Impairment of Microbial Killing and Superoxide-Producing Activities of Alveolar Macrophages by a Low Level of Ozone

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(Received February 13, 2001; Accepted March 5, 2001)

Male Wistar rats were exposed to 0.2 ppm ozone for up to 14 days, during which alveolar macrophages were collected by pulmonary lavage to assess the effect of ozone on their microbial killing and superoxide-producing activities. For rapid assessment of microbial killing activity, we measured the release of 3H-radioactivity into the supernatant by deoxycholate-lysis of the macrophages that had phagosytosed and killed 3H-uridine-labeled microbes. The killing activity against Escherichia coli and Candida albicans was reduced to 70–80% of control levels on day 3. However, phagocytosis by and the activity of lysosomal enzymes of the macrophages were not impaired. On day 14 the killing activity against E. coli had returned to control levels, whereas that against C. albicans was still reduced. Because active oxygen species plays an important role in microbial killing activity of macrophages, the effects of ozone on respiratory burst and superoxide production were examined. Aliquots of alveolar macrophages were stimulated with phorbol myristate acetate (PMA), opsonized zymosan, or lipopolysaccharide (LPS) plus cytochalasin E (Cyt.E). The respiratory burst, oxygen consumption for rapid superoxide production, was decreased to 60–80% of control levels on day 3. On day 14, the respiratory burst by opsonized zymosan was still 80% reduced, whereas that by PMA or LPS plus Cyt.E had returned to control levels. In addition, the superoxide-producing activity of ozone-exposed macrophages was 10–60% decreased on day 3. On day 14, the superoxide production by stimulation with opsonized zymosan was still 60% reduced, whereas that by PMA or LPS plus Cyt.E had returned to control levels. In conclusion, because of their decreased production of superoxide, the host defense activity of alveolar macrophages was impaired by in vivo exposure to 0.2 ppm ozone. In particular, the C. albicans-associated defect lasted throughout the exposure period.

Key words — Macrophage, host defense, ozone, superoxide, Escherichia coli, Candida albicans

INTRODUCTION

Ozone, a representative oxidant found in urban and industrial atmospheres,1) injures alveolar and bronchiolar epithelial cells. Type I epithelial cells in alveoli and the ciliated cells in terminal bronchioles are the most sensitive to this pollutant, which causes these cells to necrose and desquamate.2–4) Alveolar macrophages exist on the alveolar surface and play an important role in the host defense against inhaled microorganisms.5–7) The macrophage functions of bactericidal activity,8–10) phagocytosis,8–14) and lysosomal hydrolysis8,15,16) are impaired by high levels of ozone (e.g., 2.5 ppm). However, few studies9,10) have focused on the impairment of alveolar macrophages by low levels of ozone (e.g., 0.2 ppm).

We previously reported that after an initial impairment on day 1, the peroxidative metabolic and glycolytic enzymes of alveolar macrophages were persistently enhanced from day 3 to week 12 by in vivo exposure to 0.1 or 0.2 ppm ozone.17,18) In addition, the number of small alveolar macrophages was increased from day 3 without augmentation of DNA synthesis, suggesting an enhancement in the influx of immature macrophages. Alveolar macrophages appeared to adapt themselves to the oxidative stress by metabolic upregulation and recruitment of immature cells. However, the effect of low levels of ozone on the host defense activities of alveolar mac-

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rophages remained unclear.

In the present study, we investigated the effects of in vivo exposure to 0.2 ppm ozone on microbial killing and superoxide production by alveolar macrophages. Microbial killing and superoxide production of the ozone-exposed macrophages were markedly impaired on day 3, although they underwent the metabolic enhancement. Further, these activities associated with C. albicans were still reduced at the end of 14-d exposure.

