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Inhibition of Interferon Stimulation of Natural Killer Cell Activity in Mice Anesthetized with Halothane or Isoflurane

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Background: Basal cytotoxic activity of NK cells, a subtype of lymphocytes involved in the nonspecific immune response to viruses, tumors, and some bacteria, is altered in the postoperative period. The current study examines the effects of halothane and isoflurane on interferon-induced stimulation of NK cell cytotoxicity *in vivo* and *in vitro*.

Methods: Mice were exposed to either anesthetic on days 10, 5, 1, or -1 relative to interferon treatment on day 0. NK cytotoxicity was assessed 24 h later. Similarly, splenic mononuclear cells containing NK cells were treated with interferon, before or after *in vitro* exposure with either halothane or isoflurane, and cytotoxicity was determined.

Results: *In vivo*, isoflurane or halothane inhibited subsequent interferon-induced NK cell stimulation (>90% and 67%, respectively). No inhibition occurred if interferon was given before anesthetic exposure. Significant inhibition of interferon-induced NK cell stimulation could be observed 11 days after anesthesia. *In vitro*, both anesthetics inhibited the subsequent stimulation of NK cytotoxicity by interferon, however, cytotoxicity of NK cells treated with interferon before

anesthetic exposure was comparable to untreated interferon-stimulated NK cells.

Conclusions: Halothane and isoflurane inhibit interferon stimulation of NK cytotoxicity in naive (unstimulated) NK cells of the splenic mononuclear cell pool without affecting the cytotoxicity of previously stimulated (interferon) NK cells. This could occur directly by preventing the NK cell from responding or indirectly by altering other cells in the splenic mononuclear cell pool (T cells, macrophages), which then inhibit NK cell induction. (Key words: Anesthetics, volatile: halothane, isoflurane. Immune response: interferon α/β , NK cells.)

THROUGHOUT the past two decades, a plethora of isolated, transient, immunologic defects in patients recovering from surgery (or trauma) have been described. Anesthesia and/or the stress of surgery has been associated with: 1) decreased lymphocyte recirculation,¹ 2) decreased lymphocyte responsiveness to mitogens such as Concanavalin A,² 3) decreased blastogenic responses to microbial antigens,³ 4) decreased delayed hypersensitivity reactions,⁴ 5) decreased natural killer (NK) cell activity,⁵⁻⁸ 6) decreased mixed lymphocyte responses,⁹ and 7) enhanced antibody responses.¹⁰ All of these aberrations of immune system functions have been employed as possible mechanisms behind the clinically observed state of transient postoperative immunosuppression.⁴

Over the past few years, we have been exploring the effects of anesthesia and surgery on NK cell functions. NK cells are a subpopulation of lymphoid mononuclear cells morphologically classified as "large granular lymphocytes." These cells have been described as possessing spontaneous cytolytic activity against numerous bacteria, virus-infected cells, and tumor cells in a non-adaptive, non-major histocompatibility complex restricted fashion.¹¹⁻¹⁵ As a major effector cell of the innate, first-line immune defenses, postoperative depression of NK cell activity may be a likely contributing factor to postoperative immunosuppression.

Previously, we have reported that mice anesthetized

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with tribromoethanol, with or without surgery, had a decrease in the ability of a standard NK cell stimulator (interferon) to induce NK cytotoxicity for up to 14 days following anesthesia.¹⁶ No significant effects on baseline NK activity were observed in the group that were anesthetized without surgery. With the addition of surgical trauma, there was depression of preexisting baseline, as well as interferon-stimulated NK cell activity. Interestingly, interferon treatment before administration of the anesthetic allowed maintenance of increased levels of NK cell activity into the perioperative period.

The goal of this study was to expand our experiments to a model more relevant to clinical practice and examine mechanisms involved in this inhibition. In the first set of experiments, we examined basal interferon-stimulated cytotoxic activity of NK cells isolated from mice undergoing halothane or isoflurane anesthesia. In a second set of experiments, to begin to determine the mechanisms involved in inhibiting NK cell responsiveness, we examined basal and stimulated cytotoxicity in cells exposed to the anesthetics *in vitro*.

