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Apoptotic lymphocytes induced by surgical trauma in dogs

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Abstract
Purpose. An investigation was made of the population and function of lymphocytes in canine peripheral blood, in animals with or without laparotomy under inhalation anesthesia.
Methods. Fourteen healthy beagles were allocated to two experimental groups: laparotomy (group A) and without laparotomy (group B). Induction of anesthesia in both groups was carried out with an intravenous injection of thiopentone, and was maintained by isoflurane inhalation. Ten blood samples, consisting of 10 ml of venous blood withdrawn by venipuncture into syringes containing 200 units of preservative-free heparin, were taken, from before anesthesia to the 28th postoperative day. The blood samples were collected, and the number of lymphocytes, the lymphocyte subpopulations, the proportion of apoptotic lymphocytes, and plasma cortisol level were measured, and the blastoid transformation of lymphocytes was observed.
Results. Lymphopenia was observed in both groups after anesthesia. Flow cytometry indicated a greater reduction in the proportion of T lymphocytes than of B lymphocytes. Blast transformation was also depressed in both groups. Progression of apoptosis after anesthesia was demonstrated in both groups, with a higher percentage of apoptotic cells being observed in group A at 12 h after anesthesia (28.5 ± 3.2% by TUNEL assay). Plasma levels of cortisol were elevated to a greater extent in group A at the end of anesthesia (10.3 ± 0.8 μg/dl) than in group B (7.8 ± 1.9 μg/dl).
Conclusions. These results indicate that surgical trauma concomitant with anesthesia could impair immunocompetence by reducing the number and function of lymphocytes.

Key words Anesthesia · Apoptosis · Dog · Laparotomy · Lymphocyte

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Introduction

It is well recognized that host defense capacity is impaired in patients who receive surgical treatment [1–3]. A reduction in the number and function of lymphocytes in peripheral circulating blood has been thought to be one of the causative factors in postoperative infection. It is difficult to reveal the real effect of surgical trauma on lymphocyte activity, because surgical procedures are carried out on patients treated with analgesic and/or anesthetic agents that are reported to have suppressive effects on lymphocyte function [1–3].

In the present study we examined the population and function of lymphocytes in peripheral blood in two populations of dogs, the first group being subjected to laparotomy under inhalation anesthesia and the second group receiving inhalation anesthesia alone.

Materials and methods

The experimental protocol was approved by the Bioethics Committee at Nippon Veterinary and Animal Science University.

Fourteen healthy beagles (7 males, 7 females), with a mean age of 4.0 ± 2.0 years and a mean body weight of 10.6 ± 2.0 kg, were used. The dogs were routinely vaccinated and checked for internal parasites prior to the study, and were kept indoors and maintained on a commercial diet. The dogs were allocated to two experimental groups: laparotomy (group A) and a control group without laparotomy (group B).

Induction of anesthesia in the animals in both groups was carried out with an intravenous injection of thiopentone sodium (Ravonal; Tanabe Pharmaceutical, Tokyo, Japan) (12.4–16.5 mg·kg^{-1}). An endotracheal tube was placed into the trachea to facilitate the control of respiration, which was controlled with a mechanical ventilator. Anesthesia was maintained for 3 h through

the inhalation of 1.2–1.5 maximum allowable concentration (MAC) of isoflurane vapor (Forane; Dainihon, Tokyo, Japan) carried in 100% oxygen. The animals were placed in the ventro-dorsal recumbent position, and, in group A, a laparotomy, extending from the xiphisternum to the pubis, was aseptically performed with the animals under anesthesia. The wound was closed with 3-0 silk sutures. After recovery from anesthesia, the animals were extubated and placed in the lateral recumbent position on a blanket. The dogs in group B received anesthesia in the same manner as the animals in group A, and underwent controlled ventilation for 3 h, without laparotomy. After recovery from anesthesia, the animals were positioned in the lateral recumbent position in the same manner as the animals in group A. During the experiment, no dogs were given any kind of transfusion. After recovery from anesthesia, the animals were allowed to take food and water ad libitum. To evaluate cardiopulmonary function in the dogs while they were under anesthesia, a 7-French size catheter was inserted in the right femoral artery with dogs placed in the right lateral recumbent position. To obtain hemodynamic parameters, heart rate (HR), mean arterial blood pressure (MAP), diastolic arterial blood pressure (DAP), systolic arterial blood pressure (SAP), and electrocardiogram were recorded simultaneously. The hemodynamic parameters were measured using a transducer (Life kit; Nihon Koden, Tokyo, Japan) and recorded using a Life-Scope 9 (model OMP 7201; Nihon Koden, Tokyo, Japan). To evaluate the respiratory state, the respiration rate and end-tidal CO_2 ($EtCO_2$) were measured by capnography (Datex-Ohmeda; IMI, Saitama, Japan).

