Alterations in Splenic Lymphocyte Subpopulations and Increased Mortality from Sepsis Following Anesthesia in Mice

John F. Hansbrough, M.D., F.A.C.S.,* Ramon L. Zapata-Sirvent, M.D.,† Edward J. Bartle, M.D.,‡
Julie K. Anderson, B.S.,§ Lance Elliott, M.D.,¶ M. Ashraf Mansour, M.D.,** Walter H. Carter, Ph.D.,††

The authors evaluated the potential of a variety of anesthetics in mice to produce subsequent alterations in host defenses. Specific monoclonal antibodies and immunofluorescent microscopy were used to enumerate splenic helper/inducer: suppressor/cytotoxic lymphocyte ratios (HSLR), and resistance to bacterial challenge was evaluated by a cecal ligation and puncture (CLP) model. Two hours of anesthesia with the intravenous agents ketamine and pentobarbital and with the inhalational agents isoflurane, enflurane, halothane, and halothane-nitrous oxide, were utilized. All anesthetics produced marked depression in the HSLR, measured 24 h postanesthesia (P < 0.05); with all agents, helper T-cell populations were decreased and suppressor populations increased. The HSLR remained depressed 72 h postanesthetic, following both ketamine and halothane anesthesia (P < 0.05). A dose-response curve was determined with enflurane; increasing the anesthetic time from 1 to 6 h resulted in progressively greater depression of the HSLR 24 h later. Changes in lymphocyte subtypes of similar magnitude were found in mice after burn injury or hind limb crush injury and amputation, whereas simple laparotomy did not produce such changes. Serum corticosterone levels were not elevated 24 h postanesthetic with enflurane, suggesting that the alterations were not nonspecific stress reactions. Resistance to sepsis was determined by measuring survival for 96 h after CLP. With CLP performed 24 h following 2 h anesthesia, mortality was increased from normal: control mortality 36.5%; ketamine 65.0% (P < 0.023); isoflurane 69.5% (P < 0.006); enflurane 84.2% (P < 0.0002). Anesthesia produces dose-related alterations in splenic helper/inducer and suppressor/cytotoxic lymphocyte populations in mice, which persist for at least 72 h; resistance to subsequent bacterial challenge also is reduced, although the two effects are not proven to be related. (Key words: Anesthetics, intravenous: ketamine; pentobarbital. Anesthetics, volatile: enflurane; halothane; isoflurane. Blood: lymphocytes. Immune response: lymphocytes. Infection: septicemia.)

* Associate Professor, Department of Surgery, U.C.S.D. Medical Center.
† Post-Doctoral Research Fellow, Department of Surgery, U.C.S.D. Medical Center.
‡ Assistant Professor of Surgery, University of Colorado Medical School, Denver, Colorado.
§ Research Assistant, Department of Surgery, University of Colorado Medical School, Denver, Colorado.
¶ Assistant Professor, Department of Anesthesiology, University of Colorado School of Medicine, Denver, Colorado.
** Research Fellow, Department of Surgery, University of Colorado Medical School, Denver, Colorado.
†† Professor and Chairman, Department of Biostatistics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia.

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Address reprint requests to Dr. Hansbrough: Department of Surgery H640A, University of California at San Diego Medical Center, 225 Dickinson Street, San Diego, California 92103.

The occurrence of immunosuppression after anesthesia and surgical procedures has been controversial. While numerous studies have demonstrated anesthetic-induced depression of various aspects of the immune systems in both animals and humans, many other reports have failed to find consistent immunosuppressive effects following anesthesia.

Cell-mediated immunity is a specific arm of immunity that is important in various aspects of the host defense systems. The clinical significance of suppressed cell-mediated immunity in the perioperative period is important; it has been suggested that such suppression contributes to postoperative dissemination of carcinoma1-4 and to postoperative infections.1,3-8

However, the majority of studies of cellular immunity following anesthesia have utilized in vitro evaluations of lymphocyte functions. There are limitations of such tests, in part because of their requirement for the removal of immunocompetent cells from the host environment; these limitations may be particularly important in the study of the effects of anesthesia on immune responses, since hormonal, stress, and other host factors may be important in determining in vivo immune potential. For example, suppressed lymphocytes in surgical patients may function normally when taken out of the anergic environment.††

In the studies reported here, mice were anesthetized with various anesthetic agents, and in vivo cellular immunity was evaluated with the use of specific monoclonal antibodies to measure splenic helper and suppressor lymphocyte populations in the postanesthetic period. Identification of functional lymphocyte subpopulations by such antibodies has been shown to be an accurate and reproducible measure of cell-mediated immunity. In addition, using a colon perforation and sepsis model in mice, the effect of prior anesthetic administration on survival following subsequent intraabdominal sepsis was determined.