Materials and Methods

Animals

Male, 4–6-week-old CB6F1 mice ((BALB/c × C57BL/6)F1) were used in all experiments (Charles River Laboratories, Wilmington, MA). The animals were kept in microisolator cages in a barrier facility. Appropriate approval was obtained from institutional animal use committees for the use and care of laboratory animals. The animals were fed autoclaved food and water *ad libitum*. All animal handling procedures were performed in accordance with the guidelines published by the United States Department of Health and Human Services.[§]

Interferon

Murine interferon α/β was obtained from Lee Biomolecular Research Laboratories (San Diego, CA). The preparation was purified further by affinity chromatography using immobilized sheep anti-L929 cell interferon globulin.¹⁷ The interferon titer was determined by measuring protection from cytopathic effect of en-

cephalomyocarditis virus on L929 cells. This method is a modification of the microplate method described previously by Havell and Vilcek.¹⁸ The titer was calculated in reference to a standard (G002-904-571) distributed by the Reference Reagents Board of the National Institute of Allergy and Infectious Disease (Bethesda, MD). The specific activity of the final interferon preparations was in the order of 8×10^7 μ /mg protein. For *in vivo* experiments, 5×10^4 μ interferon α/β per mouse was injected intraperitoneally 24 h before examining NK cell activity cytotoxicity. This concentration was selected because it was the smallest dose that produced consistent maximal stimulation of NK cell cytotoxicity (data not shown).

Preparation of Splenic Mononuclear Cells

Spleens were isolated and homogenized to single cell suspensions using a ground glass homogenizer. The homogenate was layered on to a density gradient (Lymphopaque, 1088, Nyegaard, Oslo, Norway) and centrifuged at 800 g for 15 min at room temperature. Mononuclear cells at the gradient interphase were collected, washed twice in RPMI-1640 (Mediatech, Washington, DC), counted in Trypan Blue, and resuspended to the appropriate concentration for further use. NK cells accounted for 16–19% of this monocyte preparation as determined by the number of cells staining positive for the asialo GM1 marker (data not shown).

Exposure of Mice to Volatile Anesthetics

Mice were anesthetized for 2 h with either 1.5% halothane or 2.1% isoflurane in glass-covered containers measuring $6 \times 12 \times 30$ cm. These concentrations were selected based on previous studies that demonstrated a less than 20% decrease in blood pressure in the anesthetized mouse. The anesthetic agent was administered through the appropriate vaporizer and delivered by compressed 50% O₂/50% air at the rate of 5 L/min. These gases were used to prevent hypoxia (data not shown). Anesthetic gas and carbon dioxide concentrations were analyzed continuously with a Rascal Raman spectrophotometer (Ohmeda, Arlington Heights, IL). Ambient temperatures were maintained at $36 \pm 0.5^\circ$ C with the aid of a warming blanket and an overhead infrared lamp.

Exposure of Splenic Mononuclear Cells In Vitro to Halothane or Isoflurane

Cells (2×10^6 cells/ml) were suspended in RPMI-1640 solution and exposed to anesthetic vapor for 2

[§] United States Department of Health and Human Services. Guide for the Care and Use of Laboratory Animals. Washington, DC, United States Government Printing Office, 1985.

h as previously described.¹⁹ Briefly, cell suspensions in 60-mm Petri dishes were placed in a metal and glass atmospheric chamber. The anesthetic vapor was generated by directing a 95% air/5% CO₂ mixture (carrier gas) at 5 L/min through the appropriate vaporizer to the input part of the atmosphere chamber and the vapor scavenged from the output. There were no changes in cell viability following 5% CO₂ carrier gas. The buffering system in the media was adjusted to provide optimal pH. The atmosphere chamber was humidified and kept at 37° C during the experiments. The chambers containing the cell suspensions were equilibrated for 20 min and the anesthetic concentrations assessed with a Rascal Raman spectrophotometer. Cells were washed twice following exposure to the anesthetics. Viable cells were counted using Trypan Blue exclusion technique. There was no significant change in overall injury by this technique or by examining these cells morphologically.

Cytotoxicity Assay

Levels of NK activity were determined in a ⁵¹Cr release assay in which splenic mononuclear cells (1 × 10⁷ cells/ml) were reacted for 4 h with ⁵¹Cr-labeled 10⁴ YAC-1 target cells (labeled with 200 μCi/3 × 10⁶ cells overnight at 37° C in 5% CO₂) at different effector cell-to-target cell ratios (from 100:1 to 25:1). Following this incubation, supernatants were harvested (Supernatant Collection System, Skatron, Sterling, VA) and their radioactive content determined in a gamma-counter (TM Analytic, Elk Grove Village, IL). The magnitude of splenic NK cell activity was determined by the level of ⁵¹Cr released into the supernatants from the lysed YAC-1 target cells. Levels of NK cell activity were expressed as "percent cytotoxicity" calculated using the following equation:

% Cytotoxicity

$$= \frac{\text{cpm (test)} - \text{cpm (spontaneous)}}{\text{cpm (maximum)} - \text{cpm (spontaneous)}} \times 100$$

Values for cpm (maximum) and cpm (spontaneous) were determined by incubating labeled YAC-1 cells with triton X-100 or media alone, respectively. The ratio of cpm (spontaneous) to cpm (maximum) was always in the range of 0.05–0.15, with maximum incorporation being in the range of 4,000–5,500 cpm. Each cytotoxicity assay utilized 3 mice per group and was repeated 2–4 times.

