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Effect of Enflurane, Isoflurane, and Nitrous Oxide on the Microbicidal Activity of Human Polymorphonuclear Leukocytes

William D. Welch, Ph.D.*

The effect of enflurane and isoflurane with and without nitrous oxide (N₂O) on human polymorphonuclear leukocyte (PMNL) microbicidal function was studied. Bacterial killing was determined using a standard pour-plate technique and by oxidative activity as measured by chemiluminescence (CL). No killing of *Escherichia coli*, *Klebsiella pneumoniae*, or *Staphylococcus aureus* was seen at bacteria:PMNL ratios of 1 or 10:1 with either enflurane or isoflurane at concentrations greater than normally used (3%). However, at a clinically relevant concentration of 2%, a significant inhibition of bacterial killing was observed with enflurane but not isoflurane at a bacteria:PMNL ratio of 100:1. The CL response also was decreased significantly (28% inhibition) by 2% enflurane. N₂O 70% plus O₂ 30% had no effect on bactericidal activity or chemiluminescence alone or in combination with enflurane or isoflurane. The inhibition of bacterial killing and CL following enflurane exposure could be reversed by exposing the enflurane treated PMNL to air for 30 min. These results suggest that enflurane is able to inhibit PMNL microbicidal activity only when the PMNL are stressed with a large bacterial challenge or stimulus. This inhibition is temporal and only occurs during enflurane exposure. (Key words: Anesthetics, gas; nitrous oxide. Anesthetics, volatile: enflurane; isoflurane. Blood: microbial function; polymorphonuclear leukocytes.)

POLYMPHONUCLEAR LEUKOCYTES (PMNL) constitute a vital component in host defenses against invading microorganisms. Potential adverse effects on PMNL microbicidal function by frequently administered general anesthetic agents is thus an important consideration. Halothane, a widely used volatile anesthetic, recently has been shown to significantly reduce human neutrophil bacterial killing.¹ The mechanism responsible for this reduction may be due in part to an inhibition of oxygen dependent microbicidal systems.² In the present study the effects of two other commonly used volatile anesthetics, enflurane and isoflurane, and nitrous oxide (N₂O) on human PMNL bactericidal and oxidative activity were investigated.

* Assistant Professor, Departments of Pathology and Anesthesiology, Associate Director of Clinical Microbiology, Parkland Memorial Hospital.

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Address reprint requests to Dr. Welch.

Material and Methods

PREPARATION OF HUMAN PMNL AND POOLED HUMAN SERUM

Venous blood was obtained from normal healthy adult volunteers. PMNL were separated from heparinized blood by sedimentation with plasmagel (Roger Laboratories, Neuilly, France) at a ratio of 1:4 plasmagel to blood for 45 min at room temperature. The PMNL-rich supernate then was processed further in Hanks buffer pH 7.40 to yield approximately 85% to 90% PMNL as described previously.³ For pooled human serum, venous blood from six donors was allowed to clot for 30 min at room temperature and then centrifuged at 270 × g for 10 min. The individual donor serum was removed, combined in equal volumes, and kept at -80° C in 0.5-ml aliquots until needed.

BACTERIAL ISOLATES

Escherichia coli 0101 K nontypable is a blood culture isolate from the University of California, Los Angeles, Medical Center serotyped by Dr. Bertil Kaijser, Institute of Microbiology, University of Göteborg, Sweden. *Klebsiella pneumoniae* and *Staphylococcus aureus* are blood culture isolates obtained from the University of California, Irvine, Medical Center. The bacterial isolates were not killed in the presence of 15% human serum for 90 min at 37° C. All isolates were cultured on blood agar plates and stored in whole human blood at -80° C. For bactericidal assays, the isolates were subcultured to blood agar plates, inspected for purity, transferred to brain-heart infusion broth, incubated for 2-3 h at 37° C, washed twice with sterile saline solution, and adjusted to a concentration of 1 × 10⁸ bacteria/ml by comparing with an appropriate McFarland standard.