Materials and Methods

Figure 1 outlines the overall experimental design.

ANIMALS AND ANESTHETIC ADMINISTRATION

Female, inbred CF1 mice, 8–14 weeks of age, were anesthetized with various agents as follows. Ketamine hydrochloride (six mice) (Parke Davis Co., Morris Plains, New Jersey) and sodium pentobarbital (six mice) (Fort Dodge Laboratories, Fort Dodge, Iowa) were given by intraperitoneal (ip) injection in a small amount of saline (0.1–0.2 ml). Injections were repeated every 20–30 min, or whenever any animal showed evidence of waking. Animals given ip anesthetics breathed room air. The total amount of drugs administered for 2-h anesthesia times were as follows: ketamine, 8–10.5 mg; and pentobarbital, 1.28–1.92 mg. The inhalational agents were delivered with a total carrier gas flow of 5 l/min into a 40-l chamber, which allowed gas to exit at one end. Anesthetic depth was maintained at a level at which the mice exhibited only sleeping behavior. An occasional staggering journey by a mouse followed by somnolence was considered acceptable. The various agents were delivered in the following manner: isoflurane 1.4% (five mice) (Ohio Medical Anesthetics, Madison, Wisconsin) and enflurane 1.56% (five mice) (Ohio Medical Anesthetics) were delivered by oxygen flow through calibrated vaporizers (Ohio Medical Products, Madison, Wisconsin) calibrated by a mass spectrometer (Perkin Elmer, Pomona, California) standardized against calibration gases. Halothane 1.28% in oxygen (five mice) and halothane 1.04% (Halocarbon Laboratories, Hackensack, New Jersey) in 50% nitrous oxide/50% oxygen (five mice) were delivered from the main vaporizer of an Ohio 2000® (Ohio Medical Products) anesthesia machine calibrated by the same mass spectrometer noted above. These halothane concentrations represent equal MAC at this altitude when the presence of nitrous oxide is considered. Sham-treated animals received ip saline injections only or were allowed to breathe 100% oxygen in the chamber for 2 h. The number of animals in each group is indicated in table 1.

At the end of all experiments with inhalation agents, a 5 l/min oxygen flow was maintained through the chamber until arousal was noted. All guidelines of the American Physiological Society were followed.

LAPAROTOMY, BURN INJURY, LIMB CRUSH INJURY, AND AMPUTATION

Using brief (4–5 min) methoxyflurane vapor (Abbott Laboratories, North Chicago, Illinois) anesthesia, nine mice underwent laparotomy with the use of a long midline incision, irrigation of the abdominal cavity for 4 min with lactated Ringer’s solution, and wound closure with a running suture. A second group (five mice) received a 20–25% total body surface area full-thickness