Stimulation of NK Cell Activity In Vitro

Mononuclear splenocytes were incubated *in vitro* with interferon α/β (1 × 10⁴ μ/ml × 10⁶ cells) for 1 h at 37° C in 5% CO₂ in wells of a 96 well round-bottom microtiter plate (Flow Labs, McLean, VA). At the end of the incubation, cells were washed three times in RPMI-1640 (Mediatech, Washington, MD) supplemented with 5% decompartmented FCS (Whittaker, Walkersville, MD). After the last wash, cells in the wells were resuspended to 100 μl in the above media and mixed with 1 × 10⁴ radiolabeled YAC-1 cells to assess NK cell activity in a 4-h cytotoxicity assay.

Statistics

Data are presented as mean ± SEM. Student's *t* test for paired and unpaired data were employed for the determination of statistical significance of differences among compared sets of data.²⁰ Analysis of variance with Tukey *post hoc* analysis was used for multiple comparisons.

Results

Effects of the Anesthetic Exposure (In Vivo) on Baseline and Interferon-Induced Splenic NK Cell Activity

Mice were anesthetized on 11, 6, or 2 days before conducting NK cell assay for cytotoxicity. Interferon treatments to previously anesthetized and untreated mice were administered 1 day before the NK cytotoxicity assay. The assay of NK cytotoxicity activity, expressed as percent cytotoxicity, demonstrated the following (fig. 1): 1) baseline cytotoxic NK cell activity of 4.5% is not affected by either halothane or isoflurane, 4.8% and 2.2%, basal cytotoxic activity, respectively; 2) both halothane and isoflurane demonstrate significant inhibition of interferon-induced NK cell stimulation when administered up to 10 days before interferon treatment 1 day after anesthesia; 3) isoflurane appears to be more effective in inhibiting interferon-mediated stimulation of NK cytotoxicity than does halothane. Cytotoxicity had only returned to 44% of maximum in the isoflurane-exposed animals 11 days after anesthesia, whereas halothane-anesthetized animals were 66% of maximum.

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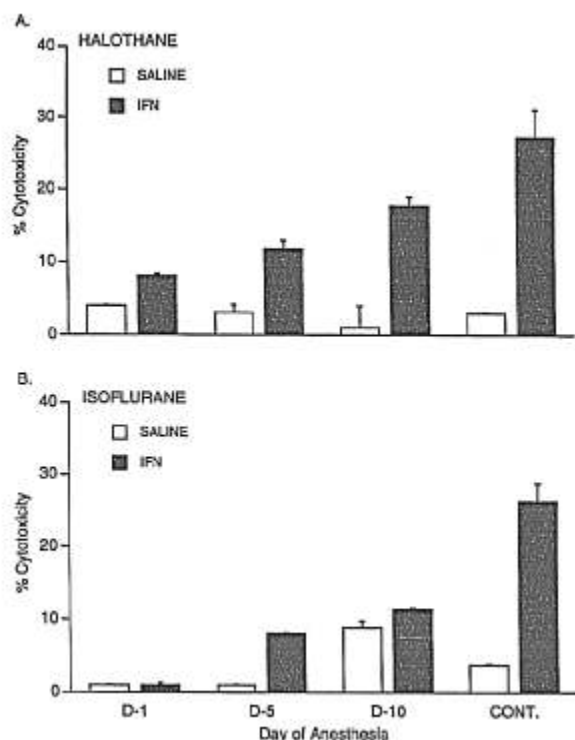


Fig. 1. Levels of NK cytotoxicity of interferon-treated mice previously anesthetized with halothane or isoflurane. In a representative experiment, groups of mice ($n = 3$) were anesthetized with either halothane (A) or isoflurane (B) on days -10, -5, or -1 before intraperitoneal interferon ($5 \times 10^4 \mu$ per mouse) treatment on day 0. Assessment of NK cell activity was performed 24 h after interferon treatment (effector/target ratio 50:1). Statistically significant differences ($P < 0.05$, Student's t test) were observed when comparing the levels of NK cytotoxicity in interferon-treated control mice to those of interferon-treated preanesthetized mice (both halothane and isoflurane). For any given day of anesthesia treatment, levels of interferon-induced NK cytotoxicity were significantly lower ($P < 0.05$) with isoflurane than those with halothane.

