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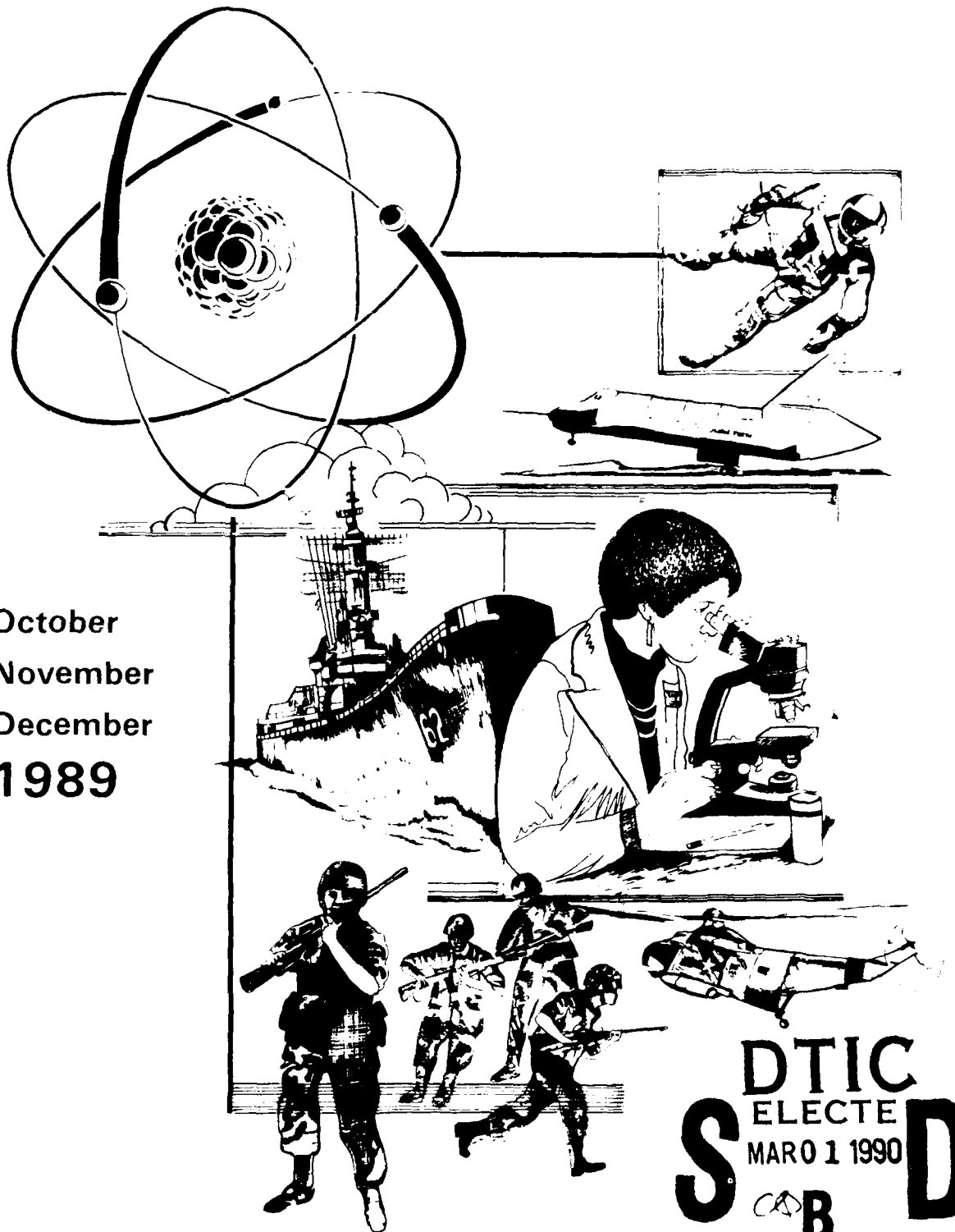
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Preparation and Care of the Area Postrema-Lesioned Cat

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Abstract *The area postrema (AP) is being widely studied to delineate its role in such varied functions as blood pressure regulation, conditioned taste aversion, water and energy balance, and radiation-induced emesis. This paper describes the preoperative preparation, surgical procedure, and postoperative care of cats kept long-term in which the AP was lesioned by electrocautery. A dorsal midline approach under gas anesthesia allowed access to selectively lesion the AP. Cats fully regained consciousness the same day and many became homeostatic within 24-48 h. Results of experiments using this model demonstrate the usefulness and effectiveness of the technique for model preparation.*

The area postrema (AP) is a small circumventricular organ located near the junction of the 4th ventricle and the spinal canal.^{1,2} It is recognized as an emetic chemoreceptor trigger zone,^{3,4} and has been studied for its role in widely varied functions including blood pressure regulation,⁵ conditioned taste aversion,^{6,7} water and energy balance,⁸ and radiation-induced emesis.⁹

Previous descriptions of the preparation of AP-lesioned animals have given only brief coverage of the preoperative preparation, surgical procedure, and post-surgical care.^{10,11} This technique was developed during the preparation of 31 cats for AP studies with three primary objectives in mind: to minimize the pain or stress experienced by the subject animal, to reduce the number of animals required for AP studies, and to increase the success rate of the preparation for more valid, consistent research subjects. An evolution of improvements in anesthesia, surgical technique, and postoperative care occurred during the course of this

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study. This report describes the preferred approach for successful development of the AP-lesioned cat.

Materials and Methods

Thirty-one purpose-bred cats^a (*Felis domesticus*) of either sex, weighing 2.0–5.5 kg, were quarantined for 2 weeks on arrival and screened for evidence of disease. All cats received a thorough physical examination, including hematology, parasitology, and serum chemistries. The cats were maintained in an AAALAC^b accredited facility in stainless steel individual cages with litter boxes and resting benches. The cats were provided commercial dry cat chow and tap water ad libitum. Animal holding rooms were maintained at $21 \pm 2^\circ\text{C}$, with $50 \pm 10\%$ relative humidity, and 10 air changes per hour of 100% conditioned fresh air. The cats were on a 12:12 light/dark full spectrum lighting cycle with no twilight.

An electrocautery unit^c with a variable current control was used with a fine wire-tipped, insulated hand probe to cauterize the AP. The unit had control settings from 1 to 10. When cauterizing the AP, the control was tested at each use, but was consistently set at slightly below 1.

A standard small-animal minor surgical pack¹² was supplemented with instruments shown in Figure 1. The retractor/elevator was fashioned from a strip of surgical steel 7 mm $\times$ 18 cm $\times$ 1 mm. The ends were ground to widths of 2 and 3 mm, their edges rounded, and the tips bent as shown.

Food was withheld after 4 p.m. the day preceding surgery, but water was provided through the night. Preoperative medications provided the morning of surgery included ampicillin (6.6 mg/kg im), dexamethasone (2.2 mg/kg im), and glycopyrrolate (0.011 mg/kg sc). Anesthesia was accomplished using halothane gas delivered via a face mask. The animals were then intubated and maintained on 0.75–1.5% halothane in 67% nitrous oxide and 33% oxygen.

Once anesthetized, the hair was clipped from the surgical site and the cat was positioned in a stereotaxic apparatus with towels placed under the sternum to relieve all the body weight from the ear bars (Fig 2). An esophageal stethoscope was placed to monitor heart rate and respiratory characteristics. An ophthalmic ointment^d was placed in each eye and the surgical site was prepared for aseptic surgery.

A midline incision was made from just rostral to the external occipital protuberance caudally to the level of the third cervical vertebra. The fascia joining the superficial cervical muscles was incised and the muscles were separated. A blunt-pronged Weitlander retractor was used to provide access to the deeper cervical muscles (Fig 3). The deep cervical muscles were incised on the midline over the occipital and atlas bones. Care was taken while incising the deep cervical muscles to avoid incising the dura mater overlying the cisterna magna. The periosteum was elevated from the occipital bone using a periosteal elevator. A cross-

^aLiberty Laboratories, New Jersey.

^bAmerican Association for the Accreditation of Laboratory Animal Care, Bethesda, MD.

^cGeiger Electrocautery, Model 200, Series 183, Geiger Inc, Philadelphia, PA.

^dBacitracin–Neomycin–Polymyxin Ophthalmic Ointment, Pharmaderm, Melville, NY.

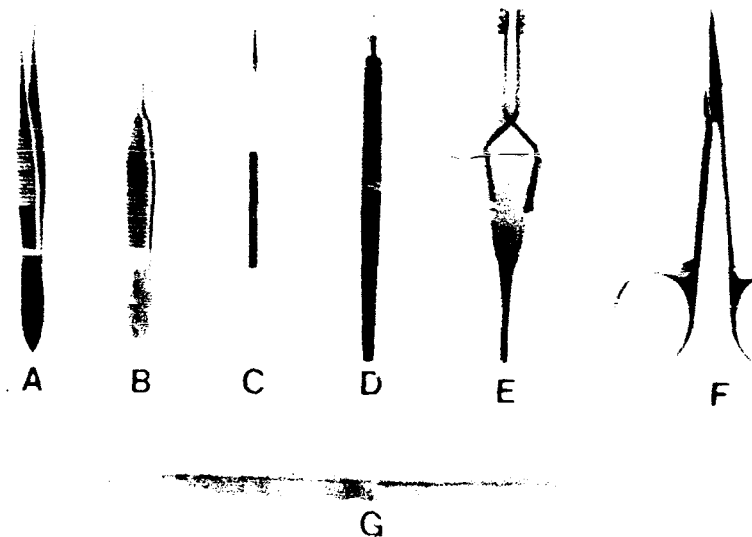


Figure 1. Special AP surgical instruments included A, iris forceps, 4-in., half curved; B, Nugent utility forceps; C, Roboz dura twist hook; D, Von Grafe cystotome; E, cross clamp retractors; F, Stevens tenotomy scissors, 4½-in.-long, straight, blunt points; G, combination retractor/elevator.

clamp 4½-in. blunt-pronged retractor (oriented 180° from the Weitlander retractor) was used to retract the deep cervical muscles.

The spine of a No. 15 scalpel blade was used to scrape the attachment of the dura mater from the occipital edge of the foramen magnum. This allowed the dura to retract from the edge of the occipital bone. The 2-mm end of the retractor/elevator was used to separate the dura from the inner plate of the occipital bone by using delicate semicircular movements. The dura was detached from the occipital bone a few millimeters at a time. A rongeur was used to remove the stripped portion of the occipital bone. At the tentorium process, the rongeur was placed at such an angle that only part of the thickness of the process was taken in each bite (Fig 4). Upon completion, the occipital bone defect was approximately 1 cm wide and extended from the foramen magnum to within 3–4 mm of the external occipital protuberance (Fig 5). Strict hemostasis was achieved using bone wax and the area was rinsed thoroughly with saline. The posterior cerebellum and dorsal aspect of the brainstem could be visualized clearly through the intact dura mater.