<p>| Table 1. Splenic Lymphocyte Subpopulations Following Anesthesia and Trauma |
|-----------------------------|-----------------------|-----------------------|-----------------------|-----------------------|</p>
<table>
<thead>
<tr>
<th>N</th>
<th>Group</th>
<th>% B</th>
<th>% Ly1*</th>
<th>% Ly2*</th>
<th>% Ly1*/Ly2* ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>Normal (control)</td>
<td>43.0 ± 0.6</td>
<td>31.7 ± 0.5</td>
<td>10.2 ± 0.2</td>
<td>3.12 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>ip Saline</td>
<td>46.6 ± 1.2*</td>
<td>29.2 ± 0.7*</td>
<td>9.0 ± 0.3*</td>
<td>3.25 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>100% O₂</td>
<td>42.6 ± 1.7</td>
<td>34.2 ± 2.1*</td>
<td>10.6 ± 0.7</td>
<td>3.24 ± 0.10</td>
</tr>
<tr>
<td>6</td>
<td>Ketamine</td>
<td>46.0 ± 1.4*</td>
<td>23.5 ± 0.9*</td>
<td>12.3 ± 0.7*</td>
<td>1.93 ± 0.07*</td>
</tr>
<tr>
<td>5</td>
<td>Halothane</td>
<td>47.2 ± 1.7*</td>
<td>23.0 ± 0.5*</td>
<td>12.6 ± 1.4*</td>
<td>1.91 ± 0.15*</td>
</tr>
<tr>
<td>5</td>
<td>Halothane + nitrous oxide</td>
<td>42.4 ± 1.7</td>
<td>24.0 ± 1.7*</td>
<td>12.6 ± 0.8*</td>
<td>1.99 ± 0.13*</td>
</tr>
<tr>
<td>5</td>
<td>Isoflurane</td>
<td>47.4 ± 1.7*</td>
<td>25.4 ± 1.2*</td>
<td>11.8 ± 0.7*</td>
<td>2.16 ± 0.04*</td>
</tr>
<tr>
<td>5</td>
<td>Enflurane</td>
<td>43.2 ± 1.7</td>
<td>24.0 ± 1.4*</td>
<td>13.6 ± 0.7*</td>
<td>1.77 ± 0.06*</td>
</tr>
<tr>
<td>6</td>
<td>Pentobarbital</td>
<td>45.3 ± 1.3</td>
<td>22.2 ± 0.6*</td>
<td>13.0 ± 0.5*</td>
<td>1.71 ± 0.03*</td>
</tr>
<tr>
<td>5</td>
<td>Burn</td>
<td>44.4 ± 1.7</td>
<td>25.0 ± 1.4*</td>
<td>13.6 ± 1.0*</td>
<td>1.86 ± 0.08*</td>
</tr>
<tr>
<td>9</td>
<td>Laparotomy</td>
<td>46.0 ± 1.3*</td>
<td>31.4 ± 1.0</td>
<td>10.4 ± 0.6</td>
<td>3.05 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>Crush-ampputation</td>
<td>46.8 ± 0.7*</td>
<td>26.4 ± 0.4*</td>
<td>15.2 ± 0.8*</td>
<td>1.77 ± 0.12*</td>
</tr>
</tbody>
</table>

Results: mean ± SEM.

*P < 0.05 (compared with normal control).
IMMUNOSUPPRESSION IN MICE FOLLOWING ANESTHESIA

Identification of Splenic Lymphocyte Subsets

Mice were killed by cervical dislocation at various times (depending on the particular experiment) after administration of the anesthetic agents. Spleen cell suspensions were prepared by pressing intact spleens through fine stainless steel screens into Mishell-Dutton balanced salt solution (BSS). Red blood cells were lysed with TRIS-NH₄Cl, and the suspensions were washed twice with BSS and centrifuged at 400 × g. The cellular pellet then was resuspended in BSS and the cell population adjusted to 2 × 10⁷ cells/ml. Twenty microliters monoclonal mouse antimonouse Lyt 1.2, binding helper/inducer T-cells (Ly1⁺,2⁺), or Lyt 2.2, binding suppressor/cytotoxic T-cells (Ly1⁻,2⁺) (New England Nuclear, Boston, Massachusetts), was added to 100 µl of cell suspension and incubated for 30 min at 4° C. The cells then were washed twice in 1 ml phosphate-buffered saline containing 0.2% sodium azide (PBS-azide) and 20 µl of fluorescein isothiocyanate-labeled goat antimonouse immunoglobulin (GAM1g; Antibodies, Inc., Davis, California) was added and incubated as above. Concurrently, a suspension of cells not incubated with monoclonal antibody was incubated with GAM1g to determine B-lymphocyte staining. A wet-mount slide was made for counting of cell subpopulations by fluorescent microscopy.

The relative cell percentages from spleens were calculated as follows: cell percentage = (monoclonal antibody bound cells minus [GAM1g alone bound cells] divided by total cells counted times 100%. Additionally, the Ly1⁺/Ly2⁺ ratio was calculated.