BACTERICIDAL ASSAY

The bactericidal assay used was performed as previously described.⁴ Briefly, the reaction mixture consisted of 0.05 ml of 1 × 10⁷ PMNL/ml, 0.01 ml of 5 × 10⁷ to 10⁹

bacteria/ml 0.01 ml of pooled human serum, and Hanks buffer to a final volume of 0.1 ml. The assay was performed in sealed plastic microtiter plates, which were mixed gently at 37° C on a Cooke microtiter shaker at the #3 setting for 30 min. After the incubation period, dilutions of the reaction mixtures were made in cold, sterile, distilled water and the number of viable bacteria (colony forming units, [CFU]) determined by growth in trypticase soy agar pour plates. Gas chromatographic analysis¹ of the air space above the sealed reaction wells for enflurane and isoflurane before and after the incubation period revealed a 3.2 ± 0.3% and 2.8 ± 0.1% v/v decrease in the enflurane and isoflurane concentrations, respectively (after exposure to anesthetic and a 30-min incubation). The percentage of bacteria killed was calculated by the following equation:

per cent bacteria killed

$$= \left[\frac{\text{CFU at beginning of assay} - \text{CFU at end of assay}}{\text{CFU at beginning of assay}} \right] \times 100.$$

Per cent inhibition of air-exposed CL response

$$= \left[\frac{\text{Integrated area from air-exposed CL response} - \text{Integrated area from anesthetic-exposed CL response}}{\text{Integrated area from air-exposed CL response}} \right] \times 100.$$

The emission spectrum of CL is in the range of 350–600 nm. Enflurane is a clear colorless liquid that was not found to absorb light in this wavelength using a Beckman® 34 spectrophotometer. The direct oxidation of luminol by H₂O₂ in the presence of high concentrations of enflurane, 10% v/v, also did not affect the emission of light as compared with controls (no enflurane present). Thus, the quenching of CL by enflurane via oxidative metabolite interaction or inactivation of the amplifier luminol were not responsible for the lowered CL responses observed following enflurane exposure.

EXPOSURE OF BACTERICIDAL AND CL ASSAY TO ENFLURANE, ISOFLURANE AND N₂O

Bactericidal and CL assay reaction components were placed in a gas-tight glass chamber and exposed to the desired concentrations of vaporized enflurane or isoflurane in air (Ohio® DM 5000 vaporizer) at a flow rate of 400 ml/min via inflow and exit valves. In experiments using 70% N₂O, 70% N₂O plus 30% O₂ was given with enflurane or isoflurane using an Ohio Kinet-O-Meter®.² Enflurane or isoflurane and N₂O were combined by connecting the exit tubes from both vaporizer and Kinet-O-Meter®.² The anesthetic mixture in this tube then was used to gas the bactericidal or CL reaction mixture in the air-tight chamber.

LUMINOL-DEPENDENT CHEMILUMINESCENCE (CL) ASSAY

The CL reaction mixture consisted of 1 × 10⁵ PMNL, 1 × 10⁻⁷ M luminol, and Hanks buffer, pH 7.40 to a total volume of 2 ml. The CL reaction was initiated after air or anesthetic exposure by addition of 0.05 mg of pooled human serum opsonized zymosan to the reaction mixture. Serum-opsonized zymosan was prepared by washing the zymosan twice with Hanks buffer and incubating it with 50% pooled serum in a mixture of 2.5 mg zymosan per milliliter for 30 min at 37° C in a water bath. The reaction mixture was added to dark-adapted polypropylene scintillation counter vials under red light. The CL response was measured in a Beckman® LS100C scintillation counter in the out-of-coincidence mode for 60 min at 0.2-min intervals. The CL response was quantitated by measuring the integrated area below the CL curves during a period of 5 to 45 min, using a DEC PDP 11/34 digital computer. The effect of the selected anesthetic agents on the CL response was determined by calculating the per cent inhibition of the air exposed CL reaction mixture as follows:

Enflurane and isoflurane concentrations were monitored at the chamber exit valve using a Hewlett Packard® 5730 A gas chromatograph (50/80 Poropak Q glass column) with a 3380S integrator. For control experiments only air was administered to the chamber with CL reaction mixture components. To determine if the air enflurane, isoflurane, or N₂O–O₂ mixtures altered pH, P_{O₂}, or P_{CO₂} of the buffer, these variables were measured after exposure of the buffer to air or anesthetic for 1 h, using an IL 213 pH blood–gas analyzer. No significant difference in pH, P_{O₂}, or P_{CO₂} was seen between the anesthetic agents and air-exposed buffer. No effect of enflurane, isoflurane, or N₂O on *E. coli*, *K. pneumoniae*, or *S. aureus* in the presence or absence of 10% serum was observed at the concentrations used.

STATISTICAL ANALYSIS

Mean, standard deviation, and one-way analysis of variance were determined for all experimental studies.

