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Exposure to Ozone Reduces Influenza Disease Severity and Alters Distribution of Influenza Viral Antigens in Murine Lungs

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Exposure to ambient levels of ozone (0.5 ppm) was shown to alter the pathogenesis of respiratory infection after aerosol infection of mice with influenza A virus. A semiquantitative method for determination of the sites of virus replication by direct immunofluorescence indicated that exposure to ozone reduced the involvement of respiratory epithelium in the infectious process and resulted in a less widespread infection of the alveolar parenchyma. Furthermore, the ozone-mediated alteration in viral antigen distribution was consistent with significantly reduced influenza disease mortality and prolonged survival time, but only when the oxidant was present during the course of infection. Reduced disease severity in ozone-exposed animals appeared to be independent of peak pulmonary virus titers, pulmonary interferon titers, and pulmonary and serum-neutralizing antibody titers. These studies suggested that the distribution of influenza virus in the murine lung was a key factor in disease severity.

Influenza virus is a clinically important agent widely distributed throughout human and animal populations. The pathogenesis of this viral infection can be modulated by chemical agents, and the presence of these agents in the atmosphere has potentially important implications in the incidence and severity of infection as well as in proposed methods for disease prevention.

Oxidizing pollutants in the atmosphere have been implicated in increased incidence of respiratory infections in humans living in urban areas (10, 16, 28). However, the severity of some of these respiratory infections has been reported to be diminished by exposure to ozone, the most abundant oxidant pollutant, or to sulfur dioxide (2, 5, 12, 13, 20).

This study was undertaken to assess the effects of exposure to ambient levels of ozone on the course of influenza virus infection in a murine model. Mice were exposed to various regimens combining ozone or filtered air or both with aerosolized infection by influenza virus. Through these manipulations, it was possible to monitor some of the parameters associated with virus infection and to determine the mechanisms by which ozone exposure influences the pathogenesis of this respiratory disease. It was found that animals exposed to ozone during infection showed (i) a reduced severity of disease, as measured by decreased mortality and delayed time of death, and (ii) a significantly different distribution of viral antigens in pulmonary tissues when compared with animals housed in non-polluted atmospheres.

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MATERIALS AND METHODS

Virus. Influenza A virus (WSN strain) was propagated in Madin Darby bovine kidney (MDBK) cells according to Choppin (8). Virus suspensions were clarified by centrifugation at 5,000 × *g* and stored at −70°C.

Experimental design. Specific pathogen-free, Swiss-Webster, female mice (Hilltop Laboratories, Scottsdale, Pa.) were 8 weeks of age at the time of initiation of each experiment. Four hundred mice in each of two experiments were divided evenly among four groups designated A through D. Groups A and B were exposed to 0.5 ppm ±10% (standard error of the mean) ozone, and groups C and D were exposed to filtered air for a 2-week period. All mice were then infected with influenza virus by aerosol. Half of the ozone-adapted animals continued in an ozone environment (group A) for a 2-week period, and half were moved to filtered air (group B) for 2 weeks. Half of the filtered-air-adapted animals were moved into ozone (group C), and half continued in a filtered-air environment (group D) for a 2-week period to serve as the infected control group.

Environmental control chambers. Mice were housed in stainless steel and glass chambers (3.5 m^3) provided by the California Primate Research Center (Davis, Calif.). One chamber received 30 volume changes per h of tandem Chemical, Bacteriological and Radiological (CBR)-filtered air (Mine Safety Appliances Co.,

Pittsburgh, Pa.). The second chamber received 30 volume changes per h of tandem CBR-filtered air mixed with ozone. Ozone was generated from vaporized medical-grade oxygen by silent electric arc discharge and monitored at 10-min intervals with a UV photometric analyzer (Dasibi Environmental Corp., Glendale, Calif.).

Aerosol exposure to influenza virus. An equal number of mice from each of the four groups was simultaneously exposed to an aerosol of influenza virus in a Tri-R Airborne Infection Apparatus (Tri-R Instruments, Inc., Rockville Centre, N.Y.). Stock virus suspensions were diluted in Eagle minimal essential medium (GIBCO Laoratories, Grand Island, N.Y.) to a concentration of 1.6×10^5 PFU/ml, and 64 mice were exposed to an 8.0-ml virus sample nebulized over a 25-min period. The total time required for transport, infection, and handling of animals outside the environmentally controlled chambers was less than 1.5 h.