Measurement of Septic Mortality Using Cecal Ligation and Puncture

The method of Baker et al.¹⁰ was used. Mice were anesthetized briefly with methoxyflurane, and their abdomens were sprayed with 70% ethanol. A small lower abdominal incision was made into the peritoneal cavity. The cecum was grasped and withdrawn with forceps and ligated at the base with a 3-0 silk suture, with care not to produce a bowel obstruction. A through-and-through puncture then was made of the cecum with a 23-gauge needle and the cecum gently squeezed to express a tiny amount of feces. One milliliter of normal saline then was placed in the abdominal cavity, the cecum replaced and the wound closed with a running 3-0 silk suture. The entire procedure took 3–4 min, and the animals awakened within several minutes from the anesthetic. No additional parenteral fluids were administered. Mice were observed closely and daily mortality determined for a 96-h period after surgery. Laparotomy alone without cecal ligation and puncture produced no mortality. For the septic mortality studies, animals received 2 h of treatment with either ketamine, isoflurane, or enflurane, and cecal ligation and puncture was performed 24 h later.

Measurement of Serum Corticosterone Levels

Blood was drawn from mice after methoxyflurane anesthesia, immediate laparotomy, and cecal puncture with a needle and syringe; samples were drawn within 3 min of initiation of anesthesia to minimize immediate stress responses. All blood was drawn at the same time of day (approximately 0900 h) to minimize diurnal variations in steroid levels. Serum corticosterone levels were determined by radioimmunoassay with the use of rabbit antiserum to corticosterone and ¹⁴C-hormone (Inter Sci Diagnostics, 2000 Cotner Avenue, Los Angeles, California), using six known concentrations of corticosterone to establish a standard curve. All samples were run in duplicate.

Statistical Methods

All data on lymphocyte populations are presented as the mean ± 1 SE of the mean. To determine statistical significance, the Student's t test for independent means was used for comparing cell populations. Contingency table analysis was used for comparing mortality in different groups of animals. An overall comparison of the groups was conducted, and each group was compared with the control using Fisher's exact test. In all analyses, statistical significance was claimed at the 5% level (P < 0.05).

Results

All animals appeared to recover quickly following cessation of anesthetics, and fed and drank subsequently in normal fashion. Five mice died during ketamine anesthesia (probably from overdose), and none died following the other anesthetics. No animals died during or after the performance of laparotomy, crush injury/amputation, or burn injury. Finally, there were few deaths beyond 96 h following cecal ligation and puncture, and although a certain percentage of these animals presumably would go on to have chronic intraabdominal abscesses develop, this aspect was not studied.

Changes in lymphocyte populations following administration of various anesthetics, simple laparotomy, burn
and traumatic injury are summarized in table 1. In all groups in table 1, lymphocyte analysis was performed 24 h after the indicated treatment. All of the anesthetic agents tested, including both the inhalational and intravenous agents, produced statistically significant decreases in helper/inducer lymphocyte (Ly1+) populations and increases in suppressor/cytotoxic (Ly2+) populations 24 h after a 2-h administration of anesthesia. The final column denotes the helper/inducer:suppressor/cytotoxic ratio, which has become accepted as a measure of the status of cellular immunity. As can be seen, administration of all of the anesthetic agents for a 2-h period resulted in marked and statistically significant decreases in the lymphocyte ratio 24 h postanesthetic. Control groups also are shown in table 1. Two-hour administration of 100% oxygen produced a slight increase in Ly1+ populations and ip injections of saline produced slight changes in both Ly1+ and Ly2+ cell numbers; in both control groups, however, the Ly1+/Ly2+ ratio was unchanged. The short duration of anesthesia with methoxyflurane, used for the traumatic injury models, also did not produce changes in lymphocyte populations 24 h later (data not shown).

Also in table 1, the effects of anesthesia on lymphocyte populations are compared with corresponding effects after various types of trauma, including laparotomy, hind-limb crush injury and amputation, and a 20–25% body surface area burn injury. In the latter three experimental groups, mice were anesthetized for only several minutes with methoxyflurane while trauma was induced. As demonstrated, laparotomy alone did not produce subsequent changes in lymphocyte populations, but burn injury and limb crush injury/amputation produced changes equivalent to those seen after 2 h anesthetic administration. These results suggest that immunologic changes following anesthesia may transiently be similar in “magnitude” to corresponding changes following severe trauma.

Figure 2 demonstrates the time course for the development of immune depression, indicated by a decreasing helper/inducer:suppressor/cytotoxic T-cell ratio, following 2 h of ketamine or halothane anesthesia. Suppression with ketamine was equally great at 24 and 48 h postanesthetic; by 48 h the lymphocyte ratio following halothane administration was significantly improved (increased) from 24 h (P < 0.05). Although lymphocyte ratios were returning toward normal at 72 h postanesthetic, ratios with both agents remained statistically depressed from normal (P < 0.05).