Ten blood samples, consisting of 10 ml of venous blood withdrawn by venipuncture into syringes containing 200 units of preservative-free heparin (heparin sodium; Takeda Pharmaceutical, Osaka, Japan), were taken from before anesthesia to the 28th postoperative day. The collected blood was used for counting lymphocyte numbers, analyzing lymphocyte subpopulations, observing blastoid transformation, measuring the proportion of apoptotic cells, and quantifying the plasma cortisol level.

Lymphocyte subpopulations, such as cells positive for CD3, CD4, CD8, CD21, CD45, and TCRαβ, were observed by flow cytometry analysis, using an auto cell screener (Cyto Ace-150; Jasco, Tokyo, Japan) after staining was performed with monoclonal antibodies (provided by Dr. F. Moore, University of California, Davis) (Table 1). Goat anti-mouse IgG antibody (Cappel, Aurora, OH, USA) was used as the secondary antibody, as described elsewhere. An index of B cells was obtained, using fluorescein isothiocyanate (FITC)-conjugated goat anti-dog IgG and IgM antibodies (Cappel) [4,5].

Table 1. Monoclonal antibodies used for staining of lymphocyte surface antigens

Surface antigens in dogs	Monoclonal antibody clones
CD3	CA17.2A12-IgG1
CD4	CA13.1E4-IgG1
CD8	CA15.4G2-IgG1
TCRαβ	CA15.8G7-IgG1
CD21	CA2.1D6-IgG1
CD45	CA12.10C12-IgG1
Anti-dog IgG	AQA645FS
Anti-dog IgM	AQA155FN

Lymphocytes transformation was measured in terms of the incorporation of tritiated thymidine when the cells were stimulated with *phytohemagglutinin* (PHA) and *concanavalin* A (Con A) in vitro. The collected blood was mixed with an equal volume of phosphate-buffered saline (PBS) and centrifuged at 400*g* in a Conray-Ficoll medium (IBL; Gunma, Japan; density, 1.077) for 20 min at room temperature. The cell layer in the medium was pipetted and washed with PBS. The mononuclear cells were suspended in RPMI-1640 medium (IBL), and were supplemented, at a concentration of 5×10^5 cells/ml, with 10% fetal bovine serum (FBS; Biocell, Rancho Dominguez, CA, USA), and cultured for 64 h at 37°C in a 5% CO_2, 95% air atmosphere. PHA (Difco Laboratories, Detroit, MI, USA) or Con A (E.Y. Laboratories, San Mateo, CA, USA) was added to a final concentration of 20 μl/ml. Transformation was quantified by determining the incorporation of ^{3}H-thymidine over a 3-h labelling period. ^{3}H-Thymidine (Amersham Pharmacia Biotech, Buckinghamshire, England), 30 μ Ci, was added to each tube and nuclear protein was precipitated, allowing measurements of radioactivity with a scintillation counter (1205 betaplate type; LKB, Turku, Finland).

Apoptotic changes in lymphocytes were assessed by three methods: chromatin staining, DNA fragmentation assays, and the TUNEL method [6]. Lymphocytes in the collected blood were separated by centrifugation of the blood with Conray-Ficoll medium; as mentioned above. The cell layer was removed, suspended in 20 μl of buffer and washed with PBS.