Calculation of mortality. Mortality was calculated by a formula which compensated for animals that would have died from influenza infection if they had not been sacrificed for sampling and which accounts for different death frequencies on different days after infection.

$$\text{Mortality} = D + \sum_{i=1}^{n}\left(\frac{di}{Xi} \cdot Si\right)$$

where D = the total number of observed deaths in the group due to influenza infection; i = the sample number; n = the number of the last sample; di = the number of deaths in the group which occurred between sample i and sample (i+1); Xi = the number of animals remaining alive in the group after the removal of sample i; and Si = the number of animals sacrificed in sample i.

The frequency of mortality observed between successive samples (di/Xi) was used as an estimate of the frequency of mortality expected in the sample group (Si). The number of expected deaths for each sample (di/Xi · Si) was summed and added to the number of observed deaths to obtain the estimate for total mortality in the group. This formula provided a reasonable estimate of the mortality when the sample size was the same for all groups under study and there was a large number of animals in each group.

Virus assay. The lungs from two mice per group on each sampling day were harvested aseptically, the trachea and main bronchus were removed, and a 10% lung homogenate was made in Hanks balanced salt solution. Homogenates were clarified by centrifugation at 5,000 × g, and supernatant fluids were stored at −70°C. Virus was titrated by plaque assay on MDBK cells under 2% agar (Difco Laboratories, Detroit, Mich.) mixed with an equal volume of double strength (2×) Eagle medium. Titers were expressed as PFU per lung.

Interferon samples. Four mice from each group were sacrificed on each sampling day to determine interferon titers in lung lavage fluids collected by the lavage method previously described (23). The fluids were adjusted to pH 2.0 with 1.0 M perchloric acid, refrigerated for 4 days, centrifuged for 1 h at 100,000 × g, and adjusted to pH 7.0 with 1.0 M NaOH. A 50% cytopathic effect reduction assay in microtiter was used to titrate interferon activity on mouse L cells treated with the lavage fluid for 24 h before challenge with 200 PFU of the Indiana strain of vesicular stomatitis virus.

Neutralizing antibody samples. The sera from mice were obtained by severing the renal artery and were pooled according to group by days post-infection. Individual samples of neutralizing antibody accumulated in the lung were obtained from four mice in each group on each sampling day by the pulmonary lavage technique (23). Serum samples were stored at 4°C and inactivated at 56°C before assay. Influenza-neutralizing activity was titrated on MDBK cells by 50% cytopathic effect reduction in microtiter.

Fluorescein-labeled antiserum to influenza A WSN virus. Virus propagated in 11-day-old embryonated eggs was used to immunize New Zealand white rabbits (29). An ammonium sulfate-precipitated globulin fraction of the serum was conjugated to fluorescein by the method of Clark and Shepard (9). The conjugate gave bright, apple-green fluorescence on influenza-infected MDBK cells but not on mock-infected MDBK cells or vesicular stomatitis virus-infected mouse L cells. The conjugate did not specifically stain lung tissues from uninfected mice housed in filtered air or in ozone environments.

Preparation of lung tissue for immunofluorescence. After intraperitoneal anaesthetization with pentobarbital sodium and exsanguination, the lungs of each mouse were perfused with 0.75 ml of Tissue Tek (Lab-Tek Products, Naperville, Ill.). Individual lobes were immersed in Tissue Tek-filled embedding molds and frozen in liquid nitrogen. The right apical lobe of each mouse lung was step-sectioned in a cryostat (Harris International Equipment, Needham Heights, Mass.) at a thickness of 4 μm. Sections were taken from all areas of one entire lobe, leaving at least 80 μm and not more than 120 μm between sections to avoid sampling of the same area of the lung lobe in adjacent tissue sections. Sections were fixed in acetone for 10 min and stored at −70°C. Tissues were stained with the conjugate for 1 h at 37°C in a humidified chamber, rinsed three times in 0.01 M phosphate-buffered saline (pH 7.3) and washed in double-distilled water. Samples were mounted in glycerol–phosphate-buffered saline (1:1) after air drying and observed under epi-illumination in a Zeiss fluorescence microscope.