The duration of enflurane anesthesia and resultant immunodepression is depicted in figure 3. Increasing suppression, measured by a decrease in the lymphocyte ratio measured 24 h postanesthetic, occurred as the anesthetic time was lengthened from 1 to 6 h. The lymphocyte ratio 24 h after 2 h administration of enflurane was decreased from the ratio following 1 h of anesthesia (P < 0.05), and the ratio after 6 h of enflurane was decreased from the ratio following 2-h administration (P < 0.05). Actual values for the lymphocyte ratios are denoted in the figure 3 legend.

Mortality data in mice receiving 2 h of anesthesia
with ketamine, isoflurane, or enflurane followed by cecal ligation and puncture 24 h later, is shown in figures 4 and 5. Administration of all three anesthetic agents resulted in greatly increased mortality following intraabdominal sepsis (P < 0.05 for all agents). Although mortality was measured over a 96-h period following cecal ligation and puncture, almost all deaths were within the first 72 h after induction of sepsis, as demonstrated in figure 4. Animals surviving for 96 h appeared healthy, and few died after this time. Autopsies were performed on selected animals; mice dying of sepsis were found to have a fulminating, exudative peritonitis, and survivors had walled-off abscesses.

Determination of serum corticosterone levels in mice demonstrated that levels 24 h following 2 h administration of enflurane were not different from levels in normal, unanesthetized mice. Individual values in five normal mice were 0.057, 0.020, 0.020, 0.013, 0.013, mean 0.027 µg/dl; values in three anesthetized mice were 0.010, 0.010, 0.020, mean 0.0133 µg/dl, P not significant).

Discussion

The effects of surgical procedures and of anesthetic administration on the various components of the immune system remain in question. Although many reports have documented immune defects following anesthesia and surgery,1,5,6,8,12–14 other investigators have failed to confirm postoperative suppression.15–19

Several factors appear to contribute to the controversy concerning the effect of anesthesia on cell-mediated immunity. Many reports of cellular immune function following surgery and anesthesia have employed various stimulatory tests of lymphocyte function; such tests are performed in vitro and depend upon interactions between various cell populations, including lymphocytes and monocytes.20 Since some cell populations probably die rapidly in culture conditions, true in vivo lymphocyte reactivity may well be altered after even short periods of culture. In addition, in vitro conditions remove cells from the host environment, and the effect on lymphocytes of various circulating factors, including hormones, stress factors, and other substances, may be lost. Finally, there are large statistical variations associated with lymphocyte stimulation tests that can make interpretations difficult.

Direct measurement of lymphocyte subpopulations, utilizing staining and quantitation of cells by antibodies specific for lymphocyte subclasses, appears to provide an accurate measure of cellular immune competence.21 A decrease in the ratio of helper/inducer:suppressor/cytotoxic cells has been shown to correlate with depressed cellular immunity. Such measurements appear to accu-

![Graph showing survival after sepsis](image)

**Fig. 4.** Time-course of survival following septic challenge (cecal ligation and puncture, CLP), 24 h after 2 h of anesthesia with various agents. Cages were inspected each 24 h and survivors noted.

rately reflect host immune potential in various disease states.22–24 and following surgery and trauma.25,26

Depression of the lymphocyte ratio and of the immune response in various disease states may be a result of either decreased helper activity or increased suppressor activity.21 Our laboratory has demonstrated both decreases in helper populations and increases in suppressor populations following abdominal surgery in humans,26 and Antonacci et al.25 reported that burn injury produced decreases in helper populations without changes in suppressor numbers.

![Graph showing percent mortality](image)