Chromatin staining assay

The cells were fixed with 1% glutaraldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 30 min at room temperature. After being washed with PBS, the cells were stained with 0.2 mM Hoechst 33342 (Calbiochem, La Jolla, CA, USA). Nuclear morphological changes were observed by fluorescence microscopy (Olympus BX60; Olympus Industries, Tokyo, Japan).

DNA fragmentation assay

The cells were suspended in an ice-cold lysis buffer (50mM Tris-HCL, pH7.8, 10mM ethylenediaminetetraacetic acid · Na, 0.5% sodium-N-lauryl-sarcosinate). The DNA was precipitated with ethanol and separated by centrifugation. Fragmented DNA was analyzed by electrophoresis through 2% agarose gels.

TUNEL method

A Mebstain Apotosis kit II (MBL Nagoya, Japan) was used to detect and quantify apototic cell death by enzymatic labelling of DNA strand breaks with fluorescein (dUTP) and terminal deoxy-nucleotidyl transferase (TdT), as described by the manufacturer. Lymphocytes were incubated with 1mM DEVD-fmk (MBL, Nagoya, Japan). The cells were fixed with 4% paraformaldehyde at 4°C for 30min and incubated with 70% ethanol at −20°C for 30min, followed by incubation with TdT reaction agent at 37°C for 1h. After being washed with PBS, the cells were resuspended in PBS and subjected to flow cytometry analysis.

Concentrations of cortisol in plasma were determined by a double-antibody radioimmunoassay system with a ^{125}I-labelled radioligand. Plasma was placed in tubes with anti-glucocorticoid antibody (Immunotech, Marseilles, France). Competitive binding of the plasma to authentic corticosteroid was determined with a gamma counter (ARC950 type; Aloka, Tokyo, Japan); radioactivity was determined after the washing of the soluble antibody.

The values for the numerical data obtained are presented as means and standard errors. The significance of the differences between the two groups was tested by the Mann-Whitney *U*-test. Differences between the preanesthetic and postanesthetic periods were assessed by analysis of variance (ANOVA), followed by the Dunnet test. A probability lower than 0.05 was considered to be significant.

Results

The hemodynamic and respiratory conditions of the two groups varied similarly within normal ranges during anesthesia. Namely; in both groups, heart rate was 100 ± 20 /min, MAP was 105 ± 10mmHg, DAP was 80 ± 6mmHg, SAP was 110 ± 25mmHg, respiration rate was 10 ± 2 /min, and $EtCO_2$ was 52 ± 6mmHg.

The number of peripheral lymphocytes decreased most significantly at 6h in group A and immediately postanesthesia in group B. Peripheral lymphocyte values recovered to the preanesthesia levels after 3 days in group A and after 1 day in group B (Fig. 1). Group A experienced a greater reduction in lymphocyte numbers

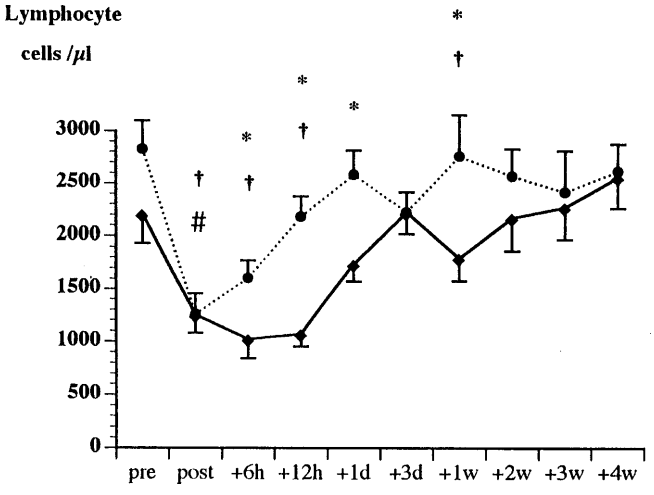


Fig. 1. Numbers of peripheral lymphocytes before (*pre*) and after (*post*) surgical trauma. *Vertical bars* show the SEM. Group A ($n = 7$), *diamonds/solid line*; group B ($n = 7$), *closed circles/dotted line*. *Statistically significant difference at $P < 0.05$, between group A and group B. †Statistically significant difference between pre and each of the post values in group A. #Statistically significant difference between pre and each of the post values in group B

than group B, showing significant differences from group B at 6h, 12h, and 1 day after recovery from anesthesia (Fig. 1).