The dura was incised by using the Roboz dura twist hook to elevate the dura over the cisterna magna and making a stab incision with a No. 11 scalpel blade. The incision was then extended cranially and caudally with a pair of tenotomy scissors. While incising the dura over the vermis of the cerebellum, careful lifting of the dura with the lower jaw of the scissors separated the dura and vermis and prevented bruising or cutting of the vermis.

Once the dura was incised, a stereoscopic operating microscope (4–6×) was

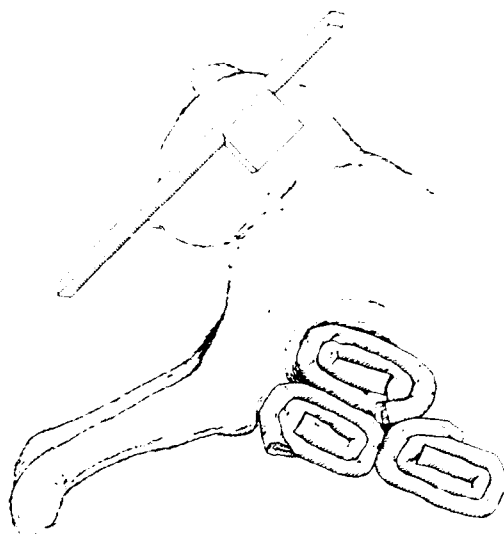


Figure 2. Proper stereotaxic positioning for AP surgery. It is important to maintain a very stable position of near maximal flexion of the atlantooccipital joint to provide adequate visualization of the area postrema. Access must be available to the anesthetist to monitor vital signs.

positioned over the patient. The 3-mm end of the retractor/elevator was used to gently elevate the vermis. The caudal medullary vellum then became apparent as a thin film. For the most part, it pulled apart as the vermis was elevated. Thicker strands were cut using a 4¼-in. Von Graefe cystotome with a 1-mm blade, taking care to avoid blood vessels. Elevation of the vermis exposed the tela choroidea which was incised using the cystotome. The cerebrospinal fluid (CSF) was sponged from the fourth ventricle using dental sponge spears or prewetted, squeezed, and shaped 4-mm-diameter surgical cotton pledgets.

After the CSF was removed, the AP was visualized as a gray/tan, slightly elevated, wing-shaped structure on the ventrocaudal wall of the fourth ventricle, extending bilaterally from the obex for 2–3 mm (Fig 6). Vascularization of the AP was much more prominent than in the surrounding tissue. Prior to cauterizing the AP, one or two prewetted and squeezed 4-mm-diameter cotton pledgets were placed between the fourth ventricle and the cerebellum to elevate the vermis and absorb CSF coming from the cerebral aqueduct.

Following a method demonstrated by Borison,⁴ the electrocautery was set such that the tip did not become red hot but just barely produced a slight pinpoint "tanning" when touched against muscle. The cautery tip was then carefully swept across the AP until all parts had become denatured to the desired degree. Generally, one controlled pass was satisfactory for lesion creation. Control subjects were prepared identically to the AP-lesioned animals except the electrocautery tip was not heated while touching the AP. To allow some intraoperative estimate of the AP damage effected by cautery, a grading system (0 to 5) was developed by the authors, as shown in Table 1. Although subjective, this provided a means of estimating the extent of the lesion intraoperatively. With the cautery tip set as described, one slow pass over the AP created a grade 1 or grade 2 lesion.

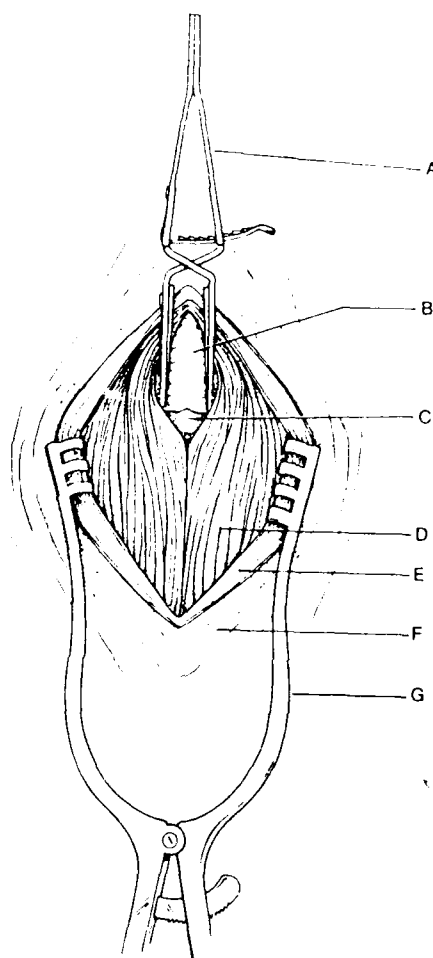


Figure 3. AP surgical field: superficial level. The handles of the Weitlander retractors are oriented ventrocaudally, while those of the cross clamp retractors are oriented dorsally. A, cross clamp retractors; B, occipital bone; C, dura mater overlying the cisterna magna; D, deep cervical muscles; E, superficial cervical muscles; F, skin; G, Weitlander retractors.

Three or more passes were required to create a grade 5 lesion. Histopathologic features of lesions created have been previously described by Rabin et al.^{6,7}

After cauterizing the AP, hemostasis was checked, cotton pledgets were removed, and the surgical area was flushed gently with saline. The incision through the dura was left open. The first layer of closure included only the deep cervical muscles, while the second layer included superficial cervical muscles. Both layers were closed with a simple continuous suture pattern using a synthetic absorbable suture with a swedged-on atraumatic needle. The skin was closed using an absorbable suture with a swedged-on reverse cutting needle in a continuous subcuticular pattern. The anesthetic gases were removed and the animal was left on 100% oxygen for 5 min before returning it to room air.

The cats were extubated as laryngeal reflexes returned. They were placed in

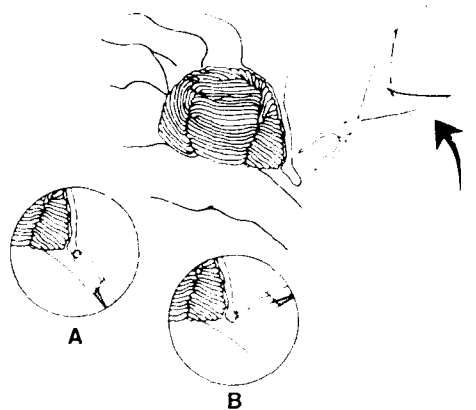


Figure 4. Rongeur angles during AP surgery. It is important that the angle of the rongeurs be rotated to bite through only part of the tentorium process of the occipital bone or bruising of the cerebellum may occur. (A) Rongeur angle at the foramen magnum; (B) rongeur angle at the tentorium process.

their cage when they could maintain sternal recumbency. During recovery an anti-inflammatory ointment^e was applied in each ear. Supplemental cage heat (40 °C) was provided via a recirculating hot water blanket and withdrawn when the cat's rectal temperature remained 38.6 ± 1 °C for 48 h.

Postoperative medications included ampicillin, dexamethasone, and when indicated, butorphanol tartrate. Ampicillin (6.6 mg/kg) was administered twice daily for 6 days. It was given im until the appetite returned, and was then given orally. Dexamethasone was administered once daily as an im injection at a rate of 2.2 mg/kg for 2 days postoperatively, followed by 1.1 mg/kg for 2 days. Butorphanol tartrate^f (0.055 mg/kg, sc) was provided at the first sign of discomfort.

The remaining postoperative care involved monitoring the rectal temperature, food, and water intake. Cats that were anorectic 48 h after surgery were given 20–50 mL of a 3:1 mixture of water and a high-caloric liquid food supplement^g by gastric gavage twice daily.

Results and Discussion

With increasing federal, social, and scientific pressures to refine experimental techniques and reduce the number of animals required in experiments, it was felt that an improved technique could be achieved.

In the authors' experience, the use of pentobarbital anesthesia for AP surgery requires mechanical ventilation during surgery, results in an extended recovery time, delays self-feeding, and retards adequate thermoregulation. Induction with an ultra-short-acting anesthetic, such as thiamylal sodium 2.5%, and maintenance on halothane gas will reduce recovery time, but may be accompanied by transitory apnea. The use of halothane for induction and maintenance eliminates the need for mechanical ventilation and significantly reduces recovery time.

^ePanalog Ointment, E.R. Squibb & Sons Inc, Princeton, NJ.

^fButorphanol Tartrate, Bristol Laboratories, Syracuse, NY.

^gSTAT-VME, Pharmacia Inc, Pensacola, FL.

The cats generally regained laryngeal reflexes and were extubated 5–7 min after skin closure, regained sternal recumbency within 20 min, and were able to walk within 30–50 min. The cats were generally alert and active in the morning after surgery and began self-grooming within 48 h. Four cats began eating the first day postoperative, although most did not begin spontaneous feeding until day 2, 3, or 4. Two cats were observed to initiate self-feeding on day 5. Almost all cats could thermoregulate normally within 24 h, and none required supplemental heat after 48 h. Except for anorexia, all cats were fully recovered within 3 days with no discernable effect on locomotion, respiration, heart rate, behavior, or other clinically observed parameters.

A total of 5 cats were lost during the entire study, all of which occurred early in the development phase of this technique. Two animals experienced intraoperative death and three other animals were euthanized following aspiration of vomitus related to gastric gavage.