Effects of Anesthetic Exposure on Splenic NK Cell Activity in Mice Previously Treated with Interferon

Since the volatile anesthetics did not appear to affect preexisting (baseline) levels of NK cell activity (fig. 1), we hypothesized that interferon stimulation of NK cell activity before induction of general anesthesia would not be altered by the anesthetics. The data from the subsequent experiments confirmed the hypothesis (fig. 2). Stimulation of NK cytotoxicity with interferon 24 h before anesthesia with either halothane or isoflurane resulted in levels of NK cytotoxicity similar to those of unanesthetized NK controls, 28.1% compared to

24.9% for halothane and 26.8% for isoflurane. The volatile anesthetics had no effect on preinduced NK cytotoxicity.

Effects of In Vitro Exposure of Splenic Mononuclear Cells to Volatile Anesthetics on NK Cell Cytotoxicity

To more closely study the mechanisms of the stimulation-inhibiting effects of halothane and isoflurane on NK cells, splenic mononuclear cells were isolated and exposed *in vitro* to the anesthetics. Splenic mononuclear cells were exposed to either 1.5% halothane or 2.1% isoflurane for 2 h, the same conditions and duration as were used for the intact mice. After this incubation, cells were washed and incubated with interferon for 1 h prior to their use as effector cells in a NK cytotoxicity assay. The results of these experiments (fig. 3) demonstrate that both halothane and isoflurane inhibit interferon induction of NK cytotoxicity *in vitro*, 81% and 82% of maximum stimulation, respectively. As before, neither of the anesthetics affected baseline NK cell activity. These data paralleled our previous observations *in vivo* (fig. 1).

Effects of In Vitro Anesthesia on Splenic Mononuclear Cell NK Activity Previously Stimulated with Interferon In Vitro

Based on our observation that neither halothane nor isoflurane anesthesia was able to affect preexisting

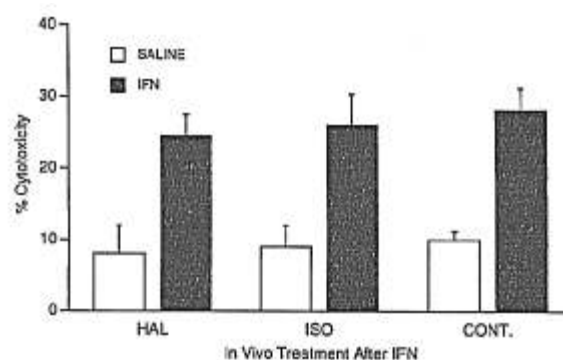


Fig. 2. Levels of NK cytotoxicity in interferon-treated mice subsequently exposed to halothane or isoflurane. In a representative experiment, groups of mice ($n = 3$) were treated with intraperitoneal interferon ($5 \times 10^4 \mu$ /mouse) 24 h prior to anesthesia with halothane or isoflurane. NK cytotoxicity was assessed 24 h later. Significant increases ($P < 0.05$, Student's t test) of NK cytotoxicity due to interferon treatment were observed in all groups (naive or anesthetized) relative to their respective interferon untreated controls. The control group was not exposed to any anesthetic. There were no significant differences ($P > 0.05$) in NK cytotoxicity among the interferon-treated mice across all groups.

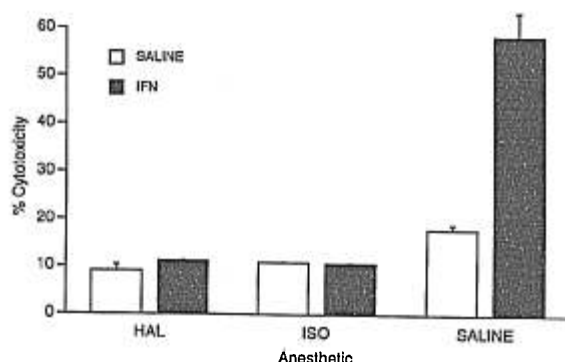


Fig. 3. Levels of NK cytotoxicity of splenic mononuclear cells exposed *in vitro* to halothane or isoflurane before *in vitro* interferon treatment. In a representative experiment, different cultures ($n = 3$) of splenic mononuclear cells (1×10^7 cells/ml) were exposed to either halothane or isoflurane for 2 h at 37°C before interferon treatment (1×10^4 $\mu\text{g/ml} \times 10^6$ cells.) Levels of interferon-stimulated NK cytotoxicity (effector/target ratio of 50:1) of carrier gas-treated splenic mononuclear cells was significantly greater ($P < 0.01$, Student's t test) than that of cells treated with either halothane or isoflurane.