Flow cytometry showed clear redistributions of the lymphocyte subpopulations in both groups. In group A, CD8+ cells had decreased significantly at 3h and 1 week postsurgery in comparison with preanesthesia values, and this reduction was accompanied by reductions in TCRαβ + lymphocytes at 3, 6, 12, and 24h, and 1 week after recovery of anesthesia. However, CD21+, IgG+, and IgM+ lymphocytes did not change during the study. In group B, CD8+ lymphocytes were significantly decreased at 3h and 3 days after recovery from anesthesia, with concomitant decreases in CD4+ cells at 12h and 3 days, and CD3+ cells at 6h after recovery from anesthesia. TCRαβ+ cells were also decreased in group B (Fig. 2).

The incorporation of ^{3}H-thymidine was significantly diminished by anesthesia in both groups. In group A, the PHA response was decreased at 6 and 12h, and the Con A response at 6, 12h, and 3 days after anesthesia. In group B, the PHA response was decreased at 12h and at 1, 2, and 3 weeks after anesthesia. Responses to Con A were also reduced at recovery from anesthesia, and at 6 and 12h, and at 1, 2, and 3 weeks after anesthesia. Differences between group A and group B were noted in PHA and Con A responses immediately after recovery from anesthesia (Fig. 3).

The proportion of apoptotic cells in normal lymphocytes was detected by morphological and biochemical techniques, with marked increases being shown in both