DETERMINATION OF THE PARTITION COEFFICIENT BETWEEN ENFLURANE AND ISOFLURANE AND HANKS BUFFER

The partition coefficient between enflurane, isoflurane, and Hanks buffer was determined using the method of

TABLE 1. Effect of a One-hour Exposure of Enflurane (E), Isoflurane (I), and N₂O on the Bactericidal Activity of Human PMNL

PMNL Ratio	Air Only	1% E	2% E	3% E	3% E, then Air (30 min)	3% E + 70% N ₂ O	3% I	3% I + 70% N ₂ O	70% N ₂ O
<i>E. coli</i>									
100:1	93 ± 6	84 ± 4	73 ± 4†	68 ± 3‡	82 ± 4	69 ± 4‡	94 ± 5	93 ± 4	94 ± 6
10:1	91 ± 5	90 ± 5	88 ± 3	81 ± 4	87 ± 6	80 ± 5	95 ± 5	96 ± 5	90 ± 7
1:1	91 ± 8	92 ± 3	93 ± 5	81 ± 4	87 ± 8	83 ± 6	95 ± 6	92 ± 6	93 ± 6
<i>K. pneumoniae</i>									
100:1	90 ± 8	85 ± 7	73 ± 5†	65 ± 4‡	86 ± 5	67 ± 5‡	94 ± 6	91 ± 5	90 ± 5
10:1	88 ± 6	86 ± 8	84 ± 8	74 ± 5	88 ± 5	73 ± 6	90 ± 7	96 ± 5	91 ± 7
1:1	89 ± 6	83 ± 7	85 ± 7	82 ± 7	88 ± 7	84 ± 5	92 ± 5	94 ± 7	89 ± 7
<i>S. aureus</i>									
100:1	83 ± 6	79 ± 6	66 ± 5†	57 ± 6‡	77 ± 5	56 ± 7‡	84 ± 5	83 ± 4	84 ± 5
10:1	82 ± 4	76 ± 5	74 ± 5	69 ± 5	79 ± 6	70 ± 7	81 ± 7	84 ± 4	82 ± 7
1:1	82 ± 6	79 ± 8	72 ± 6	75 ± 5	79 ± 6	77 ± 5	85 ± 4	82 ± 6	83 ± 5

* Mean ± standard deviation of eight separate experiments.
† $P < 0.05$, compared with air only control.

‡ $P < 0.01$, compared with air only control.

Fink and Morikawa.⁵ The partition coefficients were found to be 1.88 ± 0.39 ($n = 14$) for enflurane and 1.22 ± 0.08 ($n = 8$) for isoflurane at 24° C.

Results

EFFECT OF ENFLURANE, ISOFLURANE, AND N₂O ON HUMAN PMNL BACTERICIDAL ACTIVITY

Exposure of human PMNL to 1, 2, or 3% enflurane or isoflurane for 1 h did not alter their ability to kill *E. coli*, *K. pneumoniae*, or *S. aureus* blood culture isolates when in a bacteria:PMNL ratio of 1 or 10:1 (table 1). However, when this ratio was increased to 100:1, a significant decrease in bacterial killing was seen with 2 and 3% enflurane but not isoflurane. For *E. coli*, the per cent bacteria killed was decreased to 73 and 68%, respectively, from 93% bacteria killed in experiments without exposure to enflurane (table 1). Interestingly, even at the highest concentration of enflurane given, 3%, the inhibitory effect on PMNL (at a 100:1 bacteria:PMNL ratio) could be reversed by exposing the enflurane treated PMNL to air for 30 min. In these experiments, the enflurane-treated then air-exposed PMNL was able to kill *E. coli*, *K. pneumoniae*, or *S. aureus* at levels not statistically different from non-enflurane treated PMNL (table 1). No effect on bactericidal activity was seen with 70% N₂O plus 30% O₂ alone or in combination with isoflurane or enflurane (table 1).

EFFECT OF ENFLURANE, ISOFLURANE, AND N₂O ON THE CL RESPONSE OF HUMAN PMNL

No inhibition of the CL response was seen with 0.5% enflurane (1 h exposure), (fig. 1A) or 0.5, 1, 2, or 3% isoflurane (fig. 2). Although there was some inhibition

of CL following 1% enflurane ($12 \pm 4\%$), it was not statistically different from the control (no enflurane) CL response, $P > 0.05$ (fig. 1B). However, exposure of PMNL to 2% enflurane resulted in a significant inhibition of CL, $28 \pm 4\%$, $P < 0.001$ (fig. 1C). Exposure of PMNL given 1 or 2% enflurane to air for 30 min resulted in a significant increase in CL comparable to control (no enflurane) CL responses (Figs. 1B and C). No effect on PMNL CL was seen with 70% N₂O plus 30% O₂ alone or in combination with isoflurane or enflurane (data not shown) PMNL CL was not changed following exposure to isoflurane in concentrations from 0.5 to 3% (fig. 2).