Semi-quantitative determination of sites of virus replication. Lung tissue sections were initially scanned at 160× magnification in S-shape pattern, and the number of airways with and without fluorescence in the lining epithelium was counted. Antigen-positive airways were defined to be airways with specific fluorescence in the visible lining epithelium. Typically, the entire length of visible epithelium was fluorescing or completely void of fluorescence. However, in a few instances in groups B and C, it was necessary to subjectively designate an airway as antigen positive when fluorescence appeared in patches. The percentage of antigen-positive airways was calculated for each group according to sampling day. The same tissue sections were then scanned in S-shape pattern at 400× magnification. The alveolar area of each microscopic field was 0.159 mm^2. Each alveolar field which contained at least one fluorescent cell was defined as an antigen-positive field. The number of antigen-positive fields counted for each group according to sampling days was expressed as a percentage of the total

TABLE 1. Influenza disease severity in mice exposed to ozone and filtered air[a]

Group[b]	Cumulative % mortality	Significance[c]	Mean day of death	Significance[d]
A (O_3-V-O_3)	6.5	$P < 0.0005$	10.9	$P < 0.025$
B (O_3-V-F)	19.8	$P < 0.005$	9.8	NS[e]
C (F-V-O_3)	3.5	$P < 0.0005$	12.3	$P < 0.0005$
D (F-V-F)	30.4	Control	9.9	Control

[a] Data were derived from two experiments of 100 animals per group per experiment. Mortality was calculated as described in the text.
[b] O_3, Ozone; V, influenza virus; F, filtered air.
[c] Based on reduction from control group by χ^2 analysis.
[d] Based on increase from control group by the Student t test.
[e] NS, Not significant.

number of alveolar fields examined. The number of alveolar cells expressing fluorescence in each antigen-positive field was counted without differentiating between cell types. The mean number of antigen-positive cells per antigen-positive field was calculated and defined as antigen density within a positive field.

Statistical analyses. Differences in mortality were analyzed by the chi-square (χ^2) test. Differences in mean survival time were analyzed by the Student t test. Four-way and paired χ^2 tests were used to analyze data on fluorescence in the airways and in the alveolar parenchyma. An analysis of variance was performed on data from determination of the mean number of antigen-positive cells per antigen-positive field (antigen density).

RESULTS

Mortality. Exposure to ozone after infection significantly reduced influenza virus mortality (compare groups A and C with control group D; $P < 0.0005$; Table 1). Animals moved from ozone into filtered air after infection (group B) showed variable mortality in two experiments. Exposure to ozone after infection also resulted in a significant increase in mean survival time (group C, $P < 0.0005$ and group A, $P < 0.025$) when compared with survival time in the infected control group D. There was no effect on mean survival time in ozone-adapted animals moved into filtered air after infection (group B) in either of two experiments, despite the variability in cumulative mortality mentioned above.

Pulmonary virus production. Figure 1 shows titers of influenza virus in homogenates of pulmonary tissues. Slight reductions in virus titers were observed during the first 2 days of infection in animals exposed to ozone. Peak virus titers on days 4 and 6 post-infection were lowest in animals newly exposed to ozone (group C), whereas peak titers in the other three groups were similar.

Interferon titers. Interferon was measurable in the lung lavage fluids of all groups during the early days of infection. There were no significant differences in interferon titers among the four groups (Fig. 2) over doubling dilutions of lavage fluid. The titer of the interferon standard included in each assay did not vary (data not shown).

Virus-neutralizing antibody. Serum-neutralizing antibody titers appeared earlier and reached

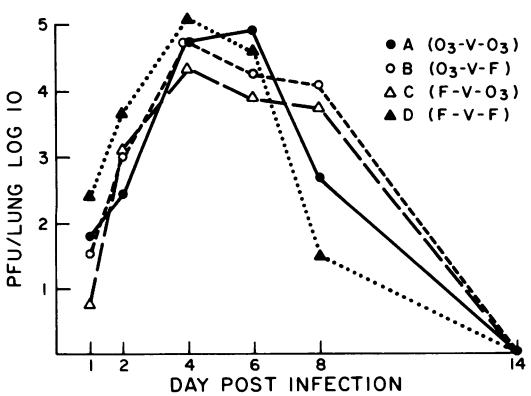

FIG. 1. Titers of influenza A virus in lung homogenates from four groups of mice (A, B, C, and D). O_3, Ozone; V, influenza virus; F, filtered air.