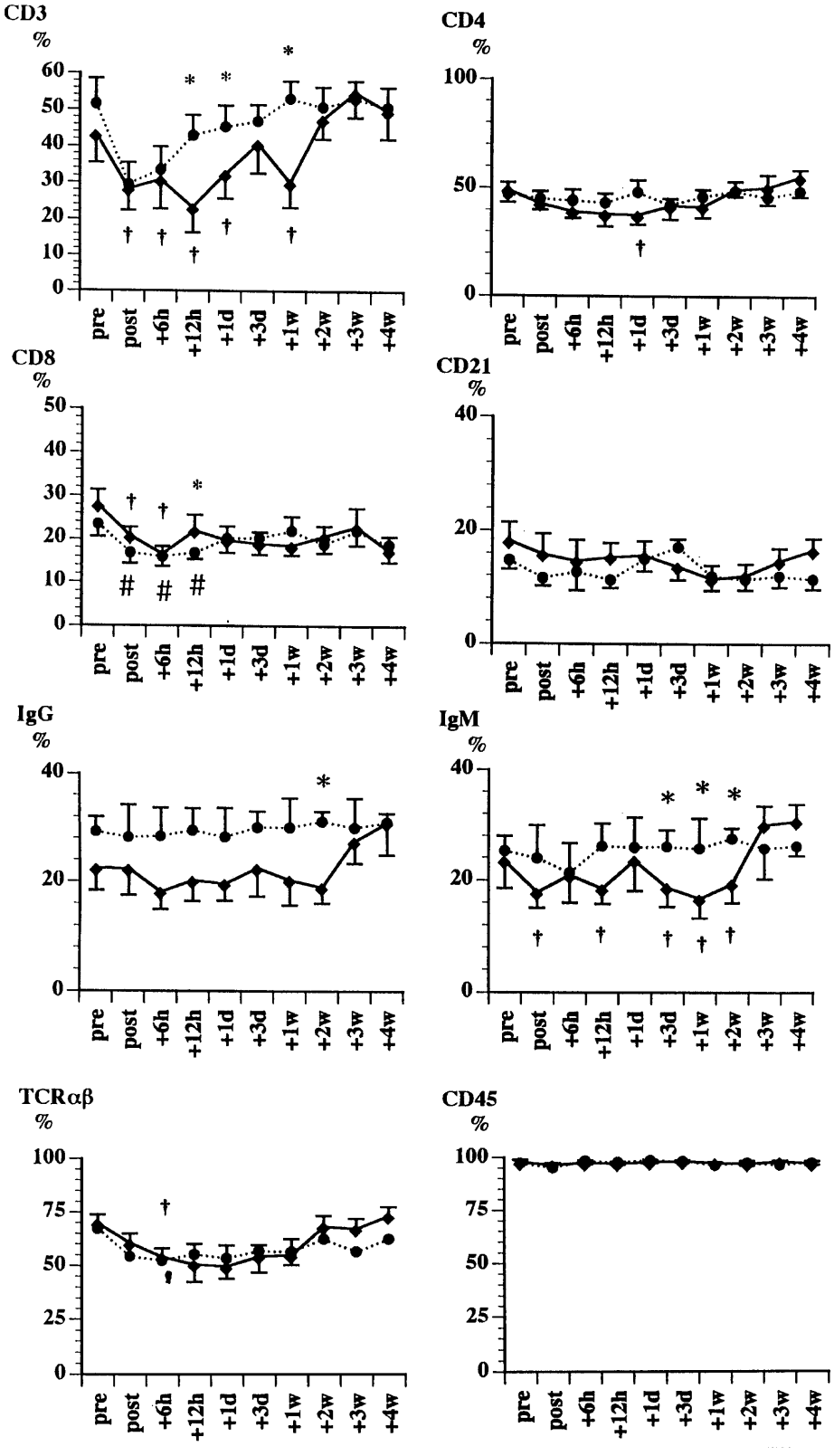


Fig. 2. Lymphocyte subpopulations in peripheral blood before and after surgical trauma. *Vertical bars* show the SEM. Group A ($n = 7$), *diamonds/solid line*; group B ($n = 7$), *closed dots/dotted line*. *Statistically significant difference, at $P < 0.05$ between group A and group B. †Statistically significant difference between pre and each of the post values in group A. #Statistically significant difference between pre and each of the post values in group B

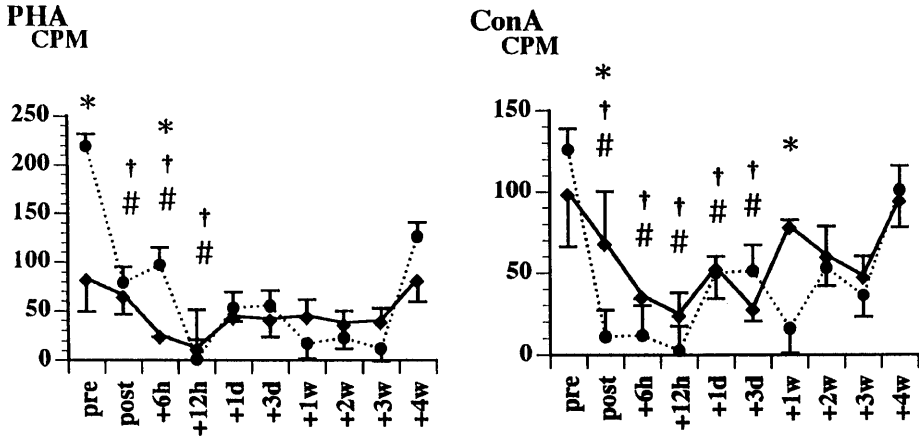
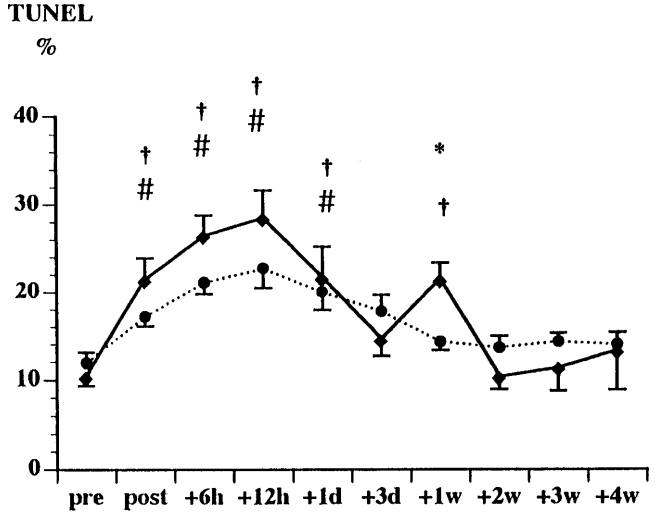
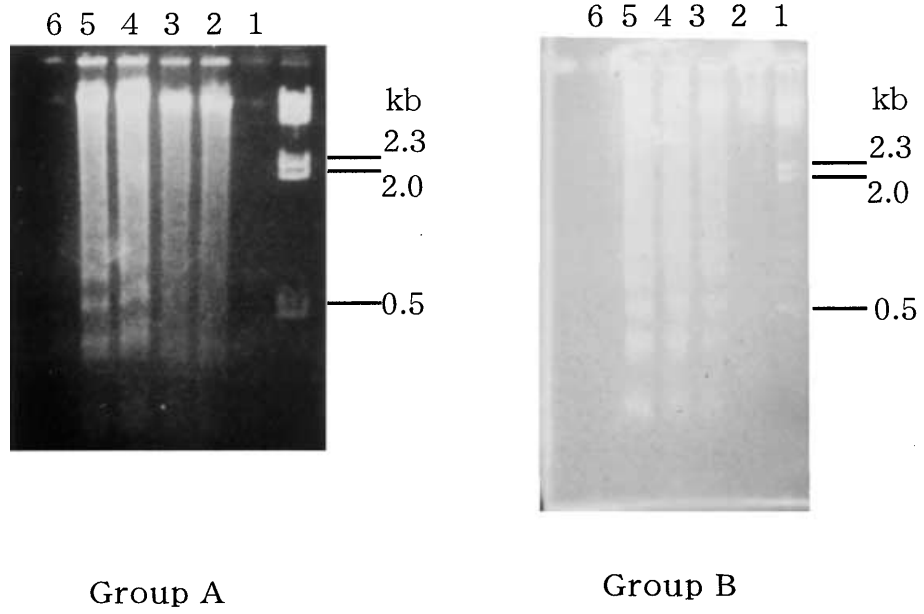