Objective determinations of pain or discomfort are difficult in the cat. We monitored appetite, ataxia, scratching at the incision line, lethargy, eye squinting, and head shaking. Analgesics were not generally required. Most cats were alert and active by the morning following surgery. Analgesic relief was provided to those few exhibiting signs of discomfort. Inflammation of the ear canals due to the stereotaxic apparatus was minimal. Focal mild hyperemia near the external opening of the horizontal ear canal was observed in a few cases. An anti-inflam-

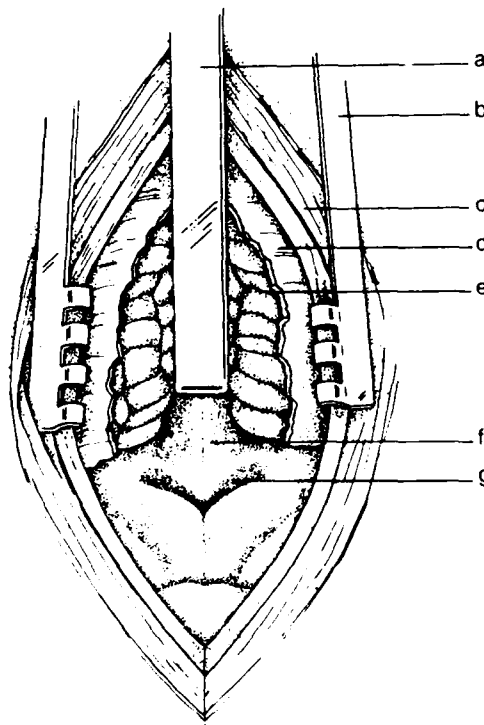


Figure 5. AP surgical field: deep level. The AP appears as a wing-shaped structure on the posterior portion of the 4th ventricle. a, retractor/elevator; b, cross clamp retractors; c, superficial cervical muscles; d, occipital bone; e, vermis of the cerebellum; f, floor of the 4th ventricle; g, AP.



Figure 6. Normal feline AP (arrows) extending anteriolaterally from the obex as a bilaterally symmetrical, wing-shaped structure. It is darker than surrounding tissue and more highly vascularized. The posterior limit is well demarcated and centered in the obex, while the anterior limits are less defined, blending with the floor of the 4th ventricle in a delta-shaped pattern.

matory ointment was administered and the hyperemia disappeared within 24–48 h. Inflammation around the surgical site was minimal. There were no cases of dehiscence.

Early in the study the cats were fed a gruel of canned cat food and water by gastric gavage. This frequently resulted in vomiting. We altered our nutrition regimen and began using a dilute solution of a high-caloric liquid feed supplement. Emetic episodes were nearly eliminated. For the few remaining animals exhibiting emesis, there may have been some effect of the procedure itself upon emesis immediately postoperatively. The benefit of these feedings, compared to the potential for complications due to inadvertent endotracheal instillation of feed mix-

Table 1
Intraoperative Grading System for Area Postrema Lesions

Grade	Appearance
0	No lesion—control
1	Slightly tan over most of AP
2	Tan to brown over all of AP
3	Brown with occasional black tags
4	Brown with frequent blackened areas
5	Majority of AP blackened by cautery

ture or aspiration must be considered. An alternative approach might include appetite stimulation using a low dose of diazepam intravenously.¹³

In our experience, this surgery is most easily performed in subadult males and females weighing approximately 4 kg. Larger, more fully developed males tended to have more extensive occipital diploe requiring longer surgical times to achieve hemostasis. Although total hemorrhage was seldom more than 3 mL from the entire procedure, time and total blood loss were both reduced if the opening was carefully but confidently enlarged to its full size before stopping to achieve hemostasis with bone wax. Cats smaller than 2.5 kg had corresponding smaller cranial vaults, which made it more difficult to gain adequate exposure of the AP.

When the preparation and care of AP-lesioned cats is performed as described above, the animals experience minimal to no apparent discomfort, but a consistent difference in conditioned taste-aversion learning and radiation-induced emesis is exhibited between AP-lesioned cats and those receiving the control surgery.^{6,7}

Acknowledgments

We gratefully acknowledge the assistance of Herbert L. Borison, who generously shared his knowledge of the procedures for making area postrema lesions in the cat with us. We also acknowledge Frances Langley, the artist of all figures in this paper, and the secretarial support provided by Roberta Bindman in the preparation of this manuscript.

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***In vitro* effects of leukotriene B₄ (LTB₄) on canine PMN effector function(s)**

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Abstract

The canine has become an accepted research model for the examination of a number of human clinical conditions. Despite its status as a research model, little is known regarding the peripheral effects of inflammatory mediator substances. Products of arachidonic acid metabolism (leukotrienes) are reported capable of altering leukocyte functions. Because of the emerging importance of the canine research model and leukotrienes we examined the effects of leukotriene B₄ (LTB₄) on several *in vitro* functions of isolated canine peripheral polymorphonuclear leukocytes (PMN). Changes in forward angle light scatter properties of the cells were used as one measure of PMN activation. Other functional changes examined following LTB₄ pretreatment included chemotactic capability, the electrophysiological state of the cell plasma membrane, and the metabolic oxidative response (i.e. H₂O₂ production). Random cellular movement of PMNs increased by 120% and 72% following preincubation with 10⁻⁷ and 10⁻⁹ M LTB₄, respectively. LTB₄ between 10⁻⁷ and 10⁻¹³ M did not significantly alter cellular resting membrane potential. Between 10⁻⁷ and 10⁻⁹ M LTB₄ elicited significant levels of cellular H₂O₂ production. Although significant, H₂O₂ production was < 40% that induced by phorbol myristate acetate (PMA). In numerous respects, canine *in vitro* PMN responses parallel previous reports of human cell function(s) in the presence of inflammatory mediators and may represent an attractive alternative for investigation of PMN dysfunctions.

Abbreviations: PMN, polymorphonuclear leukocyte; PMA, phorbol myristate acetate; FBS, fetal bovine serum; PBS, phosphate buffered saline; HPLC, high performance liquid chromatography; BRM, baseline random migration; HBSS, Hank's balanced salt solution

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Introduction

The canine, an attractive research model for a number of clinical investigations, has been used extensively in studies of developmental hematopoiesis, septic shock, and immune physiology. It was our intent to investigate functional responses of canine peripheral PMNs following preincubation with LTB₄, a well-known inflammatory modulator. PMNs represent the primary cellular elements of host defense and, as such, constitute a significant proportion of the host's circulating nonspecific im-

mune capabilities. The general process(es) by which PMNs respond to alterations of homeostasis, or episodes of infection, have been collectively termed inflammation. Conceptually, inflammation consists of an orderly series of functional events including increased PMN adhesiveness, diapedesis, chemotaxis, phagocytosis and production of an oxidative burst. Because PMNs are capable of multiple functions, they are subject to numerous serum-mediated and microenvironmental influences. The eicosanoids are metabolites of arachidonic acid produced through either cyclooxygenase or lipoxygenase metabolic pathways, and they represent one class of important inflammatory mediators, the leukotrienes. One of the more effective eicosanoid PMN modulators is 5(S),12(R)-dihydroxy-eicosatetraenoic acid, leukotriene B₄ (LTB₄) which reportedly modulates chemotaxis [1-5], adherence [6, 7], aggregation [1, 2, 8, 9], degranulation [10, 11], and oxidative metabolism [12-15]. Unfortunately, very little is known of LTB₄'s effects on canine PMNs.

Because of the physiological and inflammatory importances of LTB₄, we examined its *in vitro* abilities to activate and alter the *in vitro* functions of canine PMNs including chemotaxis, resting membrane potential and oxidative burst capability. The sensitivities of canine *in vitro* PMN functions to LTB₄ were compared to those induced by phorbol myristate acetate (PMA), a potent modulator of numerous PMN activities.

Materials and methods

Reagents

Reagents used were phorbol myristate acetate, dimethylsulfoxide (Sigma Chemical Company, St. Louis, MO), Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), HEPES buffer, and trypan blue (Grand Island Biological Company, Grand Island, NY), dichlorofluorescein diacetate (DCFH-DA, Eastman Kodak Company, Rochester, NY), ammonium chloride (Fisher Scientific, Silver Spring, MD), propidium iodide (Calbiochem, LaJolla, CA), and DiOC₅(3) (Molecular Probes, Junction City, OR). Stock solutions of DCFH-DA (5 mM) and DiOC₅(3) (1 mM) were stored in absolute ethanol. PMA was dissolved in dimethylsulfoxide at 0.01 M and stored at -70 °C.

Fetal bovine serum (FBS, Hyclone Labs, Logan, UT) was heat-inactivated (56 °C, 60 minutes) and filtered (0.45 µm) before use.

Animals

Eight 1-2 year old Hra beagles (*Canis familiaris*, 10-12 kg) were used in these experiments. Dogs were quarantined on arrival and screened for evidence of disease before being released to experiments. Dogs were kennelled in an AAALAC accredited facility and provided commercial dog chow and tap water *ad libitum*. Animal holding rooms were maintained at 20 °C ± 1 °C with 50% ± 10% relative humidity using at least 10 air changes hour of 100% conditioned fresh air. The dogs were maintained on a 12 hour lighting cycle with no twilight.

LTB₄

LTB₄ in a stock solution of methanol was the generous gift of Dr. J. Rokach (Merck-Frosst Laboratories, Pointe Claire-Dorval, Quebec, Canada). Aliquots were evaporated under nitrogen and resuspended in appropriate assay media. Purity of the LTB₄ stock was routinely monitored (2) by reverse-phase high-performance liquid chromatography (HPLC). A Beckman HPLC system with an Ultrasphere C-18 column, 4.6 × 250 mm, packed with 5 µm particles was used. The solvent system was methanol:water:acetic acid (70:30:0.01, v:v:v) adjusted to a pH of 5.7 with ammonium hydroxide, and eluted at a flow rate of 1 ml/minute. LTB₄ solutions, based on UV (280 nm wavelength) detection methods, were free of contaminants and decomposition products.

PMN isolation

Peripheral blood was drawn from the lateral saphenous vein of canines into syringes containing preservative-free heparin (10 U/ml). Blood was washed in HBSS without Ca⁺⁺ and Mg⁺⁺ (400 × g, 10 minutes, room temperature). Contaminating red blood cells (RBC) were lysed with 0.83% NH₄Cl (10 minutes, 4 °C), and the leukocytes were pelleted. The leukocyte pellet (>85% PMNs) was minimally resuspended in HBSS without Ca⁺⁺ and Mg⁺⁺ and divided into aliquots for chemotaxis and flow cytometric analysis. Wright-

stained blood smears were prepared for differential and morphological examination(s). Complete blood counts were obtained by automated analysis (Model S + 2, Coulter Electronics, Hialeah, FL).

Chemotaxis assay

Chemotactic capabilities of PMNs were evaluated by quantitating the cells that migrated through a 10 μ m-thick polycarbonate membrane. A 48-well micro-chemotaxis chamber assembly (Neuro Probe Inc., Bethesda, MD) was used as described by Harvath et al. [16]. Lower wells received media, chemoattractant (1:100 dilution of zymosan activated plasma) or LTB₄ between 10⁻⁷ and 10⁻⁸ M. Upper wells received 50 μ l of a cell suspension at 2 $\times$ 10⁶ cells/ml. Chambers were incubated at 37 °C in a humidified environment and gassed with 5% CO₂ and air for one hour. Filters were fixed in 100% methanol and stained with Dif-Quick (Fischer Scientific, Silver Spring, MD). Seven high-power (100 $\times$) microscopic fields, across the diameter of the well, were examined and the mean cellular migration field hour determined.