MATERIALS AND METHODS

Materials —— The following reagents were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.): cytochrome c, catalase, phorbol 12-myristate 13-acetate (PMA), zymosan A, lipopolysaccharide (LPS), cytochalasin E (Cyt.E), p-nitrophenyl-N-acetyl-β-D-glucosaminide and sodium deoxycholate. We obtained N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) from Dojindo Laboratories (Kumamoto, Japan); Eagle’s MEM and RPMI 1640 from Nissui Seiyaku Co. (Tokyo, Japan); fetal bovine serum (FBS) from Flow Laboratories Inc. (McLean, VA, U.S.A.); Micrococcus lysodeikticus and DNase I from Boehringer Mannheim (Tokyo, Japan); p-nitrophenyl-β-D-glucuronide and p-nitrophenyl phosphate from Nacalai Tesque (Tokyo, Japan); and carbon ink #591017 from Rotring (Hamburg, Germany). Escherichia coli K12 and Candida albicans were obtained from the University of Tokyo. FJ and YNB culture media and casamino acids were purchased from Difco Laboratories (Detroit, MI, U.S.A.). [5-3H] uridine (TRK.178, 26 Ci/ml) was chased from Difco Laboratories (Detroit, MI, U.S.A.).

Exposure Conditions —— Male Jcl:Wistar rats (specific pathogen-free; SPF) at 30°C for 18 hr: ca. 5 × 10⁴ dpm/10⁶ cells of E. coli and ca. 5 × 10⁴ dpm/10⁶ cells of C. albicans. The radiolabeled microbes were rinsed with saline and resuspended in RPMI 1640 containing 10 mM HEPES (pH 7.4) for use in the various assays.

Alveolar macrophages were resuspended at 2.5 × 10⁵ cells/ml in Eagle’s MEM supplemented with 10% FBS. Aliquots of 2.0 × 10⁵ cells were seeded in triplicate in 24-well plates and incubated in 5% CO₂ at 37°C for 30 min. ³H-uridine-labeled 30- or 120-fold E. coli and 8- or 16-fold C. albicans per macrophage were added to the wells of precultured macrophages; the resulting cocultures were incubated for an additional 30 min (E. coli) or 2 h (C. albicans) for microbial killing by the macrophages. Then, 0.15% deoxycholate and 25 µg/ml DNase I were added to the cultures, which were incubated for another 15 min. The macrophages and dead microbes were lysed with deoxycholate and DNase I, and the lysate was spun at 8000 rpm for 10 min to collect the radioactivity released into the supernatant. The microbial killing activity was defined as the ratio of radioactivity recovered in the supernatant to that added to the culture.

Measurement of Superoxide Production —— The oxygen consumption attributable to the respiratory burst was monitored with a Clark-type oxygen ele-
trote (Model 53, Yellow Springs Instrument Co.,
Yellow Springs, OH, U.S.A.) as described by
Estabrook. Aliquots of alveolar macrophages
(3.0 \times 10^6 cells) were resuspended in duplicate in 3
ml of assay solution [Dulbecco’s phosphate-buffered
saline supplemented with 1.3 mM MgCl_2, 5.5 mM
glucose, and 5.0 mM HEPES (pH 7.4)] and
preincubated at 37°C for several minutes. Mitochon-
drial respiration was blocked with 1 mM NaCN. The
alveolar macrophages were stimulated with 0.33 µg/
ml PMA, 200 µg/ml opsonized zymosan, or 5 µg/ml
LPS plus 5 µg/ml Cyt.E. Oxygen consumption was
measured while the cell suspension was stirred con-
tinuously.

Superoxide production was measured by assaying
the reduction of cytochrome c. Aliquots of al-
veolar macrophages (1.0 \times 10^6 cells) were resus-
pended in duplicate in 1 ml of assay solution supple-
mented with 20 µM cytochrome c and 5 µg/ml cata-
lase; then the cells were preincubated at 37°C for
several minutes. The alveolar macrophages were
stimulated with 0.4 µg/ml PMA, 200 µg/ml op-
sonized zymosan, or 5 µg/ml LPS plus 5 µg/ml
Cyt.E. The increase in the absorbance at 550 nm was
calculated in terms of the increase in the reduced
form of cytochrome c with the differential molecu-
lar absorption coefficient, 21.0. The cell suspen-
sion in the cuvette was stirred continuously with a
windmill-driven stirrer.