Fig. 3. Blast transformation of lymphocytes in response to phytohemagglutinin (*PHA*) and concanavalin agglutinin (*ConA*). *Vertical bars* show the SEM. Group A ($n = 7$), *diamonds/solid line*; group B ($n = 7$), *closed circles/dotted line*. *Statistically significant difference at $P < 0.05$ between group A and group B. †Statistically significant difference between pre and each of the post values in group A. #Statistically significant difference between pre and each of the post values in group B



a



b Group A Group B

Fig. 4. a Percentages of apoptotic lymphocytes before and after surgical trauma. *Vertical bars* show the SEM. Group A ($n = 7$), *diamonds/solid line*; group B ($n = 7$), (*closed dots*)/*dotted line*. *Statistically significant difference, at $P < 0.05$, between group A and group B. †Statistically significant difference between pre and each of the post values in group A. #Statistically significant difference between pre and each of the post values in group B. **b** DNA ladder fragmentation detected in peripheral blood mononuclear cells (PBMC). *Lane 1*, Before anesthesia; *lane 2*, after anesthesia; *lane 3*, 6 h after anesthesia start; *lane 4*, 12 h after anesthesia start; *lane 5*, 1 day after anesthesia start; *lane 6*, 3 days after anesthesia start. Fragmented DNA was analyzed by electrophoresis through 5% agarose gels and ethidum bromide staining. λ DNA digested with *Hin*dIII was used as a DNA size marker