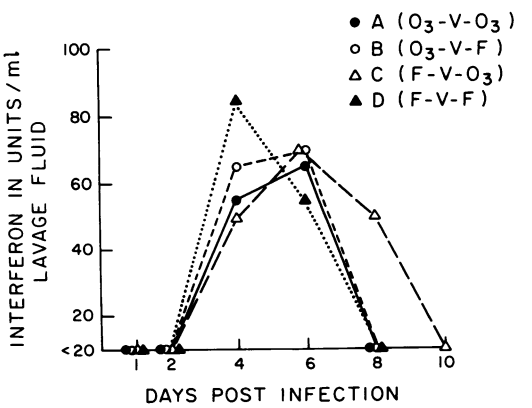

FIG. 2. Interferon titers in lung lavage fluid from four groups of mice (A, B, C, and D). O_3, Ozone; V, influenza virus; F, filtered air.

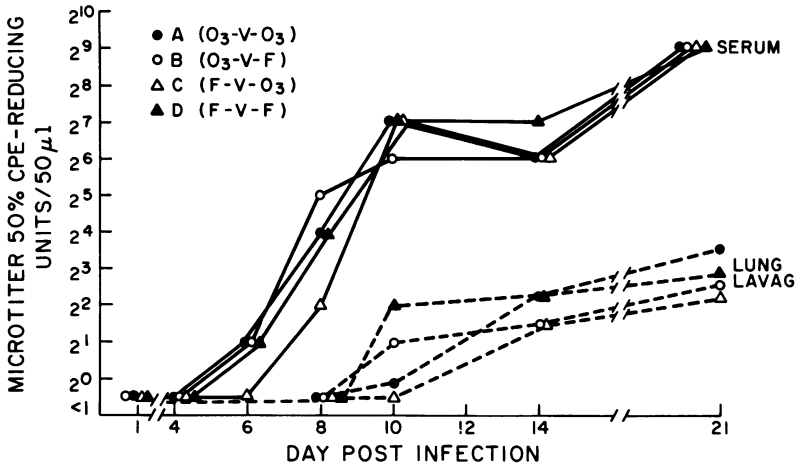

FIG. 3. Influenza-neutralizing antibody titers in serum and lung lavage fluid from four groups of mice (A, B, C, and D). O_3, Ozone; V, influenza virus; F, filtered air.

higher levels than those in the lung lavage fluids in all four groups (Fig. 3) over doubling dilutions of lavage fluid or serum. The neutralizing titer of a rabbit hyperimmune influenza antiserum included in each assay did not vary (data not shown). No significant differences in the time of onset or magnitude of the neutralizing antibody response in either sera or lavage fluids were detected among the four groups studied.

Immunofluorescence determination of viral antigens in the airways. Assessment of involvement of respiratory airways in the infectious process was based on calculation of the percentage of airways which expressed viral antigens (Fig. 4). In the environmental control group D, influenza infection predominated in the epithelium of the respiratory airways early in infection. The percentage of antigen-positive airways increased through day 4 postinfection and showed a relatively continuous distribution of viral antigens along the lining epithelium (Fig. 4). In mice housed continuously in ozone (group A), airway