**Assay of Phagocytosis** —— Phagocytosis by the
alveolar macrophages was assayed by using op-
sonized carbon particles. Carbon particles were
mixed with an equal volume of rat serum and op-
sonized at 37°C for 20 min, after which they were
diluted 300-fold with the culture medium. Aliquots
of alveolar macrophages (1.5 \times 10^6 cells) were re-
suspended in duplicate in 3 ml Eagle’s MEM supple-
mented with 10% FBS and precultured in plastic
dishes for 1 hr. Then 300 µl of opsonized carbon par-
ticles was added to the macrophage culture and al-
lowed to undergo phagocytosis for an additional 1 hr.
Excess carbon particles that were not phagocytosed
were rinsed away by using a warmed isotonic
HEPES buffer supplemented with 1.3 mM MgCl_2,
1.8 mM CaCl_2, and 5% FBS. The macrophages that
had phagocytosed carbon particles were stained
black in the cytoplasm; we counted the cells under
light microscopy. The phagocytic index was defined
as the ratio of the number of stained macrophages
to the total cell count.

**Assays of Lysosomal Enzymes** —— Aliquots of
alveolar macrophages were suspended in 0.25 M su-
crose solution containing 10 mM Tris–HCl (pH 7.4)
and 0.5 mM EDTA and homogenized on ice in a ta-
pered Potter-Elvehjem Teflon homogenizer (358133,
Wheaton Scientific, Millville, NJ, U.S.A.). The en-
zyme activities of lysozyme, β-glucuronidase, N-
acetyl-β-glucosaminidase, and acid phosphatase
were assayed at 37°C as described in Methods of
Enzymatic Analysis. 24–27) The substrates of those
enzymes were *Micrococcus lysodeikticus*, p-
nitrophenyl-β-D-glucuronide, p-nitrophenyl-N-
acetyl-β-D-glucosaminide, and p-nitrophenyl phos-
phate, respectively. The protein concentration of the
lysate was determined according to Lowry et al. 25)

**Statistical Analysis** —— Analyses of significant
differences between exposure and control groups
were performed by means of Student’s *t*-test or
Welch’s *t*-test after the analysis of variance.

**RESULTS**

**Measurement of Microbial Killing**

In the present study, we developed a convenient
method for measuring microbial killing. The mem-
branes of macrophages and dead microbes were lys-
ed by treatment with deoxycholate, which released
the radioactive RNA of the microbes that were in-
gested by the macrophages into the culture super-
manant. The difference between the radioactivity of
the supernatant from a culture containing alveolar mac-
rophages and that of one not containing was attrib-
uted to the dead microbes. Approximately 20% of
the total radioactivity was released only from mi-
crobes at 0.15% deoxycholate: the additional release
(net release) of radioactivity by the macrophages was
maximum at this concentration.

The net release of radioactivity from 3H-uridine-
labeled *E. coli* dose–dependently increased as the
ratios of microbes per macrophage increased from
30 to 180 (Fig. 1A). In the absence of rat serum, the
net release of radioactivity from alveolar macroph-
ages cocultured with labeled *E. coli* was 14 ± 2% of
the total radioactivity added. In contrast, the net re-
lease of radioactivity from these cells in the pres-
ence of rat serum was 35 ± 5% of the total quantity.
The *E. coli*-associated killing activity of alveolar
macrophages was enhanced 1.8- to 3.1-fold in the
presence of 2.6% rat serum (Fig. 1A).

The net release of radioactivity from 3H-uridine-
labeled *C. albicans* dose–dependently increased be-
tween the ratios of 2 to 18 microbes per macroph-
age (Fig. 1B). In the absence of rat serum, the net
release of radioactivity from alveolar macrophages cocultured with labeled \textit{C. albicans} was 12\% to 21\% of the total radioactivity added. In contrast, the net release of radioactivity from these cells in the presence of rat serum was 19\% to 38\% of the total quantity. The \textit{C. albicans}-associated killing activity of the alveolar macrophages was enhanced 1.4- to 2.0-fold in the presence of 2.6\% rat serum (Fig. 1B). Compared with that for \textit{E. coli}, this reduction in net release for \textit{C. albicans} is probably due to the decrease in capacity of macrophages to kill and hydrolyze \textit{C. albicans}, which has a thick cell wall of proteoglycans that must be lysed.

**Impairment of Microbial Killing**

\textit{In vivo} exposure of alveolar macrophages to 0.2 ppm ozone decreased their microbial killing activity against \textit{E. coli} to 70--80\% of that in the controls on day 3 despite the lack of a decrease on day 1 (Fig. 2). The activity had returned to control levels by day 14. In addition, the antimicrobial activity of the ozone-exposed macrophages against \textit{C. albicans} was decreased to 70--80\% of that in the controls on day 3 in the absence and presence of rat serum; however, the decrease persisted until day 14 (Fig. 3).