Flowcytometric analysis of PMN light scatter properties

Light scatter properties of the PMN were analyzed on a Coulter EPICS 541 flow cytometer (Hialeah, FL) equipped with a 2 W argon ion laser emitting 200 mW at 488 nm. Cells isolated as described above were resuspended in PBS supplemented with 0.2% FBS. Cells were equilibrated at 37 °C for 15 minutes and analyzed by flow cytometry prior to and following incubation with 10⁻⁷ M LTB₄ for 5 minutes at 37 °C. PMNs were distinguished from other mononuclear cell types, RBC's, and cellular debris based upon their characteristic forward-angle (low angle light scatter) and right-angle light scatter properties.

Membrane potential

Alterations in membrane potential were determined by measuring changes in the intracellular concentrations of DiOC₂(3), a lipophilic dye that diffuses through cell membranes. Cellular fluorescence intensity varies as a function of membrane potential, decreasing as the membrane depolarizes.

PMNs (10⁶ cells/ml) resuspended in glucose-supplemented (1 mg/ml) HBSS, were incubated with 10⁻⁸ M DiOC₂(3) for 10 minutes at 37 °C. The resultant cellular fluorescent spectra were stable for 30 minutes if cells were maintained at 4 °C. Cells were then stimulated with PMA (100 ng/ml) to demonstrate maximum membrane depolarization, or with varying concentrations of LTB₄ for 7 minutes at 37 °C. Changes in resting membrane potential ($t = 0$ minutes) were analyzed flow-cytometrically on a FACS Analyzer interfaced to a Consort 30 computer system (Becton Dickinson, Sunnyvale, CA). Green fluorescence was monitored between 515 and 545 nm after excitation by a mercury arc lamp equipped with a 485/22 nm excitation filter. PMNs were distinguished from other cellular types based on coulter volume and right angle light scatter properties. Cellular viability was assessed by vital dye exclusion of trypan blue or propidium iodide.

Intracellular H₂O₂ production

H₂O₂ production was measured as described previously by Bass et al. [17]. DCFH-DA, a nonpolar, nonfluorescent compound, diffused through cell membranes and was hydrolyzed by cellular esterases to nonfluorescent, intracellularly trapped, 2',7'-dichlorofluorescein (DCFH). The H₂O₂ produced by activated PMNs oxidizes DCFH to the fluorescent analogue 2',7'-dichlorofluorescein (DCF). PMNs (10⁶ cells/ml) were incubated with 5 μ M DCFH-DA in PBS supplemented with 0.2% FBS for 10 minutes at 37 °C and stimulated with PMA (100 ng/ml) or varying concentrations of LTB₄ (37 °C, 15 minutes). DCF levels were measured by flow cytometry of a FACS analyzer as described for membrane potential. Percent change in intracellular H₂O₂ production was determined by the following formula:

$$100 \times \frac{\text{Mean Fluorescence Intensity (FL)}_{\text{control}} - \text{Mean FL, experimental}}{\text{Mean FL, control}}$$

Statistical analysis

All data are presented as the mean $\pm$ standard error. Statistical differences were determined by

analysis of variance with Duncan's test between means for repeated measures. P values < 0.05 were considered statistically significant.

Results

PMN Light Scatter Properties: Figure 1, Table 1

By monitoring light scatter properties (i.e., forward and right-angle light scatter), changes in PMN morphology can be quantitatively assessed. Forward angle light scatter was examined before and after addition of LTB_4 . Figure 1 depicts a histogram of the normal light scatter distribution pattern representing the loss of forward-angle light scatter following incubation of PMNs with 10^{-9} M LTB_4 . Mean channel loss for control PMNs, incubated without LTB_4 , was 6.50 ± 2.13 . Table 1 demonstrates the mean loss of forward-angle light scatter following incubation with 10^{-11} to 10^{-5} M LTB_4 . The mean loss of forward-angle light scatter, a dose dependent response, was maximum at 10^{-9} M (35.88 ± 6.05) loss of mean channel numbers. No changes in the right angle light scatter properties were observed following LTB_4 incubation (data not shown).

Table 1
Effect of LTB_4 on forward angle light scatter profiles of canine PMNs.

Concentration (M)	Loss of mean channel number ($\pm$ SEM)
Control	6.50 ± 2.13
1×10^{-11}	30.69 ± 4.11
1×10^{-9}	35.88 ± 6.05
1×10^{-10}	24.34 ± 2.47
1×10^{-11}	11.33 ± 0.52

Table 2

Generation of intracellular H_2O_2 in canine PMNs following stimulation with either PMA or LTB_4 .

Stimulus	Concentration	Mean FI intensity		Increase H_2O_2 production ($\times 100$)
		0 min	15 min	
PMA	100 ng/ml	3.47 ± 0.22	54.87 ± 3.04	13.81 ± 0.80
LTB_4	10^{-11} M	4.68 ± 0.18	26.43 ± 1.22	$4.76 \pm 0.26^*$
	10^{-9} M	4.45 ± 0.26	23.01 ± 3.87	$4.49 \pm 0.52^*$
	10^{-11} M	4.64 ± 0.19	10.41 ± 1.22	$1.22 \pm 0.10^*$
	10^{-12} M	4.57 ± 0.19	10.25 ± 1.02	$1.29 \pm 0.10^*$

* $p < 0.01$ compared with stimulation with 100 ng/ml PMA

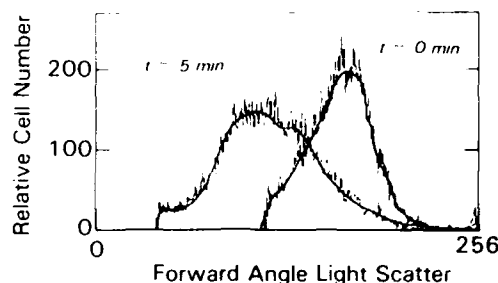


Figure 1
Representative histograms of forward angle light scatter of PMNs prior to ($t = 0$), and following incubation with 10^{-9} M LTB_4 ($t = 5$) at 37°C . The histograms represent cell number (ordinate) as a function of forward angle light scatter (abscissa) on a linear 256 channel scale.

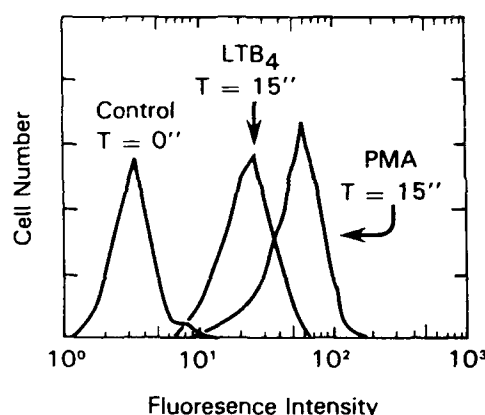


Figure 2
Histogram representing the fluorescent distribution patterns of resting, PMA (100 ng/ml), and LTB_4 (10^{-9} M) stimulated PMNs. Cells preincubated with DCFH-DA for 10 minutes were stimulated with either PMA or LTB_4 for 15 minutes at 37°C . The histograms represent cell number (ordinate) as a function of fluorescence intensity (abscissa) from 10,000 events.

H₂O₂ measurement: Figure 2, Table 2

Effects of LTB₄ on cellular H₂O₂ production are shown in Table 2. H₂O₂ production, maximal between 10⁻⁹ and 10⁻⁸ M LTB₄, was <40% of the maximal PMA response. Figure 2 shows the fluorescence histograms of unstimulated (*t*=0 minutes) PMNs and those incubated with either LTB₄ (10⁻⁹ M) or PMA (100 ng/ml). This comparison with PMA was established since maximum H₂O₂ production, without loss of cell viability, was achieved with 100 ng/ml PMA. Vital dye examination of cells after PMA or LTB₄ treatment demonstrated viabilities >95%. At, or below, 10⁻¹⁰ M LTB₄, H₂O₂ production was negligible. A uniformly responding single population was apparent with increasing levels of LTB₄ stimulation and no unresponsive population(s) were discerned based on gating parameters used.

Chemotaxis: Table 3

Chemotaxis to LTB₄ was examined between 10⁻⁷ and 10⁻¹⁷ M. The highest concentrations, 10⁻⁷ M and 10⁻⁹ M, induced cellular migrations which were 120% and 72% (*p*<0.05), respectively, over baseline random migration values (Table 3). Chemotaxis to concentrations between 10⁻¹¹ M and 10⁻¹⁷ M resulted in a 20%-36% increase over baseline random migration.

Membrane potential changes: Table 4

Levels of LTB₄ between 10⁻⁷ and 10⁻¹³ M did not significantly alter the PMN resting membrane potential(s). Loss of cellular fluorescence intensity (indicating membrane depolarization) following *in vitro* LTB₄ incubation, was generally <20% of the PMA response (Table 4).

Table 3

Comparison of the effects of LTB₄ on canine PMN chemotaxis with zymosan activated plasma (ZAP).

Stimulus	Concentration	Cell HPF (± SE) **
Media		40.0 ± 1.3
ZAP	1 × 10 ⁻²	232.4 ± 6.7
LTB ₄	1 × 10 ⁻⁷	88.4 ± 1.5 *
LTB ₄	1 × 10 ⁻⁹	67.2 ± 1.7 *
LTB ₄	1 × 10 ⁻¹¹	50.8 ± 2.0
LTB ₄	1 × 10 ⁻¹³	54.8 ± 2.3
LTB ₄	1 × 10 ⁻¹⁵	48.0 ± 0.9
LTB ₄	1 × 10 ⁻¹⁷	51.2 ± 1.2

* *p* < 0.05 when compared to control (media).

** Average number of cells which migrated per high power field (HPF).

Discussion

Inflammatory processes are complicated by the mediation of numerous endogenous factors. PMNs have been identified as both a source and a target of inflammatory factors [18]. Inflammatory responses can generally be divided into two distinct phases; the first phase involving PMN efflux, and the second, direct effector cell interaction at the inflammation site. The first phase requires directed movement of PMNs and is operationally and functionally necessary for successful resolution of inflammation. Chemotaxins, which provide the gradients necessary for directed migration, are likely

Table 4

Alterations in the resting membrane potential of canine PMNs following stimulation with either PMA or LTB₄.