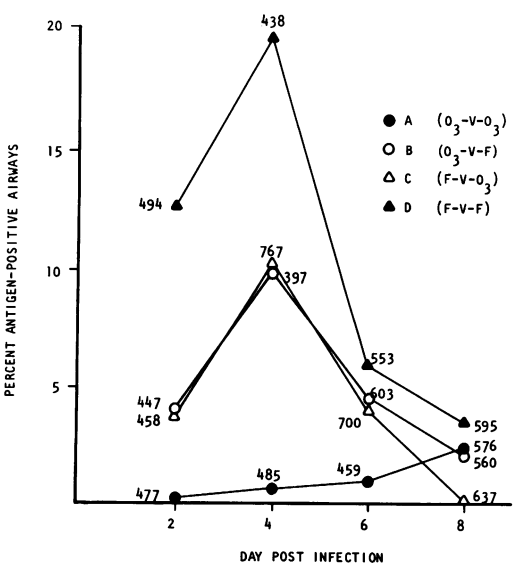

FIG. 4. Quantitation of airway fluorescence in groups A, B, C, and D. Numbers adjacent to data points indicate the number of airways observed for each determination. O_3, Ozone; V, influenza virus; F, filtered air.

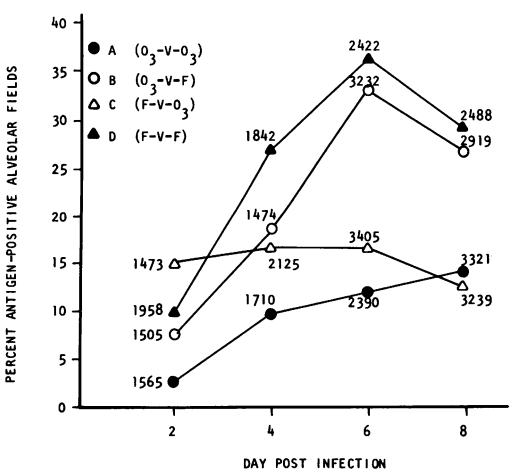

FIG. 5. Quantitation of alveolar field fluorescence in groups A, B, C, and D. Numbers adjacent to data points indicate the number of alveolar fields observed for each determination. O_3, Ozone; V, influenza virus; F, filtered air.

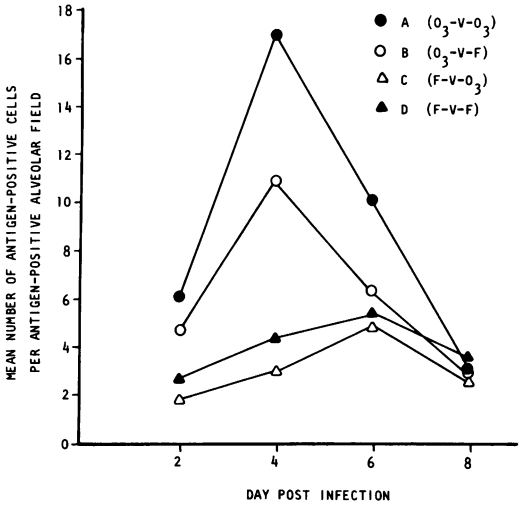

FIG. 6. Mean antigen density in each antigen-positive alveolar field from Fig. 5 for groups A, B, C, and D. O_3, Ozone; V, influenza virus; F, filtered air.

epithelium was essentially void of fluorescence on all days postinfection. Groups housed in combined atmospheres of ozone followed by filtered air (group B) or filtered air followed by ozone (group C) displayed percentages of antigen-positive airways intermediate between the two continuous-environment groups. Chi-square analysis confirmed that animals exposed to ozone had significantly fewer antigen-positive airways early in infection than did animals housed continuously in filtered air ($P < 0.0005$). Additionally, the statistical analysis confirmed that continuous exposure to ozone before and during infection (group A) resulted in the fewest antigen-positive airways of all groups during the early days of infection ($P < 0.0005$ on days 2 and 4; $P < 0.005$ on day 6).

Immunofluorescence determination of viral antigens in the alveolar parenchyma. Certain trends were apparent in the distribution of viral antigens in the alveolar parenchyma, depending on the group and the day postinfection. Fluorescence in the parenchyma was either dispersed throughout the alveoli such that relatively few fluorescent cells were observed in any given field and adjacent fields contained similar numbers of fluorescent cells, or viral antigens were concentrated into foci containing relatively large numbers of fluorescent cells surrounded by areas void of viral antigens. Two distinct measurements were used to determine the extent and nature of involvement of the alveoli in the infectious process. Alveolar fields which contained at least one fluorescent cell were defined as "antigen-positive fields" and expressed as a percentage of the total number of fields observed. The total number of fluorescent cells observed in the alveoli was tallied and expressed as the mean number of antigen-positive cells per antigen-positive field, described hereafter as "antigen density." Figures 5 and 6 show the semiquantitative determinations obtained from observations of fluorescent antibody-stained alveolar tissues by day postinfection in each environmental group.