**Fig. 5.** Ninety-six-hour mortality of mice undergoing cecal ligation and puncture 24 h after anesthesia with the indicated agents. **A**, control mice, no anesthesia; **B**, ketamine; **C**, isoflurane; **D**, enflurane. Mortality in all anesthetic groups was increased from normal (P < 0.023 for ketamine, P < 0.006 for isoflurane, and P < 0.0002 for enflurane). Numbers in bars indicate number of mice in each experimental group. Actual mortality values were as follows: control 36.5%; ketamine 65.0%; isoflurane 69.5%; enflurane 84.2%.
However, there have been few other studies of changes in lymphocyte subpopulations following surgery and administration of anesthetic agents. Hole and Bakke\textsuperscript{17} failed to demonstrate changes in lymphocyte subpopulations after hip surgery in elderly patients. Other factors may explain this difference, including the elderly age of those patients, the use of concomitant blood transfusion, differing techniques of anesthesia, and the use of a different technique for measuring cell populations (Fluorescent Activated Cell Sorter, or FACS). We have previously reported that major surgery in humans produced changes in postoperative lymphocyte populations similar to those shown in our animal studies shown here.\textsuperscript{26} The use of medications that may have immunologic effects, such as antiinflammatory and histamine-blocking agents,\textsuperscript{9} also must be defined carefully in clinical studies. Salo \textit{et al.} reported a decrease in the number of T-suppressor cells after open-heart surgery,\textsuperscript{27} although monoclonal antibodies were not utilized.

The results reported in this study indicate that the anesthetic agents studied, including both inhalational and intravenous drugs, produce a marked, prolonged depression in cellular immunity in mice, characterized by both decreases in helper/inducer lymphocytes and increases in suppressor/cytotoxic lymphocytes. In addition, we found that a 2-h duration of anesthesia resulted in greatly increased mortality following induction of intraabdominal sepsis 24 h later, compared with animals not subjected to anesthesia. Thus, anesthetic-induced suppression of cellular immunity appears to be reproducible and quantifiable and associated with reduced host resistance against bacterial challenge.

Other cell populations have immunoregulatory properties but are difficult to study at this time. For example, although suppressor lymphocytes exert a negative regulatory influence on immunity, another class of immunoregulatory T-cells exerts positive regulation by opposing suppressor cell activity; these cells have been named "contrasuppressor cells"\textsuperscript{28} and they constitute a small subset of Ly1\textsuperscript{+}2\textsuperscript{−} cells. The future availability of more specific monoclonal antibodies will make further detailed analysis of such lymphocyte subpopulations possible.

The mortality studies presented here support the role of anesthesia in depressing host defenses against bacterial sepsis. Others have shown that anesthesia (specifically halothane) produces increased mortality with fecal peritonitis in mice\textsuperscript{29,30}; the present study confirms those findings and in a more quantitative fashion. The sepsis model we used in this report has been shown to be an appropriate analog of surgical intraabdominal sepsis. Blood cultures become positive for multiple organisms in this model as early as 1 h after the procedure.\textsuperscript{10} In a similar model in rats, an early hyperdynamic phase of sepsis has been demonstrated, which deteriorated later into a hypodynamic phase\textsuperscript{31}; it is of course impossible to equate the experiments in rats with similar experiments in mice, and efforts to study hemodynamics in septic mice would be difficult.

Although the correlation of depressed cellular immunity with increased septic mortality following anesthesia is clear in these studies, other components of host defenses may be depressed, including various aspects of the nonspecific inflammatory response.\textsuperscript{3,4,7,14,32,33} For example, halothane\textsuperscript{35} and enflurane\textsuperscript{14} have been shown to inhibit bacterial killing by neutrophils, while nitrous oxide and isoflurane did not have such effects. However, the inhibitory effects occurred only during exposure \textit{in vitro} to the anesthetic agents and were rapidly reversible.

Unfortunately, the multiple factors and variables involved in critically ill surgical patients have made it difficult to clearly identify all factors contributing to suppressed immunity. Some previous studies of the effects of anesthesia on the immune response have suggested that such suppression is largely a response to surgical or anesthetic stress and that the suppression may be prevented by epidural block.\textsuperscript{34,35} However, direct effects of modern anesthetics on catecholamine and corticoid responses appear to be minimal, although anesthesia combined with surgical procedures may produce significant changes in both responses.\textsuperscript{32,36} In addition, it has been suggested that light anesthesia or anesthesia associated with complications such as hypotension or hyperventilation may produce greater changes of the neuroendocrine systems than following uncomplicated anesthesia.\textsuperscript{37}

Hole\textsuperscript{38} failed to find effects of catecholamines on \textit{in vitro} lymphocyte or monocyte function, and while adrenal corticoid production was found to be elevated to approximately three times normal levels after both halothane and epidural anesthesia, anesthesia without surgery failed to elicit this response.\textsuperscript{32} Our studies found that serum levels of corticosterone, the principal active corticosteroid in mice, were not elevated 24 h after enflurane administration in mice.

\textbf{References}