Stimulus	Concentration	Mean FL intensity (± SE)		Loss of FL intensity ** (%)
		0 min	7 min	
PMA	100 ng/ml	38.76 ± 1.54	25.85 ± 1.51	33.3 ± 1.54
LTB ₄	10 ⁻⁷ M	39.05 ± 1.59	36.73 ± 1.11	5.7 ± 1.20 *
	10 ⁻⁹ M	37.93 ± 1.67	35.27 ± 1.40	7.0 ± 0.58 *
	10 ⁻¹¹ M	36.39 ± 1.99	35.15 ± 1.94	3.7 ± 0.33 *
	10 ⁻¹² M	39.52 ± 0.60	37.33 ± 0.46	5.3 ± 0.33 *

* *p* < 0.01 when compared with PMNs stimulated with 100 ng/ml PMA.

** Loss of FL intensity indicates membrane depolarization.

to activate circulating PMNs at low concentrations. One important class of inflammatory mediator substances are the leukotrienes [19], produced by stimulated PMNs and monocytes [20]. Leukotriene B₄ (LTB₄), an important inflammatory leukotriene, modulates PMN calcium metabolism, chemotaxis, adherence and degranulation functions [21-24]. LTB₄ induced alteration in PMN accumulation have been described in rabbit [25, 26], guinea pig [27], human [28] and rat [1]. Inflammatory exudates of LTB₄ following the subcutaneous implantation of carrageenan-soaked polyester sponges have been correlated with PMN infiltration rates in rats. LTB₄ in the inflammatory exudate was determined to alter aggregation, chemotaxis, and degranulation functions *in vitro* [1]. PMA has been widely used as a reference standard for the investigation of numerous PMN cellular functions. In comparison to the induction of superoxide anion by PMA, LTB₄ has been described as a weak metabolic stimulant [14]. Previous reports of human PMN degranulation have demonstrated LTB₄ to be 35%-50% as effective as the receptor mediated response to fMLP [11]. Canines appear unresponsive to fMLP, reportedly due to a lack of fMLP receptors [29], so its use in the investigation of LTB₄ priming or other receptor dependent mechanisms remains to be determined. Our investigations, based on the intracellular production of H₂O₂, concur with LTB₄'s characterization as a weak metabolic stimulus. At high concentrations LTB₄ induces <40% of the H₂O₂ induced by PMA stimulation. At concentrations below 10⁻⁸ M, LTB₄ does not stimulate superoxide anion production in human PMNs [15]. Similarly in the canine PMN, there was no oxidative burst (i.e. H₂O₂) produced at concentrations of LTB₄ < 10⁻⁶ M.

LTB₄ may perform as a primary agonist or as a cellular priming agent. LTB₄'s role as a cellular primer is undoubtedly complex and would require examination in the presence of other inflammatory cytokines. LTB₄ has been reported to enhance the human PMN's oxidative burst to fMLP but not to PMA or zymosan [13]. Conversely, exposure to LTB₄ resulted in significant decreases in PMN aggregation and degranulation [8]. Human [30, 31] and rabbit PMNs [32] bind LTB₄. We have preliminary data indicating that canine PMNs may also specifically bind LTB₄ (data not shown). Further characterization of this binding site, to meet

the criteria for receptor designation, including Scatchard analyses to quantitate high affinity binding sites are in progress.

Inflammatory response patterns may be concentration-dependant. Low agonist concentrations may stimulate selective cellular function(s) while higher concentration levels desensitize low level responses and order higher level dependent responses. LTB₄ has been reported to be a nanomolar inflammatory chemotaxin [33, 34] in rats [1, 2], rabbits [32, 35], cats [5], humans [2, 4, 6, 10] and, as reported here for the first time, dogs. LTB₄ is a dose-dependent modulator of human PMN aggregation [2] and, between 10⁻¹¹ and 10⁻⁸ M, LTB₄'s effects are comparable to C5a or fMLP induced chemotaxis in rat, guinea-pig and human subjects [3]. Threshold concentrations for LTB₄ induced human PMN chemotaxis are in the range of 10⁻⁹ M [32, 33, 35]. The threshold concentration for canines approached significance ($p=0.07$) at 10⁻⁹ M and at 10⁻⁷ M, LTB₄ induced maximum canine chemotaxis ($p<0.05$) in accord with previously published human maxima (10⁻⁶ to 10⁻⁸ M) [6, 24, 33, 34]. These findings confirm, and extend, the ability of lipoxygenase products to mediate PMN functions and also demonstrates the considerable similarities of human and canine PMNs to the effects of LTB₄.

LTB₄, in comparison to PMA, has been reported to be ineffective in depolarizing human PMNs [36]. In the canine model, LTB₄ was likewise ineffective. PMA's ability, and LTB₄'s inability, to induce PMN membrane depolarization, are interesting since the calcium iontophoretic ability of LTB₄ for PMNs has been well documented [37]. LTB₄'s ability to induce an oxidative burst (increased production of H₂O₂) in the apparent absence of membrane depolarization might infer that depolarization may not be a necessary antecedent to oxidative burst induction. LTB₄ induced activation signals in human PMNs are reported to be very short-lived [14] and our inability to detect depolarization(s) may be due to technical limitations. Metabolic (i.e. H₂O₂) and chemotactic changes were greatest between 10⁻⁹ M and 10⁻⁷ M LTB₄, with little significant effects below these doses. Although membrane perturbations should conceptually occur at lower concentrations, none were detected within this report. Leukotrienes are naturally produced and may exist in locally high concentrations. Concentrations used in this protocol may

mimic the high localized concentrations produced *in vivo* during an inflammatory event.

As reported here, LTB₄ induced morphological changes modulated the oxidative burst capability of PMNs in a concentration dependent fashion without altering the resting membrane potential of the cell. Due to the numerous levels at which LTB₄ may exert regulatory effects, its mechanism(s) of action, in regard to PMN dysfunction(s) and host susceptibility, deserve further, carefully controlled, investigation and analysis. Further, it appears from these results that, for the investigation of LTB₄'s effects on PMN dysfunction(s), the canine PMN represents a suitable alternative to human cells.

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Interleukin-1 Enhances Survival of Lethally Irradiated Mice Treated With Allogeneic Bone Marrow Cells

By Joost J. Oppenheim, Ruth Neta, Pierre Tiberghien, Ronald Gress, James J. Kenny, and Dan L. Longo

Interleukin-1 (IL-1) enhanced the capacity of allogeneic bone marrow (BM) cells to promote survival of mice given doses of radiation (1,200 to 1,350 cGy) that are significantly higher than those generally used for BM ablation (850 to 950 cGy). Three to five times greater numbers of lethally irradiated (1,200 to 1,350 cGy) C57B1/6 (H-2b) mice given 10^7 T-cell-depleted Balb/c (H-2d) BM cells survived over 6 weeks if also treated with a single intraperitoneal (IP) dose of 10 μ g IL-1 20 hours before or from 1 to 3 hours after radiation. The spleens of these mice were reconstituted predominantly, but not exclusively, with donor cells (54% to 91%). Histologic examination of the epidermal and gastrointestinal tissues of mice surviving more than 6 weeks did not reveal any evidence of graft-versus-host (GVH) disease; however, since 10% to 43% of the mice died between days 30 and 46, the possibility of a

GVH syndrome in these mice cannot be excluded. The spleen cells from irradiated mice given BM transplants and IL-1, which consisted of $\geq 85\%$ donor cells, were able to generate specific T-cell cytotoxic killing of unrelated allogeneic donor cells but were unreactive to target cells bearing either host or donor major histocompatibility complex (MHC) class I antigens. Thus, long-term mixed chimeric survivors were tolerant to recipient and donor alloantigens but exhibited immunologic competence. These results show that IL-1 promotes survival of lethally irradiated mice and that allogeneic hematopoietic cells in such animals develop tolerance to host MHC antigens. Although there are many unanswered questions, these data suggest that IL-1 may prove clinically useful in promoting BM engraftment.

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IL-1 PROTECTS MICE from lethal doses of radiation over a dose range that usually results in a fatal hematopoietic syndrome.^{1,2} IL-1 protects mice more effectively when administered 24 hours before than after lethal doses (LD 100/30) of irradiation. When administered following middle-dose radiation (LD 95/30), IL-1 has also promoted the survival of up to 100% of mice.³ Indeed, we and others have demonstrated accelerated recovery of colony-forming unit erythroid (E-CFU)² and of granulocyte-macrophage colony-forming unit (GM-CFU), burst-forming unit-erythroid (BFU-E), and colony-forming unit-megakaryocyte (CFU-Meg)^{4,6} in sublethally irradiated, IL-1-treated mice. This suggests that IL-1 may promote and accelerate the recovery of residual hematopoietic stem cells and progenitor cells that survive sublethal radiation. The concept that IL-1 is an important stimulant of hematopoiesis has also been reinforced by data showing that IL-1 is identical to hematopoietin-1,^{7,9} a growth factor for hematopoietic progenitor cells with high proliferative potential. In addition, it is possible that IL-1 may also indirectly promote hematopoiesis by inducing expression of receptors for colony-stimulating factors (CSF)⁸ as well as the production of CSF.¹⁰

Transplantation of autologous, isologous, or allogeneic BM cells also protects animals from lethal doses of irradiation in a dose range below that inducing the gastrointestinal syndrome.¹¹ The capacity of IL-1 to enhance hematopoiesis suggested that IL-1 may also promote hematopoietic recovery following bone marrow transplants. Consequently, experiments were designed to evaluate whether (1) transplantation of allogeneic BM cells combined with IL-1 treatment results in enhanced survival of lethally irradiated mice, (2) lethally irradiated mice given IL-1 require fewer allogeneic BM cells to survive, (3) administration of IL-1 along with T-cell depleted allogeneic bone marrow cells leads to the development of tolerance, and (4) IL-1 preadministration is more effective than IL-1 therapy following irradiation and the relative capacity of either of these treatments to restore host cells or enhance donor-cell recovery.

MATERIALS AND METHODS

Mice. Inbred female C3H/HeN, C57B1/6, and Balb/c mice ranging from 8 to 12 weeks of age were obtained from the Animal Production Area at Frederick Cancer Research Facility, Frederick, MD. Mice were maintained in a pathogen-free environment and fed commercial rodent chow and acidified water (with HCl to a pH of 2.5) ad libitum. All cage cleaning, handling, injections, and care of mice for 2 weeks following irradiation was performed in a laminar-flow clean-air unit.