In the control group (D), the percentage of antigen-positive alveolar fields increased rapidly each day postinfection to day 6 (Fig. 5). Concurrently, the number of antigen-positive cells per positive field increased through day 6 (Fig. 6).

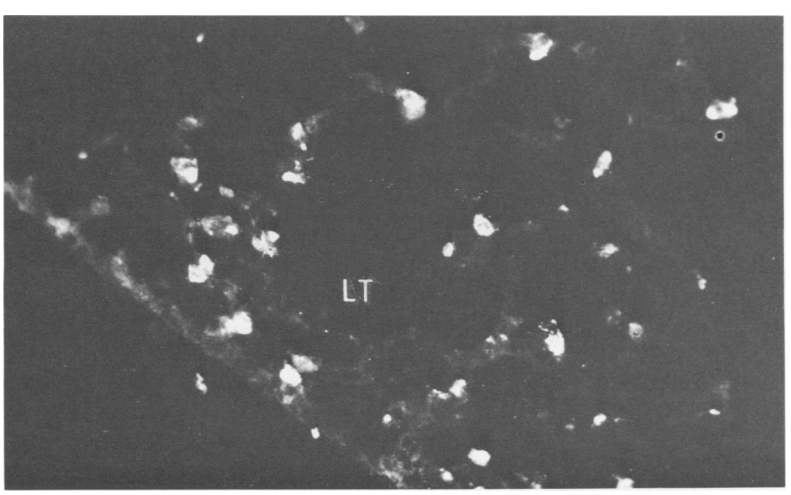

FIG. 7. Widespread fluorescence of alveolar cells in the lung parenchyma of a mouse in group D 6 days after influenza virus aerosol (× 3,000). LT, Lung tissue.

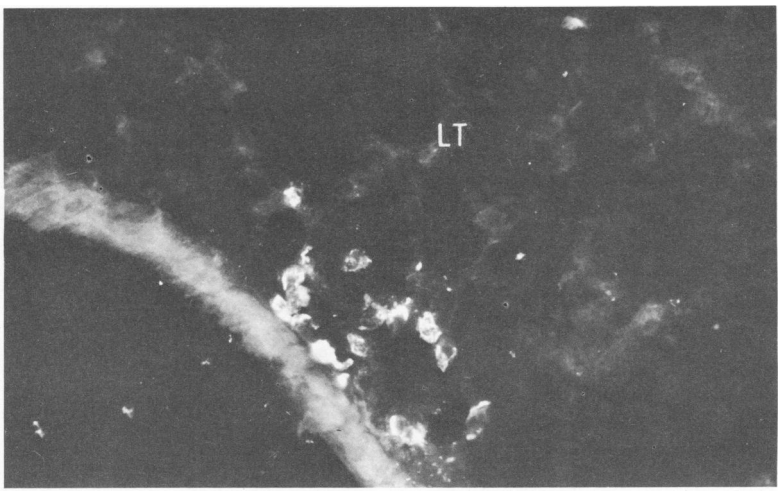

FIG. 8. A cluster of fluorescing cells adjacent to an antigen-negative airway and a large area of uninfected lung parenchyma from a mouse in group A 2 days after influenza virus aerosol (× 3,000). LT, Lung tissue.

The relatively low antigen density and relatively high percentage of antigen-positive fields was indicative that virus was dispersed throughout the parenchyma in relatively even distribution (Fig. 7).

In animals continuously exposed to ozone (group A), the percentage of antigen-positive alveolar fields was the lowest of all groups (Fig. 5), and the antigen density in each positive field was the highest of all groups (Fig. 6), peaking at day 4 postinfection. These observations indicated that viral antigens were concentrated into foci of infected cells in the parenchyma, leaving large areas of apparently uninfected tissue (Fig. 8).