Discussion

Enflurane and isoflurane, in combination with N₂O, are popular general anesthetic agents in clinical practice. The present report describes the effect of clinical concentrations of these agents on human polymorphonuclear leukocyte microbicidal activity.

The methods used to evaluate the effect of enflurane, isoflurane, and N₂O on PMNL microbicidal activity included a standard pour plate killing assay and the technique of CL or the emission of light by phagocytosing PMNL. Bacteria chosen for the PMNL killing assay consisted of the most frequently isolated gram-positive (*S. aureus*) and gram-negative (*E. coli*, *K. pneumoniae*) blood culture isolates.⁶

Increasing bacteria:PMNL ratios were used in the bactericidal assay to stress PMNL killing ability. It has previously been shown that PMNL maximally are stressed at a ratio of 100 bacteria per PMNL, with bacterial killing decreasing at higher bacterial ratios.⁷ In the present study, inhibition of bacterial killing by 2 or 3% enflurane-treated PMNL occurred only at a bacteria:PMNL ratio of 100:1. All of the bacteria tested showed a similar killing pattern.

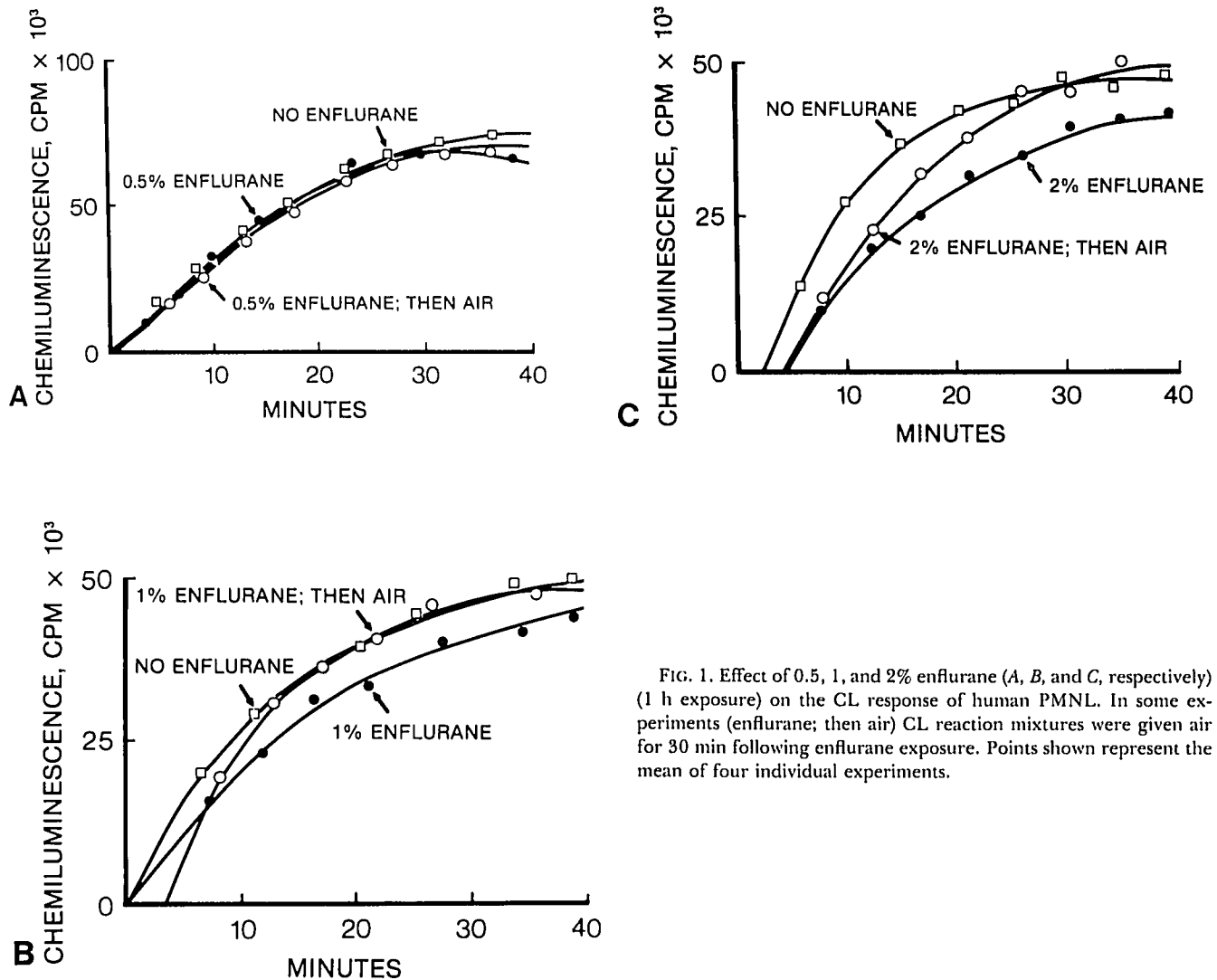


FIG. 1. Effect of 0.5, 1, and 2% enflurane (A, B, and C, respectively) (1 h exposure) on the CL response of human PMNL. In some experiments (enflurane; then air) CL reaction mixtures were given air for 30 min following enflurane exposure. Points shown represent the mean of four individual experiments.

In contrast to enflurane, halothane has been shown to inhibit PMNL bacterial killing at a low bacteria:PMNL ratio (1 or 2:1) at approximately half the equipotent enflurane concentration (0.5% halothane or 0.65 MAC).¹ The 2% concentration of enflurane required for inhibition of bacterial killing in the present study is equivalent to 1.19 MAC.