Irradiation. Mice were placed in a plexiglass container and given single doses of whole-body irradiation at 3 Gy/min using a 137 Cesium source (model 68A MARK irradiator, J.L. Shepherd, Glenoble, CA). The number of surviving mice was recorded daily for up to 150 days. Previous studies established 900 cGy to be a lethal dose for C57B16 mice within 8 to 16 days, which is the usual time course for the fatal hematopoietic syndrome.

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Cytokines. Human recombinant IL-1 β and IL-1 α were generously provided by Mr Iriye of the Otsuka Corporation, Rockville, MD, and by Dr Peter Lomedico of Hoffmann-LaRoche, Nutley, NJ, respectively. The preparations were used on a weight basis and had a specific activity of 2×10^7 U/mg by the thymocyte mitogenic assay¹² and 2.5×10^9 units/mg (Lot 1/87) by D10 assay,¹³ respectively. All reagents were diluted to the desired concentration with endotoxin-free phosphate-buffered saline (PBS) just before intraperitoneal injection in a volume of 0.5 mL. The Hoffmann-LaRoche IL-1 α preparation contained 0.125 endotoxin U/mL by Limulus amoebocyte lysis (LAL) assay. Since IL-1 α and IL-1 β have equivalent radioprotective activity, both were used for this study.¹⁻⁶

Bone marrow cells. BMC were obtained by flushing them from the femurs of mice under sterile conditions with RPMI 1640 medium. BM cells were dispersed through a 25-gauge needle washed once with RPMI 1640 at 4°C, gently centrifuged at 200 g for 6 minutes to remove aggregates and debris, counted in a hemocytometer, and the indicated cell number administered intravenously into the caudal veins of recipient mice in a volume of 0.2 mL. The allogeneic BM cells were T-cell depleted by incubating them with a 1:32 dilution of rabbit polyclonal anti-mouse brain antiserum for 30 minutes at 4°C, washing once, then incubating the BM cells once or twice again with a 1:3 dilution of fresh guinea pig complement for 30 minutes at 37°C. The cells were recounted and injected.

Experimental protocol. From 8 to 12 C57B16 mice per group were treated with 0.5 mL PBS or with 10 μ g IL-1 α or β in 0.5 mL PBS intraperitoneally either 20 hours before or 1 to 3 hours after lethal irradiation. Mice were given either no or from 2.5 to 10×10^6 Balb/c T-cell-depleted BM cells intravenously in a volume of 0.2 mL 1640 RPMI medium in their tail veins 1 to 3 hours following irradiation. The survival of mice was monitored on a daily basis for up to 6 months.

In vitro generation and assay of cytotoxic T-cell activity. Spleen cells of long-term surviving C57B16/mice (>2 months) reconstituted with Balb/c BM cells and IL-1 treatment, were assayed for their ability to kill H-2^b, H-2^d, or unrelated H-2^k target cells in vitro as previously described.¹⁴ Spleen cells at 4×10^6 /mL were cultured with 1×10^6 irradiated stimulator cells in RPMI 1640 with 10% fetal calf serum (FCS), penicillin streptomycin, nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, and L-glutamine in humidified air with 10% CO₂ at 37°C. The surviving cells were tested 5 days later for their lytic activity in a 4-hour ⁵¹Cr release assay on 3-day concanavalin-A-stimulated spleen-cell lymphoblasts. The results are expressed as percent specific lysis at various ratios of effector to target (E:T) cells as follows:

% specific lysis = 100

$$\times \frac{(\text{Experimental release} - \text{spontaneous release})}{(\text{Maximum release} - \text{spontaneous release})}$$

Cell phenotyping. The percent Thy 1 positive BM cells was determined before and after T-cell depletion using anti-Thy 1.2 monoclonal antibodies as assessed by flow cytometry (FACS analysis). In addition, the H-2 phenotype of single-cell suspensions of spleen cells reconstituting some of the long-term (over 6 weeks) surviving mice was determined using anti-H-2^d (34-2-12, IgG2a) or anti-H-2^b (5F1 IgG2b, produced by Linda Sherman, La Jolla, CA) monoclonal antibodies (kindly provided by Dr David Sachs, National Cancer Institute, Bethesda, MD), followed by FITC-labeled goat anti-mouse IgG2a or IgG2b, respectively, and FACS analysis. Mice were analyzed individually, and three mice were analyzed in each group in repeated experiments.

Statistical analysis. A chi-square analysis with Yates's correction for continuity of number of surviving mice was performed. In addition, a computer program was used to analyze and compare the

survival curves of the various treatment combinations. The methods used were those of Kaplan-Meier, Cox, and Kruskal-Wallis.¹⁵ Comparisons of donor H-2^d cells in spleen resulting from the various treatments were made with the nonparametric Wilcoxon rank-sum test.¹⁶

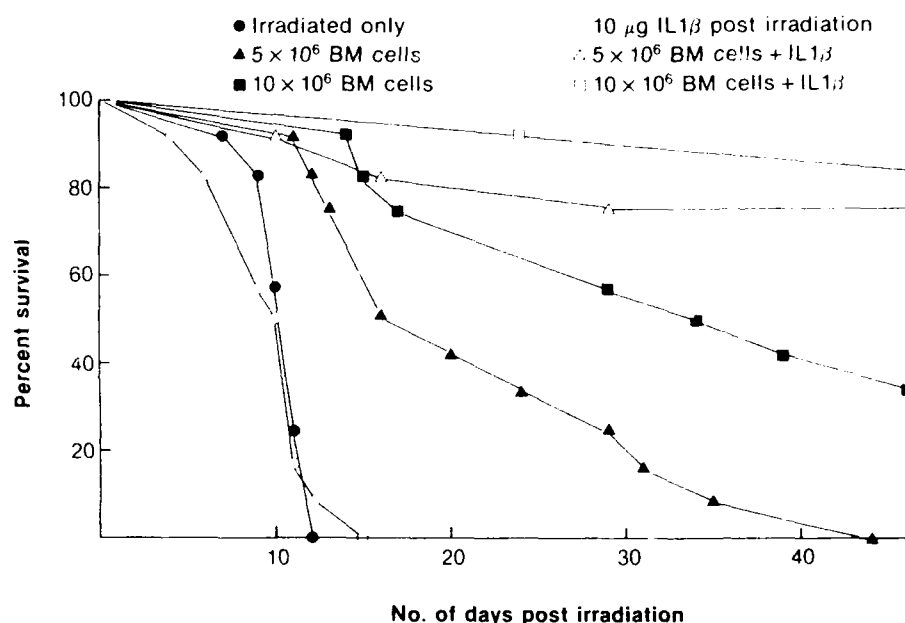
RESULTS

Effects of allogeneic BM transplantation. Since allogeneic H1-A matched BM cells are frequently not available in outbred human populations, the effect of IL-1 on survival of lethally irradiated mice reconstituted with partially T-cell-depleted allogeneic H-2 nonidentical BM cells was evaluated. Balb/c BM cells were treated once with anti-brain-cell antiserum, followed by fresh guinea pig complement, to diminish donor T cells that could generate GVH reactions. This reduced the proportion of Thy 1+ BM cells as determined by FACS analysis from 3% to 1%. The ability of IL-1 to promote survival of lethally irradiated C57B1/6 mice (H-2^b) given suboptimal numbers (less than 15×10^6) of such T-cell-depleted Balb/c (H-2^d) BM cells was tested.

Lethally irradiated C57B1/6 mice were given from 2.5 to 10×10^6 T-cell-depleted Balb/c BM cells and a single dose of 10 μ g IL-1 β 20 hours before or 1 to 3 hours after radiation. This dose of IL-1 was chosen because it was more radioprotective than other doses and schedules.³ A single high dose of 1,200 cGy was given in an attempt to suppress recovery of the host cells and favor the establishment of the allogeneic BM graft. We have previously shown that 900 to 1,000 cGy, a radiation dose usually considered to be an LD 100/30, becomes an LD 0 if the mice are pretreated with 10 μ g IL-1 20 hours before radiation. The hematopoietic systems of IL-1-treated mice recover from the damaging effects of radiation.³ Following 1,200 cGy doses of irradiation, all C57B1/6 mice that were not treated or were only given a 10 μ g IP dose of IL-1 β after irradiation died with a median survival time (MST) of 11 days (Fig 1). Although treatment of C57B1/6 mice with 2.5×10^6 T-cell-depleted Balb/c BM cells with or without subsequent IL-1 β prolonged the survival of a few mice (MST of 12 days), none survived for more than 1 month (data not shown). All irradiated mice given 5×10^6 BM cells without IL-1 β died by 45 days (MST of 15 days). In contrast, 9/12 mice treated with both 5×10^6 Balb/c BM cells and IL-1 β by 1 to 3 hours after irradiation survived beyond 45 days, after which mice stabilized and survived long-term (over 20 weeks). Only 5/12 C57B1/6 mice treated with 10^7 Balb/c BM cells without IL-1 survived beyond 46 days. In contrast, 11/12 mice given IL-1 along with 10^7 Balb/c BM cells after irradiation survived for over 45 days. Consequently, therapy with IL-1 β along with 5 or 10×10^6 allogeneic BM cells after irradiation markedly prolonged long-term survival (over 20 weeks) of lethally irradiated mice ($P < .001$). The IL-1 β treatment after irradiation markedly improved the effect of the transferred BM cells.

Mice given IL-1 β 20 hours before irradiation, even in the absence of BM cells, were radioprotected (more than 87%) (Table 1). Thus, IL-1 is much more effective in promoting survival when given by itself before rather than after a dose of 1,200 cGy irradiation ($P < .001$). The survival of mice pretreated with IL-1 that were given 2.5×10^6 BM cells

Fig 1. Survival of lethally irradiated C57B1/6 mice treated with IL-1 β and Balb/c (H-2^d) BM cells. The conditions in this experiment were as described for Fig 1, with the following modifications. C57B1/6 mice (12 per group) were irradiated with 1,200 rad, given a single dose of 10 μ g human recombinant IL-1 β and either 2.5×10^6 (data not shown), 5×10^6 , or 10×10^6 Balb/c BM cells within 1 to 3 hours following irradiation. Significantly more mice given 5×10^6 BM cells and IL-1 survived than mice given only BM cells ($P < .01$), and more mice given 10^7 BM cells and IL-1 survived than if given only 10^7 BM cells ($P < .05$).



actually decreased from 87% to 40% ($P < .001$). In contrast, none of the mice survived when given 2.5×10^6 BM cells and IL-1 after irradiation. Mice given 5×10^6 BM cells survived significantly better if given IL-1 before than after irradiation ($P < .05$). Only mice given 10^7 BM cells benefited equally from IL-1 given either before or after irradiation.