Fluorescence in the alveolar parenchymas of animals in groups B and C was a combination of trends observed for the two continuous-exposure groups. In ozone-adapted animals moved to filtered air (group B), there was initially a low percentage of infected alveolar fields with relatively high antigen density (Fig. 5 and 6). This fluorescence distribution was followed by a rapid increase in antigen-positive fields, accompanied by a decrease in antigen density. In group C animals, moved from filtered air into ozone, the high percentage of antigen-positive fields observed in infection did not increase, and antigen density remained the lowest of all groups throughout the period of observation (Fig. 5 and 6).

Chi-square analysis of immunofluorescence data derived from alveolar observations (Table 2) revealed that animals housed in combined atmospheres, groups B and C, underwent a series of stepwise significant shifts in alveolar involvement relative to the continuous-exposure groups A and D. Animals newly exposed to ozone (group C) shifted to the "left" of the chi-square table to become increasingly similar to animals that had been housed continuously in ozone (group A). Animals adapted to ozone and moved to filtered air (group B) shifted to the "right" of the chi-square table to become increasingly similar to animals housed continuously in filtered air. By days 6 and 8 postinfection, these shifts had resulted in significantly fewer antigen-positive alveolar fields in animals housed in ozone than in animals housed in filtered air, regardless of the exposure atmosphere before infection.

DISCUSSION

Exposure to ambient levels of ozone (0.5 ppm) after aerosol infection with influenza A virus significantly reduced the severity of disease in mice, as shown by decreased mortality and increased survival time. Reduction of disease severity appeared to be dependent on the continued presence of ozone during the infectious process rather than on the atmosphere before infection and did not correlate with virus, interferon, or neutralizing antibody titers recovered from the lung or with neutralizing antibody titers in the sera. When compared with control infected mice housed in filtered air, mice exposed to ozone showed a significant alteration in the distribution of viral antigens within the pulmonary tissues, resulting in a less widespread infection of the lung. The pulmonary viral antigen distribution was consistent with observed mortality and appeared to be a major factor in determining the outcome of infection.

An analysis of the kinetics of virus production in the lungs of infected animals revealed slight

TABLE 2. Chi-square analysis of immunofluorescence determinations on alveolar fields by day postinfection

Day post viral infection	Antigen-positive alveolar fields by group[a]							
2	A	$P < 0.0005$ <	B	$P < 0.0005$ <	D	$P < 0.0005$ <	C
4	A	$P < 0.0005$ <	B	NS =	C	$P < 0.0005$ <	D
6	A	$P < 0.0005$ <	C	$P < 0.0005$ <	B	$P < 0.01$ <	D
8	A	NS =	C	$P < 0.0005$	B	NS =	D

[a] P value is based on chi-square analysis of paired tests, using data from Fig. 5. NS, Not significant.

reductions in pulmonary virus titers during the early days of infection in animals housed in ozone, with the lowest peak titers observed in animals newly exposed to ozone after infection (group C). A correlation between reduced early virus titers and reduced mortality has been previously reported in mice (25, 27); however, it seems unlikely that slight reductions in early virus titers lead to large reductions in mortality by direct correlation. The early events in influenza virus infection take place predominantly in airway-associated regions of the lung (21, 29) and may influence the subsequent events in the alveolar parenchyma. High titers of virus early in infection may induce more extensive damage to the alveolar tissue by mechanisms involving increased inflammation, lung consolidation, or serum protein exudation, or they may facilitate a more widespread infection of the parenchyma.

The titers of locally accumulated interferon appeared to be largely determined by the pulmonary virus titers, analogous to observations reported by several other investigators (18, 22, 30), rather than influenced by the environmental manipulations. Elevated lung lavage interferon titers did not correlate with enhanced protection from disease.

The difference in mortality among environmental groups could not be attributed to disparity in either the time of appearance or titers of virus-neutralizing antibody recovered from the lungs or sera. When the relatively equal antigenic load among the four environmental groups is considered, these results are not unusual. This observation is substantiated by previous reports demonstrating comparable neutralizing antibody levels in cases of different disease severity in respiratory virus infections between control and SO_2- or ozone-exposed subjects (2, 20). The appearance of local neutralizing antibody was coincident with the decline in pulmonary virus titers and suggested a role for locally accumulated neutralizing antibody in virus clearance. The local immune response has been previously implicated in recovery from respiratory virus infections (1, 7, 14, 27), and immunoglobulin A has been demonstrated to facilitate reduction of pulmonary influenza virus titers (17).