PMNL killing of microorganisms is dependent to a large extent upon oxygen-dependent killing systems.^{8,9} To determine if enflurane exposure may affect such systems, the CL response of enflurane-treated PMNL was measured. A 28% inhibition of the CL response was seen after exposure to 2% enflurane. This is substantially different from the effect of halothane on PMNL CL at equipotent anesthetic concentrations where little or no effect on CL was seen.²

Isoflurane, which is a chemical isomer of enflurane, differing only in the position of Cl and F atoms on the number 1 and 2 carbons, did not demonstrate any inhibition of neutrophil function. The positioning of Cl and F atoms therefore may be critical for enflurane to perturb the neutrophil membrane and prevent a normal response to stimulation.

The approximate ratio of zymosan to PMNL in the current CL experiments was from 100 to 150:1. Taken together with the bacterial killing data, the inhibition of PMNL microbicidal function by enflurane occurs only when the PMNL is given a large bacterial challenge or stimulus. The inhibition of active or stressed PMNL by enflurane may result from an enflurane-protein (such as NADPH oxidase) or plasma membrane interaction. Such mechanisms have been proposed to explain the action of

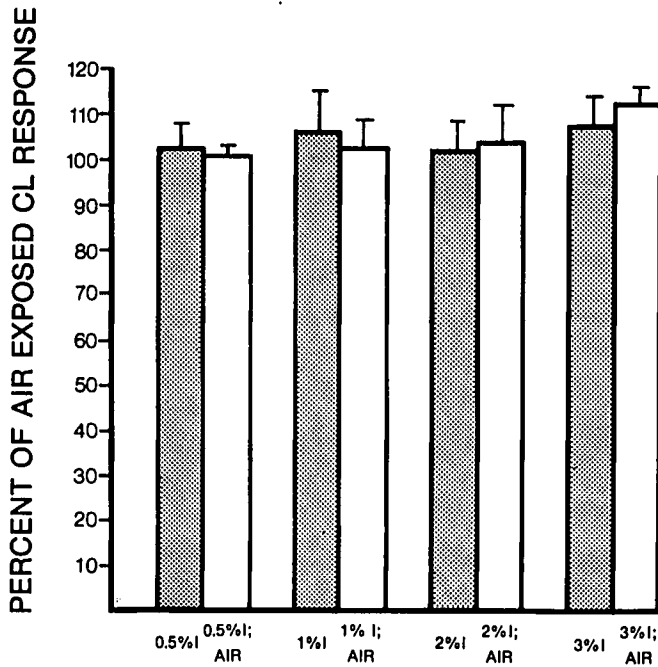


FIG. 2. Effect of 0.5, 1, 2, and 3% isoflurane (1 h exposure) on the CL response of human PMNL. In some experiments CL reaction mixtures were given air for 30 min following isoflurane exposure. Data shown represent the results from four individual experiments.

several anesthetics.^{10,11} The quantitatively different effects of halothane and enflurane then could be explained by each anesthetic having a predilection for some different aspect of the PMNL microbicidal pathways or PMNL membrane. That the inhibitory effect of enflurane can

be reversed by exposure to air implies a transient effect, by whatever mechanism of the anesthetic. The clinical significance of these results remains to be determined.

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