Phenotypic analysis of the spleen cells from mice determined from 1.5 to 3 months after treatment with 10×10^6 Balb/c BM cells and IL-1 after irradiation revealed that 8/8 mice were reconstituted predominantly (74% to 92%) with donor (H-2^d) cells but still had from 4% to 23% surviving host (H-2^b) cells (Table 1). All of the mice were mixed chimeras. Contrary to our expectations, 7 of 8 mice treated with IL-1 before 1,200 cGy and 10^7 BM cells were nevertheless also predominantly reconstituted with cells of the H-2^d (donor) phenotype. However, mice treated with IL-1 and BM cells after irradiation were repopulated with higher numbers of spleen cells (mean of 143×10^6 cells per spleen) than mice given IL-1 20 hours before irradiation (mean of 42×10^6 cells per spleen). Although pretreatment

of mice lethally irradiated with 1,200 cGy with IL-1 only favored the recovery of autologous BM cells, provided IL-1 was given together with sufficient numbers of allogeneic BM cells, reconstitution with donor cells was favored. In mice given 5×10^6 BM cells and IL-1 after irradiation, 3 of 5 spleens contained a majority of cells of the donor type, but two spleens consisted of over 90% host cells. Overall, the higher the number of donor cells administered in conjunction with IL-1, the higher the donor cell engraftment ($P < .05$). Histologic examination of the gastrointestinal and epidermal tissues of these mice did not reveal any evidence of GVH disease (data not shown).

The spleen cells from mice treated with 1,200 cGy, with or without BM transplants, plus IL-1 that survived for over 8 weeks, were tested for their capacity to generate in vitro cytotoxic T-lymphocyte reactivity against host (H-2^b), donor (H-2^d), or unrelated third-party (H-2^k) target cells (Fig 2). Pooled spleen cells from 3 C57B1/6 (H-2^b) mice that were reconstituted with 86% Balb/c (H-2^d) donor cells did not generate any lytic effect on either H-2^b or H-2^d target

Table 1. Phenotype (MHC) of Spleen Cells of C57B1/6 Mice Surviving More Than 6 Weeks After Irradiation With 1,200 cGy

Dose of Balb/c BM Cells	Treatment With 10 μ g IL-1 β								
	None*			Preirradiation			Postirradiation*		
	No. of Survivors/Total	MST	% H-2 ^d Spleen Cells	No. of Survivors/Total	MST	% H-2 ^d Spleen Cells	No. of Survivors/Total	MST	% H-2 ^d Spleen Cells
None	0/20*	11	ND†	7/8‡	58	0	0/20*	11	ND
2.5×10^6	0/22	12	ND	4/10‡	37	32 $\pm$ 29§	0/22	13	ND
5×10^6	0/24	15	ND	11/12‡	60	44 $\pm$ 15	11/24	36	50 $\pm$ 18
10×10^6	8/24	29	63 $\pm$ 12	12/12	60	71 $\pm$ 10	22/24	60	83 $\pm$ 2

Abbreviations: MST, median survival time; ND, not determined.

*Pooled data from two different experiments.

†Not determined because of lack of survivors.

‡Significantly higher survival compared with IL-1 postirradiation ($P < .05$).

§Mean and standard error of the mean percent donor-derived (H-2^d) spleen cells detected 6, 8, and 12 weeks postirradiation. The remaining spleen cells were of the host (H-2^b) type.

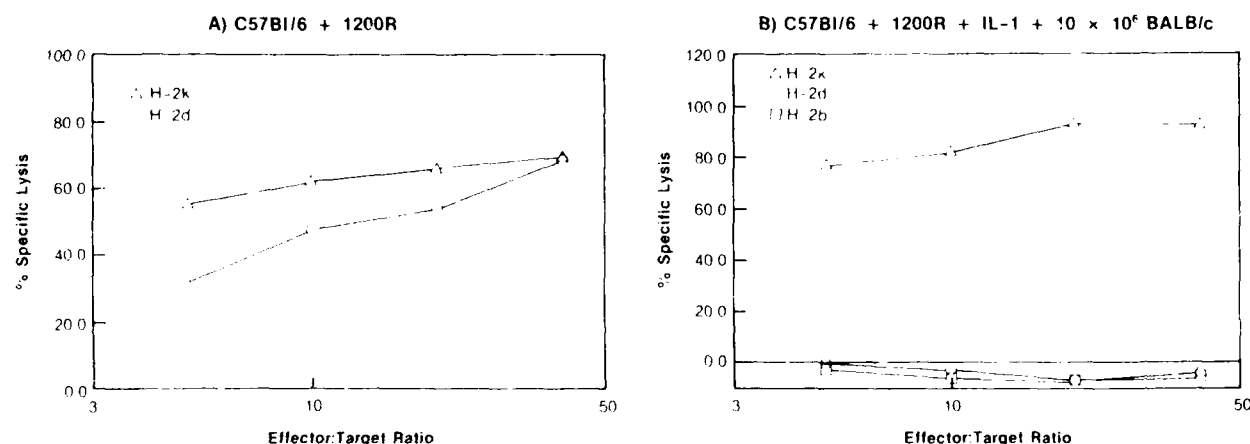


Fig 2. Generation of in vitro cytotoxic T-cell activity by spleen cells from C57B1/6 (H-2^b) mice 4 months after lethal (1,200 rad) irradiation with or without allogeneic Balb/c (H-2^d) BM transplants. Mice were given 10 μ g IL-1 β IP only before lethal irradiation (A) or IL-1 together with 10×10^6 Balb/c BM cells after irradiation (B). Spleen cells (4×10^6) from these mice were stimulated in vitro for five days with irradiated (2,000 rad) Balb/c (H-2^d), C57B1/6 (H-2^b) or C3H/HeN (H-2^k) spleen cells (1×10^6), and the resulting effector cells were mixed with ⁵¹Cr-labelled ConA-induced lymphoblasts from the same three strains. The percent specific lysis of target cells at various ratios of effector to target cells is shown. Each point represents the mean percent specific ⁵¹Cr released by triplicate wells. The spleen cells of two to three mice were pooled to obtain sufficient effector cells in this representative experiment. SE were consistently less than 5% of the mean and have therefore been omitted.

lymphoblasts but possessed cells capable of specifically lysing allogeneic H-2^k target cells (Fig 2B). Thus, cells from the irradiated animal were tolerant to both donor and host MHC specificities but were competent to recognize allogeneic MHC determinants. In contrast, C57B1/6 spleen cells that had recovered from lethal irradiation because of preirradiation treatment with IL-1 β only (ie, without allogeneic BM cells) were capable of lysing H-2^d as well as H-2^k target lymphoblasts (Fig 2A). Thus, the use of IL-1 with high doses of radiation allows recovery of some tolerant host hematopoietic elements, provided sufficient numbers of allogeneic

BM cells are also administered. Such a treatment regimen resulted in varying degrees of mixed chimerism (ie, host and donor hematopoietic recovery). The long-term surviving mice showed no evidence of GVH disease, and their T cells were tolerant to both donor and host MHC antigens.

In an attempt to eliminate more of the residual surviving recipient hematopoietic precursor cells, C57B1/6 recipients were treated with 1,350 cGy irradiation and reconstituted with more thoroughly T-cell depleted Balb/c BM cells. Treatment with anti-brain antiserum and guinea pig complement twice reduced the proportion of Thy 1⁺ cells from 4%

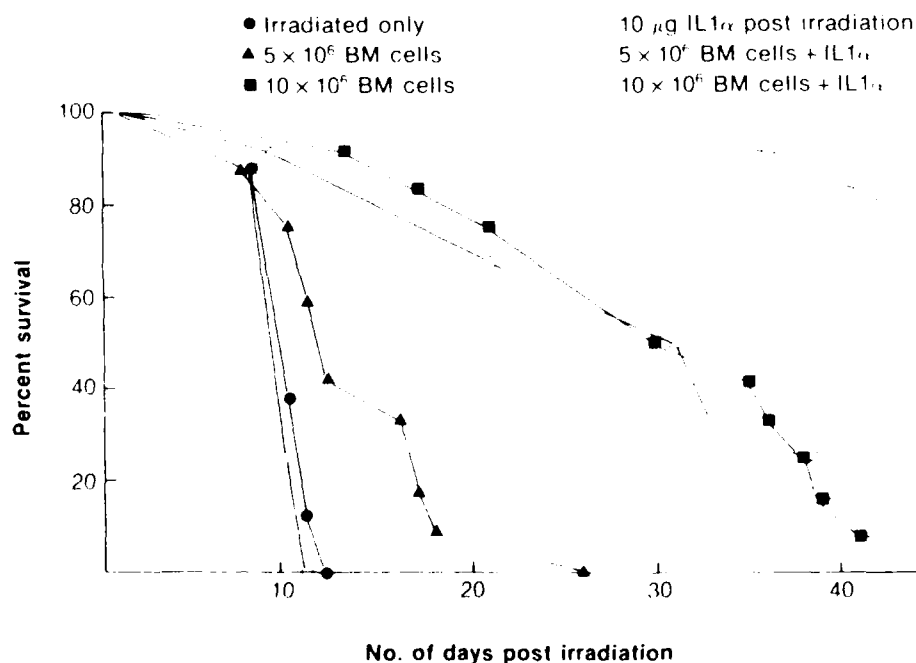


Fig 3 Survival of lethally irradiated C57B1/6 mice treated with IL 1 α and Balb/c (H-2^d) BM cells. The conditions in this experiment were similar to those described in the legend for Fig 2, except that mice were irradiated with 1,350 rad. Significantly more mice survived if given 10⁷ BM cells and IL 1 than if given 10⁶ BM cells only ($P < 0.01$).

Table 2. Phenotype (MHC) of Spleen Cells of Surviving C57B1/6 Mice (6 Weeks) After Irradiation With 1,350 Rad

Dose of Balb/c BM Cells	Treatment With 10 μ g IL-1 ^a								
	None			Irradiation			Postirradiation		
	No. of Survivors/Total	MST	% H-2 ^b Spleen Cells	No. of Survivors/Total	MST	% H-2 ^b Spleen Cells	No. of Survivors/Total	MST	% H-2 ^b Spleen Cells
None	0/8	10	ND*	0/8	14	ND	0/8	10	ND
2.5×10^6	0/10	11	ND	1/10	29†	24†	0/10	28†	ND
5×10^6	0/12	12	ND	2/12	32†	74, 71	2/12	31†	83, 74
10×10^6	1/12	31	56.2	6/12	43†	70, 56, 76	9/12	145†	76, 80, 83

Abbreviations: MST, median survival time; ND, not determined.