baseline (figs. 1 and 3) or *in vivo* interferon-induced (fig. 2) levels of NK cytotoxicity, we hypothesized that *in vitro* exposure of mononuclear cells to halothane or isoflurane would not decrease NK cytotoxicity previously induced *in vitro* with interferon. Data presented in figure 4 confirm this hypothesis. Neither *in vitro* exposure to halothane nor *in vitro* exposure to isoflurane was able to significantly inhibit preinduced levels of NK cytotoxicity: 121% and 109% of maximum stimulation, respectively. These results were in accordance with the *in vivo* findings (fig. 2).

Discussion

In this study, we investigated the effects of halothane and isoflurane on interferon-induced stimulation of mouse splenocyte NK cytotoxicity. The data clearly demonstrate that: 1) halothane and isoflurane inhibit interferon-induced stimulation of NK cytotoxicity of murine splenic mononuclear cells after either *in vivo* or *in vitro* exposure, 2) anesthesia with either of the anesthetics does not appear to significantly affect baseline or preinduced (interferon) levels of NK cytotoxicity, and 3) the duration of the anesthesia-induced inhibition lasts at least 11 days for both anesthetics and is somewhat more pronounced with isoflurane.

The exact mechanism by which the anesthetics inhibit induction of NK cell cytotoxicity cannot be delineated. The demonstration that the *in vitro* model

behaves in the same manner as the *in vivo* model strongly suggests that the anesthetic inhibition of the NK cell cytotoxicity occurs directly or indirectly (by the anesthetic's effect on another type of monocyte present in the splenic monocytic pool), or both. Indirect inhibition, from another cell type in the splenic monocyte pool, could occur as a result of direct cell-to-cell contact or by the secretion of paracrine substances such as cytokines. In addition, the observation that anesthetics can exert a direct effect on the NK cell or another monocyte cell from the splenic monocytic pool that modulates NK cell activity strongly argues against the stress of anesthesia, or surgery for that matter, being necessary to produce this type of inhibition of immune responsiveness.

Volatile anesthetic agents can significantly affect the cytotoxic activity of lymphocytes. Decreases in cell-mediated cytotoxicity and the ability of lymphocytes to kill tumor cells following halothane/nitrous oxide anesthesia have been reported.²¹ In previous studies, using the same mouse model described in the present report, we have documented the ability of interferon, when given 5 days prior to anesthesia and surgical debulking of a transplanted primary B16F10L melanoma, to produce a cure in $>50\%$ of the mice.²² *In vivo* depletion studies creating mice deficient for NK, CD4, and/or CD8 cells confirmed the primary role of NK cells as mediators of this therapeutic effect of inter-

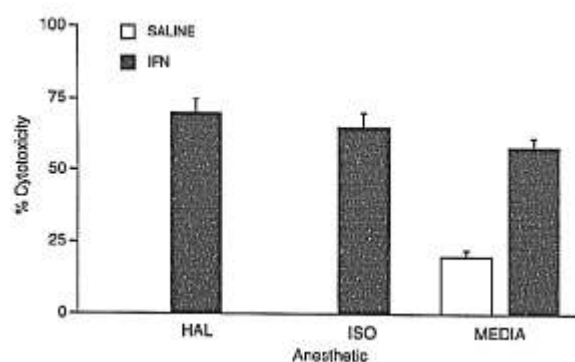


Fig. 4. Levels of NK cytotoxicity of splenic mononuclear cells exposed *in vitro* to halothane or isoflurane after *in vitro* interferon treatment. In a representative experiment, different cultures ($n = 3$) of splenic mononuclear cells (1×10^7 /ml) were treated *in vitro* with interferon (1×10^4 $\mu\text{g/ml} \times 10^6$ cells) before exposure to either halothane or isoflurane. NK cytotoxicity levels (effector/target ratio of 100:1) of unanesthetized control splenic mononuclear cells were not significantly different from those treated with halothane or isoflurane ($P > 0.05$, Student's t test). Interferon-treated splenic mononuclear cells showed significantly increased levels of NK cell activity relative to untreated controls ($P < 0.01$, Student's t test).