The determination of the sites of virus replication was made by immunofluorescent observation and by the enumeration of infected airways, alveolar fields, and alveolar cells in mice infected with influenza virus by the aerosol method. It was reasoned that the events observed throughout the entirety of a single lung lobe were representative of events which occurred in the entire lung. Numerical determinations of fluorescence were, therefore, obtained from the observation of a sequence of sections through one specified lung lobe by a step-sectioning technique that assured an overview of the entire lobe without presenting identical areas of the lobe in different tissue sections. The immunofluorescence determinations revealed extensive differences in influenza viral antigen distribution among the four environmental groups studied.

The striking difference early in infection in animals continuously exposed to ozone was a virtual absence of viral antigens from the respiratory epithelium. The removal of ozone from the airways by transfer of ozone-adapted animals to filtered air (group B) resulted in the appearance of viral antigens within the epithelium as early as 2 days postinfection, whereas no increase in infection of airways occured when animals were newly exposed to ozone after infection (group C). These observations suggest that the presence of ozone within the airway results in inactivation of virus infectivity in vivo. Our recent in vitro studies have shown that enveloped viruses such as influenza can be readily inactivated within a short period by exposure to levels of ozone comparable to that employed in this experiment (4). However, the

similar virus titers obtained in pulmonary homogenates among all groups did not substantiate a mechanism of detectable virus inactivation during the ongoing infectious process. This may be due in part to the establishment of an ozone concentration gradient within the respiratory tract (6, 11), where areas sequestered from an oxidant concentration sufficient for virus inactivation would contribute to the total pulmonary virus titer. Alternatively, exposure to ozone has been shown to induce extensive desquamation of respiratory epithelium and replacement with cells which morphologically resemble squamous epithelium (3, 11, 15, 26). These pulmonary changes may result in airway lining which is more resistant to infection by influenza virus.

The present study illustrated that the extent and nature of involvement of the alveolar parenchyma in the infectious process could be determined by two combined parameters: (i) the distribution of viral antigens, represented by the percentage of observed fields which contained viral antigens; and (ii) the concentration of viral antigens, represented by the mean number of fluorescent cells within an antigen-positive field (antigen density).

Our observation indicated that exposure to ozone during the infectious process had profound effects on the distribution of viral antigens in the alveolar parenchyma. Animals which were continuously exposed to ozone (group A) showed a concentration of viral antigens into foci in the alveolar parenchyma, leaving large areas of apparently uninfected tissue. The increase in the percentage of antigen-positive fields was not as great as that found in the infected control group (group D), although the antigen density within each positive field was significantly higher than that found in the infected control group. The percentage of antigen-positive fields in animals newly exposed to ozone (group C) did not substantially increase after infection, and these fields contained the lowest antigen density of all groups. In contrast, animals transferred from ozone into filtered air showed a rapid expansion of viral antigen through the alveolar parenchyma. These observations suggest that the spread of influenza virus infection through the alveolar parenchyma is impaired only when the oxidant is present during the infectious process. Furthermore, the reduced parenchymal spread of infection in the ozone environment did not appear to be dependent on the initial antigen density.

The limitation of spread of viral antigens in the alveolar parenchyma in groups housed in ozone during the infectious process correlated with decreased influenza disease severity, as shown by reduced mortality and prolonged mean survival time. The lowest mortality and longest survival time correlated with the lowest antigen density and a relatively low percentage of antigen-positive alveolar fields (group C). Other parameters of this study, including pulmonary virus titers, interferon titers, and neutralizing antibody responses, could not be correlated with the observed differential disease severity.

The results obtained by assessment of influenza viral antigen distribution in pulmonary tissues are supportive of previous studies which have shown that production of fewer or smaller lesions in the alveoli or reduced lung consolidation is associated with decreased disease severity in respiratory virus infections (19, 24, 25).

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