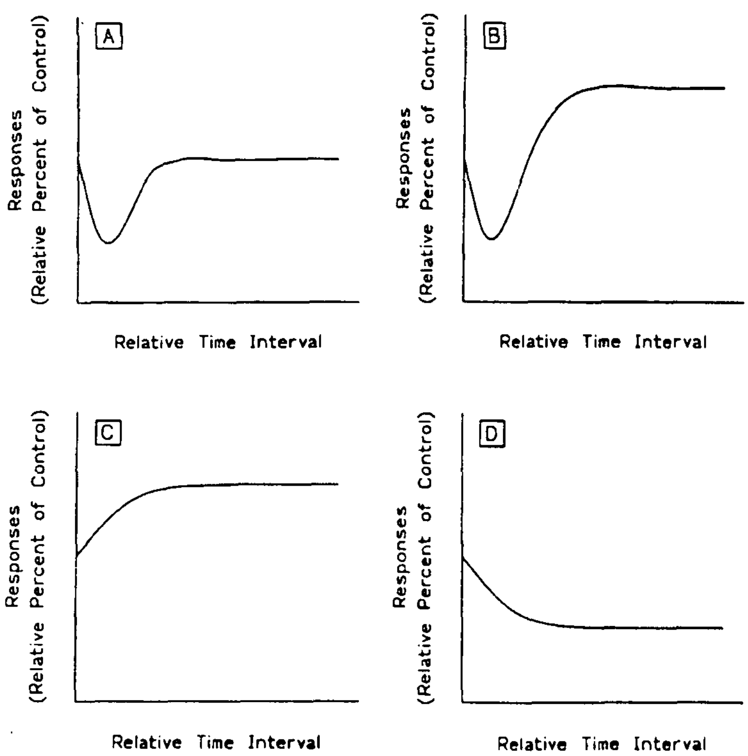


Figure 11. Relative responses of the immune apparatus (lymphoid organ weights, proliferative responses to mitogen stimulation, NK cell activity, alveolar macrophage phagocytosis, and intrapulmonary staphylococcal killing (see

of the response, as exemplified by the O_3 exposure response pattern depicted in Figure 11A. This is supported by our range-finding studies that showed that continuous 0.5 ppm O_3 exposure for 10 consecutive days had no effect on AM-dependent intrapulmonary bacterial killing of *S. aureus*, either immediately after cessation of exposure or at 1 and 5 days thereafter (GJ Jakab, unpublished observations).

Superimposed on the three exposure patterns are the O_3 concentrations and whether the exposures are continuous or intermittent. In general, it appears that the O_3 concentrations modulate the intensity and the duration of the responses. Intermittent O_3 exposures can be viewed as alternating cycles of O_3-induced injury and repair that appear to blunt the intensity of the responses.

When the effects of O_3 exposure on antigen stimulated responses are examined, the number of possibilities in exposure protocols is vastly increased. The simplest of these protocols is to expose animals to O_3 either before or after immunization. The data show that continuous O_3 exposure immediately before immunization had no effect on the DTH response to SRBCs (*28*), the DTH response to *Listeria* antigen, or the proliferative response of MLN to *Listeria* antigen (*10*), whereas O_3 exposure after immunization suppressed the responses. Using primary and secondary immunizing regimens, we have investigated the timing between immunization and O_3 exposure and found that as the interval was increased beyond 3 days, no effect was observed (*68*).

Summary

From the above discussion, it is apparent that many known and unknown factors impinge on the effect of O_3 exposure on immunologic responsiveness. The extent to which nutritional factors, health status, age, species variability, genetic background, and immune assay sensitivity interact to play a role on the effect of O_3 exposure on the immune system is difficult to unravel from the available data. Mechanistic studies will undoubtedly provide insights into the causes of the observed responses. Questions that beg answers include: *a*) Is there a level of O_3 exposure below which immune responses are enhanced and above which there is a toxic effect that suppresses immune responses? *b*) What is the mechanism for adaptation? *c*) Given the fact that O_3 itself never reaches the systemic immune compartment, what is the mechanism of O_3-induced suppression of systemic immunity? *d*) Where, in the sequence of events that leads to an antigen-stimulated immune response, does O_3 have an effect? Is it antigen availability, antigen presentation or a step in the complex cascade of events in the afferent limb of the immune response which may result in altered effector function? As alluded to before, O_3 and other oxidant gases are inflammatory when inhaled and thus cause a variety of permeability changes in the lung that could cause an increase in reactive immune tissue (BALT) or increase the availability of antigen to immunoreactive areas.

Much information is available regarding the effects of O_3 exposure on immune function. However, this information has not paved major inroads toward an understanding of the mechanisms by which O_3 exposure enhances/impairs/modulates immune function. The necessary database from which specific mechanistic hypotheses can be tested is available. The results of these studies will undoubtedly lead to a more complete understanding of the mechanisms by which O_3 alters immune function.

Although it is expectated that future experimental studies will unravel the mechanisms by which O_3 alters immune responsiveness, their relationship to human health effects will require serious consideration. The National Ambient Air Quality Standard for O_3 is 0.12 ppm for a maximum of 1 hr that is not to be exceeded more than once per year. The worst case situation for human environmental exposure is in urban areas with concentrations reported as high as 0.36 ppm O_3 (*137*). However, the ambient concentrations of O_3 do not reflect the high O_3 excursions documented in urban areas. Indeed, most experimental animal studies have had to use O_3 exposures in excess of the ambient and spike urban O_3 concentrations to demonstrate an immunotoxic effect. In this regard, the study of Selgrade et al. (*18*) found no effect on multiple immune parameters when rats were chronically exposed to O_3 in a way that simulated an urban profile. Finally, in addition to the O_3 concentrations, the inferences that can be drawn between human health effects and the battery of immune tests recommended in the National Toxicology Program guidelines for immunotoxicity evaluation in mice (*113*) need to be clarified.

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perspectives
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Environmental Epidemiology

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Perinatal Exposure to Dioxins

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