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feron.²³ Mice not treated with interferon or given interferon after surgery displayed 100% mortality due to massive lung metastases. These mice demonstrated significantly lower NK cytotoxicity than was observed in the mice receiving the interferon before anesthesia and surgery. Based on the above results, it appeared that the effectiveness of interferon therapy was dependent on a functioning pool of NK cells capable of being stimulated to higher levels of NK cytotoxicity, which could not occur following anesthesia and surgery secondary to NK cell unresponsiveness.

In a related study involving the effects of halothane on the immune response to an influenza virus pulmonary infection in mice, we demonstrated an inhibition of lymphocyte recruitment into the lungs 10 days following anesthesia.²⁴|| This decrease in lymphocyte responsiveness was associated with a decrease in the virus-specific immune mediated pathology. This effect was demonstrated histologically as well as functionally by leakage of ¹²⁵I albumin across the alveolar capillary border. The present study identifies a specific lymphocyte subpopulation (NK cells) with a decreased responsiveness to activation lasting for more than 10 days following halothane anesthesia. This observation would support the hypothesis that the mechanism by which halothane decreases influenza-virus-specific immune-mediated pathology is through inhibition of activation and recruitment of this general type of effector cells.

The clinical implications raised by these studies are intriguing. In addition to increasing our overall understanding of the phenomenon of postoperative immune suppression, these results also suggest potential treatment strategies that may be beneficial in certain subclasses of patients requiring surgery, such as patients with suppressed immune systems secondary to a disease processes, drug therapy, or age. Surgical morbidity and mortality secondary to complications that can be related directly to a depressed immune response may be decreased in this manner.

In the area of oncology, our results have implications involved in the timing of immune therapy for the surgical patient. Immune therapy for solid tumors that cannot be completely surgically excised generally has not been successful. Our results may not only explain why this may occur but also suggest a possible mechanism to improve the success rate of these experimental

treatments. Stimulating immune response before anesthesia and surgery may be more efficacious than starting immune therapy following surgery, which has been the practice previously.

We have, thus far, limited our studies to murine NK cells. However, a large portion of the work in this field of "anesthesiology-immunology" has utilized human peripheral blood obtained from patients recovering from surgery as a source of immunocompetent cells. Most of the published data concerning NK cell activity demonstrate a transient decrease in NK cell baseline cytotoxicity in postoperative recovery.⁶ Recognizing this as a possible immunosuppressive event, Tønnesen *et al.* suggested use of extradural analgesia *versus* neuroleptanesthesia whenever possible as an avenue by which to prevent compromising the immune defenses.^{5,6} These authors discussed the possible clinical importance of the presence of elevated levels of NK cell activity in postoperative recovery with respect to postoperative morbidity. While we can hypothesize on the clinical effects of strategies of elevating postoperative levels of NK cell activity, specifically with preoperative interferon treatment, the clinical significance of NK cell activity in association with postoperative morbidity remains to be addressed directly.

In the study of the effects of anesthetics alone on cells of the immune system, Stevenson *et al.* demonstrated that *in vitro* exposure of peripheral lymphocytes to halothane did not affect existing levels of NK cytotoxicity but did decrease monocyte hydrogen peroxide generation.²⁵ Interferon- γ activation of the monocytes decreased the inhibitory effect of halothane. It is noteworthy that halothane did not appear to affect baseline phagocytosis or tumor growth inhibitory properties of the monocytes. These findings, in accordance with our own, suggest that it is the activational properties and not the "housekeeping" functions of immune cells that are inhibited by anesthesia.

In summary, numerous questions remain unanswered concerning the mechanism(s) behind the phenomenon that we refer to as the "anesthetized NK system." The important clinical benefits of the study of the effects of anesthesia on the immune system are self-evident. Understanding the mechanisms behind the clinically described transient postoperative immunosuppression may generate studies of therapeutic strategies by exploring means of maintaining or enhancing the immune system, thereby, it is hoped, decreasing postoperative morbidity and mortality that may be associated with anesthetic and surgical immunosuppression.

|| Tait AR, Davidson BA, Johnson KJ, Remick D, Knight PR. Halothane alters the virus-specific immune response to influenza infection in mice (abstr). ANESTHESIOLOGY 73:A340, 1990.

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