**Reduction of Superoxide Production**

The respiratory burst of ozone-exposed alveolar macrophages was measured in the presence of 1 mM NaCN, which blocked mitochondrial respiration. Despite no impairment on day 1, the respiratory burst was reduced to 60\%, 79\%, or 70\% of that in the controls on day 3: the macrophages were stimulated with PMA, opsonized zymosan, or LPS plus Cyt.E, respectively (Fig. 4). By day 14, the respiratory burst had returned to control levels in case of PMA- or LPS plus Cyt.E-stimulation, whereas in case of opsonized zymosan-stimulation the respiratory burst remained reduced.

The superoxide production of ozone-exposed alveolar macrophages, that were stimulated with PMA, opsonized zymosan, or LPS plus Cyt.E, was measured by assaying the reduction of cytochrome \textit{c}. Despite no reduction on day 1, superoxide production decreased to 12\%, 31\%, or 60\%, respectively, of that in the controls on day 3 (Fig. 5). By day 14, superoxide production had returned to con-
control levels in the cells stimulated with PMA or LPS plus Cyt.E, whereas that in the cells stimulated with opsonized zymosan remained reduced.

**Effect on Phagocytosis**

The phagocytic index of the ozone-exposed alveolar macrophages was measured by using opsonized carbon particles. The phagocytic index showed no impairment on day 3, even though the microbial killing and active oxygen-producing activities were markedly reduced. On day 14, the phagocytic activity was slightly decreased (Table 1).

**Effect on Activities of Lysosomal Enzymes**

The activities of lysosomal enzymes, such as lysozyme, β-glucuronidase, N-acetyl-β-glucosaminidase, and acid phosphatase in the ozone-exposed alveolar macrophages, were measured. None of the lysosomal enzymes examined showed a significant decrease in activity during the exposure period (Table 1).

**DISCUSSION**

In the present study, we developed a convenient method for measuring the microbial killing activity of alveolar macrophages. Quie et al. reported a procedure for measuring the bactericidal activity of polymorphonuclear leukocytes. In their method, uningested bacteria have to be thoroughly rinsed away before the leukocytes are lysed by hypotonic treatment. To apply the method of Quie et al. to alveolar macrophages, the cells should be adherent during rinsing, otherwise the loss of weakened and detached cells would positively affect the phagocytic index. Lehrer et al. proposed the advantage of using radiolabeled microbes so that the radioactivity released into the culture supernatant could be counted instead of counting microbial colonies on agar plates. However, when using the method of Lehrer
et al., free microbes still need to be rinsed away completely. In the present study, we eliminated the rinse procedure by using a detergent (i.e., deoxycholate). At the doses evaluated, the net radioactivity released from 3H-uridine-labeled *E. coli* and *C. albicans* because of microbial killing by macrophages showed a cellular ratio-dependent increase and no saturation.

Goldstein *et al.* reported that the bactericidal activity of alveolar macrophages against *Staphylococcus aureus* was decreased by in vivo exposure to 2.5 ppm ozone for 4 hr. Approximate 64% of the total *S. aureus* that were intratracheally inhaled was cleared after 5 hr from the lung in the control, whereas the exposure to ozone adversely increased the number of *S. aureus* in the lung by 15%. Gilmour *et al.* reported that ozone exposure to 0.4 ppm for 3 hr or 0.5 ppm for 1 to 3 days impaired the clearance of inhaled *Streptococcus zooepidemicus* or *S. aureus*, respectively. In addition superoxide production of alveolar macrophages was reduced by 2-hr exposure to 1 ppm ozone. In our study, the microbial killing activity against *E. coli* and *C. albicans* was decreased by in vivo exposure to a low level of ozone (0.2 ppm) for 3 days. At the same time, the superoxide-producing activity was impaired markedly, whereas the phagocytic index and the activities of various lysosomal enzymes were unaffected. Therefore the decrease in microbial killing activity of the ozone-exposed alveolar macrophages on day 3 is attributable to the decrease in superoxide production.