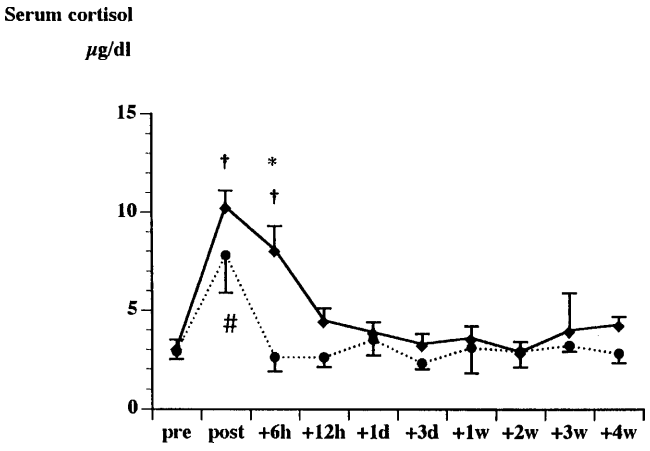


Fig. 5. Changes in serum cortisol level. *Vertical bars* show the SEM. Group A ($n = 8$), *diamonds/solid line*; group B ($n = 6$), (*closed dots*)/*dotted line*. *Statistically significant difference, at $P < 0.05$, between group A and group B. †Statistically significant difference between pre and each of the post values in group A. #Statistically significant difference between pre and each of the post values in group B

groups at recovery, and 6 and 12 h after recovery from anesthesia. In group A, the values rose from 10.4% before anesthesia, to 28.5% 12 h after anesthesia, and in group B, from 11.9% before anesthesia, to 22.7% 12 h after anesthesia, producing a significant difference between the two groups at 12 h and 1 day after recovery from anesthesia (Fig. 4a). DNA fragmentation was visible at recovery and at 6 and 12 h postanesthesia in both groups, and additionally at day 1 in group A (Fig. 4b).

Serum levels of cortisol demonstrated significant increases at recovery from anesthesia and 6 h after recovery from anesthesia in group A, and at recovery from anesthesia in group B ($P < 0.05$). There was a significant difference between the two groups at 6 h after recovery from anesthesia (Fig. 5).

Discussion

The present study in animals with laparotomy showed that: (1) peripheral blood lymphocyte counts, and in particular, the counts of T lymphocytes, markedly decreased; (2) the blastogenic function of lymphocytes was diminished; and (3) the progression of programmed cell death was promoted. The results seen in these dogs were the similar to those seen in humans [6,7,15]. Although barbiturates as anesthesia-inducing agents have been reported to decrease the mitogenic and antibody-producing ability of lymphocytes, their action at clinical dose levels is not strong [15,16]. There have been few studies of the effects of isoflurane on blast formation and antibody production by lymphocytes [8,13,14]. In contrast, halothane, a related agent, has been studied extensively, and a variety of observations have been made of decreased blast formation, suppression of antibody production, and suppression of the cytotoxic action of natural killer (NK) cells [2,7,9,15,16]. We postulate that isoflurane has effects similar to those of halothane. The prolonged decrease in peripheral blood lymphocyte counts and the greater decrease in the percentage of CD3+ cells in the group with laparotomy than in the group with anesthesia alone indicate that surgical procedures influence the number, constitution, and function of lymphocytes.

In both groups of dogs, the plasma levels of cortisol were markedly low before anesthesia and was elevated at recovery from anesthesia [15]. The high plasma cortisol levels were attributed to stress. The stress was not induced by the venipuncture procedure, but, rather, by the surgical procedures and anesthesia, as venipuncture sampling prior to the anesthesia and surgical procedures did not produce a marked elevation in cortisol levels. We conclude that these changes in dogs were caused by the surgical procedures, which induced severe stress, which causes changes in the nervous, endocrine, and local cellular systems. In particular, it is suggested that the secretion of cortisol from the adrenal cortex influences lymphocyte apoptosis. It is well known that the administration of exogenous corticosteroid preparations rapidly reduces numbers of peripheral blood lymphocytes [17]. The mechanism by which this reduction in lymphocyte counts occurs has been reported to be apoptosis.

In conclusion, in dogs, as in humans, the number of peripheral lymphocytes decreased and the proportion of apoptotic lymphocytes increased as immunity was reduced by surgical stresses such as anesthesia and the surgical procedure itself. The decrease in the number of peripheral lymphocytes and the increase in the induction of apoptosis in lymphocytes were highly correlated with the results for lymphocyte transformation test (i.e., the findings when cells were stimulated with PHA and C or A), suggesting that the quantification of apoptotic lymphocytes could be a useful index of the immunity estimated by the lymphocyte transformation test. These findings strongly indicated the usefulness of apoptotic lymphocyte detection as a quantitative index of immunity in dogs under surgical stress.

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