According to Gilmour *et al.*, a reduction in the clearance of *S. aureus* disappeared during a prolonged exposure of 14 days. In our study, we found a full recovery of the microbial killing activity against *E. coli* by day 14, whereas the impairment concerning *C. albicans* remained reduced. On day 14 superoxide production of the ozone-exposed alveolar macrophages, when stimulated with LPS from *E. coli* cell wall, had returned to a control level. However, superoxide production by the stimulation with opsonized zymosan, proteoglycan of *C. cellevieic* cell wall like one of *C. albicans*, remained reduced. Corresponding to the reduction in superoxide-producing activity, the killing activity against *C. albicans* also remained reduced. Lehrer and Cline found that polymorphonuclear leukocytes from patients with chronic granulomatous disease lack the ability to produce superoxide anions and cannot kill *C. albicans*; they presumed that active oxygen molecules played an important role in the killing of *C. albicans*. We similarly found that the persistent reduction in the microbial killing activity against *C. albicans* was attributed to reduced production of superoxide.

Interestingly, both microbial killing and superoxide production were decreased on day 3, although they showed no reduction on day 1. In contrast, the short term-exposure of 0.4 to 2.5 ppm ozone rapidly impaired the clearance of *S. aureus* and *S. zooepidemicus*. We have reported that the alveolar macrophages of rats exposed to 0.2 ppm ozone showed a significant reduction in the peroxidative metabolic and glycolytic pathways on day 1. Those pathways were enhanced on day 3 and after, and the macrophages seemed to have physiologically adapted to the ozone. We cannot explain the apparent discrepancy between the metabolic enhancement on day 3 and the impairment of antimicrobial and superoxide-producing activities in the present study. However, the low level of
ozone we used seems to require a prolonged exposure time to initiate injury and recovery of the host defense mechanism.

In the present study, we observed no significant decrease in the activity of the measured lysosomal enzymes during the exposure period. However, Hurst et al. reported the decrease in activities of acid phosphatase, β-glucuronidase, and lysozyme in the alveolar macrophages of rabbits exposed to 1 to 7 ppm ozone for 3 hr.15) Further, the alveolar macrophages of rabbits that inhaled aerosolized S. aureus lacked or had reduced the lysosomal enzyme activities after exposure to 2.5 ppm ozone for 4 h.8,16) The differences between the findings in those previous reports and the present study are likely due to the 5- to 10-fold difference in the concentration of ozone used.

In conclusion, in vivo exposure to 0.2 ppm ozone impaired the microbial killing activity of alveolar macrophages by reducing their superoxide-producing activity. The C. albicans-associated detrimental effect lasted throughout the exposure period.

Acknowledgements The authors thank Dr. Katsuko Kakinuma at the Departments of Inflammation Research and Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, for her advice on measuring superoxide production of alveolar macrophages.

Table 1. Effects of 0.2 ppm Ozone on the Activity of Lysosomal Enzymes and Phagocytosis of Alveolar Macrophages

<table>
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<th>Control</th>
<th>Ozone</th>
<th>Exposure Time (days)</th>
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<td>Lysozyme8)</td>
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<td></td>
<td>63.5±7.5</td>
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<td></td>
<td>62.8±6.4</td>
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<td></td>
<td>59.2±9.2</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>36.4±2.8</td>
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<tr>
<td>N-Acetyl-β-glucosaminidase9)</td>
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<td></td>
<td>12.0±0.8</td>
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<td></td>
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<td></td>
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<td>Acid Phosphatase9)</td>
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<td></td>
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<td></td>
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<td></td>
<td>74.7±4.1</td>
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<tr>
<td></td>
<td>71.7±2.5</td>
<td>63.4±4.3 3c)</td>
<td>14</td>
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</table>

a) Rats were exposed to 0.2 ppm ozone for a maximum of 14 days, during which alveolar macrophages were harvested by pulmonary lavage (n = 6/group). b) 10^3 unit/min/mg protein of macrophage homogenate (mean ± S.D.). c) nmole/min/mg protein of macrophage homogenate (mean ± S.D.). d) Ratio of the number of macrophages containing carbon particles to the total number macrophages during 1-hr culture (%; mean ± S.D.). e) Significant at p < 0.05.

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