*Not determined because of lack of survivors.

†Significantly longer survival in comparison with mice not given IL-1 ($P < .05$). The survival of mice given IL-1 after 10^7 BM cells was significantly greater ($P < .05$) than that of mice pretreated with IL-1.‡The percent donor-derived spleen cells was determined 6 weeks after BM transplantation. The remaining spleen cells were predominantly of the host (H-2^b) type.

to 0%. All irradiated mice that were untreated or treated only with 10 μ g IL-1 β IP before or after irradiation died within 8 to 12 days (Fig 3, Table 2). All mice given 2.5×10^6 Balb/c BM cells without IL-1 died in 8 to 14 days (Table 2). The survival of mice given 2.5×10^6 Balb/c bone marrow cells intravenously (IV) and 10 μ g IL-1 β IP after 1,350 cGy was prolonged to 10 to 38 days, with a median survival time of 28 days. Irradiated mice given 5×10^6 Balb/c BM cells without IL-1 died within 8 to 26 days, with a median survival time of 12 days, and all but one of 12 mice given 10×10^6 Balb/c BM cells without IL-1 died within 13 to 41 days (Fig 3). In contrast, 17% of mice given 5×10^6 BM cells plus IL-1 after irradiation survived for over 45 days, and 9 of 12 mice treated with 10^7 BM cells followed by IL-1 have survived beyond 45 days. In these allotransplanted mice given higher doses of 1,350 cGy, the beneficial effects of giving IL-1 as a radioprotector 20 hours before irradiation together with 10^7 BM cells was less effective than the therapeutic effects of IL-1 given after irradiation (Table 2). The overall survival of mice given IL-1 before or after irradiation along with allogeneic BM cells was lower after 1,350 than 1,200 cGy (Tables 1 and 2).

Phenotypic analysis of spleen cells of mice surviving 1,350 cGy for over 6 weeks revealed that the majority of spleen cells from 4 and 7 of the mice that had received IL-1 α and 5×10^6 or 10^7 Balb/c BM cells, respectively, were of donor (H-2^b) origin (Table 2). However, the spleen cells still contained from 17.4% to 44.2% recipient cells. The spleen cells from one surviving mouse that had received IL-1 and 2.5×10^6 BM cells was small and contained only 24% donor cells. Consequently, even after 1,350 cGy, some of the host cells survived, and the hematopoietic cells of the mice were still chimeric.

DISCUSSION

Preliminary studies of C57B1/6 mice that were irradiated with 1,100 rad and treated with 5×10^6 T-cell depleted Balb/c BM cells along with 2 μ g IL-1 β daily for 6 days, beginning immediately after irradiation, resulted in long-term survival of only 40% of the mice, in comparison with only 10% survival of mice given BM cells only. Furthermore, by flow cytometry, the spleen cells of most of these surviving

mice were reconstituted predominantly with host (H-2^b) cells. Since none of the lethally irradiated mice given these doses of IL-1 only survived, we speculated that in this pilot study, the allogeneic cells transiently promoted survival until replaced by the hosts' own BM cells. These results led us to study the effects of higher doses of irradiation, a higher dose of IL-1, and more BM cells in order to decrease the likelihood of host-cell recovery and improve the probability of donor-cell engraftment. These experimental modifications clearly showed that IL-1 can markedly enhance the survival of lethally irradiated mice treated with allogeneic bone marrow cells. IL-1 promoted the survival of C57B1/6 (H-2^b) mice irradiated with 1,200 to 1,350 cGy and reconstituted with T-cell depleted Balb/c (H-2^d) allogeneic cells, despite major differences in MHC. The numbers of long-term surviving mice (over 1.5 months) treated with 5×10^6 or 10^7 allogeneic BM cells plus IL-1, far exceeded the numbers of surviving mice given only BM cells. Thus, IL-1 can promote either the recovery of host BM cells or accelerate the engraftment and proliferation of donor bone marrow cells or both.

Radioprotection by prior treatment with IL-1 without BM cells was completely ineffective, as was IL-1 given by itself after 1,350 cGy irradiation. The failure of IL-1 treatment after this higher dose of radiation is presumably due to an insufficient number of residual host hematopoietic stem cells and progenitor cells for the IL-1 to act on. Furthermore, few mice given only BM cells survived 1,350 cGy. IL-1 pretreatment and IL-1 therapy (ie, after radiation) were each equally effective in promoting the survival of mice irradiated with 1,350 cGy and given 10^7 allogeneic BM cells. The pretreatment with IL-1 improved the capacity of the host to support the engraftment with allogeneic cells given 24 hours later. Even at 1,200 cGy, most of the mice that were pretreated with IL-1 were reconstituted predominantly with allogeneic donor cells. Perhaps their enhanced survival was based on a transient recovery by host cells, which were then replaced by the unirradiated donor cells.

The capacity of IL-1 to promote hematopoiesis may be based on several recognized biologic activities of IL-1. IL-1 is known to stimulate stromal cells to produce GM-CSF and G-CSF¹⁷ and to induce receptors for M-CSF on hematopoietic precursor cells⁸; it is possible that the bone marrow

graft-promoting effects of IL-1 are mediated by the colony-stimulating factors (CSFs). Furthermore, IL-1 can act directly on hematopoietic stem cells and stimulate their proliferation in synergy with GM-CSF or IL-3.¹⁹ That such synergy also occurs *in vivo* was shown by combining suboptimal nonradioprotective doses of IL-1 with GM-CSF to achieve optimal radioprotection.²⁰

It is also possible that IL-1-induced recovery of nonhematopoietic tissue may contribute to the improved survival of mice whose hematopoietic tissues were restored with donor BM cells. IL-1 does increase systemic scavenger activity through the induction of acute-phase proteins such as metallothionein and ceruloplasmin²¹ and intracellular scavenger molecules such as mitochondrial manganese-superoxide dismutase.²² These scavengers may reduce the free oxygen radicals produced in extensively damaged tissues. Indeed, treatment with superoxide dismutase (SOD) alone following irradiation has been reported to enhance survival.²³ However, although the gastrointestinal tissues of lethally irradiated mice treated with a combination of allogeneic BM cells and IL-1 showed less evidence of damage than those of untreated mice, we were unable to conclude whether IL-1 rather than the BM cells were directly beneficial to nonhematopoietic tissues. The restorative effect of IL-1 may also involve its capacity to promote resistance to infectious organisms.²⁴ Thus, IL-1-induced restoration of hematopoiesis, induction of scavengers, and host-defense mechanisms may indirectly contribute to the improved recovery of the allogeneic BM recipients.

GVH reactions are a potentially serious problem in mice given incompletely T-cell depleted allogeneic BM cells,²⁵ especially when the mice exhibit partial reconstitution with their own cells. Indeed, from 10% to 42% of mice treated with allogeneic BM cells and IL-1 died between 30 and 45 days following irradiation (1,200 cGy). In contrast, lethally irradiated mice treated with isologous BM cells and IL-1 stabilize after 30 days and do not perish between 30 and 45 days.²⁶ These late deaths could be due to inadequate immune function, inadequate bone marrow engraftment, or GVH reactions. Although several of the mice that were phenotyped at 6 to 8 weeks postirradiation had increased numbers of spleen cells (2×10^6 cells), which is compatible with an active GVH, histologic examination failed to reveal any other evidence of GVH in the epidermal, gastrointestinal, or

hepatic tissues of asymptomatic long-term survivors. Since we did not autopsy the mice that died before 6 weeks, we have not established whether they had acute GVH.

There have been reports that mice treated with IL-1 to counter the toxic doses of chemotherapeutic agents such as cyclophosphamide did show better hematopoietic than immunologic recovery.²⁷ It is therefore possible that IL-1 may promote granulocytopoiesis and erythropoiesis at the expense of lymphopoiesis and could therefore either reduce the incidence or delay the onset of GVH. On the other hand, our studies show that donor-cell reconstituted spleen cells from mice surviving 1½ to 5 months have the capacity to generate T cells that are cytotoxic for unrelated (H-2^k) but not donor (H-2^d) or recipient (H-2^b) target cells. This suggests that the T cells in the chimeric mice that survive for over 6 weeks have not only become tolerant to both donor and recipient MHC antigens but are also immunologically competent, as judged by their ability to muster a specific CTL response against an unrelated allogeneic target cell.

Many other questions concerning the contribution of IL-1 to survival of lethally irradiated BM-engrafted mice remain to be answered. The optimal dosage, schedule, and route of administration of IL-1 need to be established in rodents as well as in larger animal models. The hematologic recovery, immune competence, and occurrence of GVH in IL-1-treated BM-cell engrafted mice must be evaluated in greater detail. The capacity of IL-1 to interact with other agents, such as anti-asialo GM-1 antiserum (which suppresses natural killer [NK] cell activity and reduces hybrid resistance to promote bone marrow engraftment)²⁸ is being studied.²⁹ Our findings do suggest that IL-1 may permit the use of higher doses of radiation therapy in the conditioning regimens used to treat malignancies currently managed by allogeneic BM transplantation. IL-1 may allow the delivery of more effective therapy, either by itself or with fewer donor BM cells.

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Interaction of Haloperidol and Area Postrema Lesions in the Disruption of Amphetamine-Induced Conditioned Taste Aversion Learning in Rats

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RABIN, B. M. AND W. A. HUNT. *Interaction of haloperidol and area postrema lesions in the disruption of amphetamine-induced conditioned taste aversion learning in rats.* PHARMACOL BIOCHEM BEHAV 33(4): 847-851, 1989. — Two experiments were run to determine the mechanisms underlying the acquisition of an amphetamine-induced conditioned taste aversion. In the first experiment, it was shown that pretreatment with haloperidol (0.1–0.5 mg/kg, IP) attenuated, but did not prevent, taste aversion learning produced by amphetamine (3 mg/kg, IP). In the second experiment, combining area postrema lesions with haloperidol (0.5 mg/kg) pretreatment completely blocked the acquisition of an amphetamine-induced taste aversion. The results are interpreted as indicating that amphetamine-induced taste aversion learning has both a central component, which is mediated by dopaminergic receptors, and a nondopaminergic peripheral component, which is mediated by the area postrema.

Conditioned taste aversion Amphetamine Haloperidol Dopaminergic Area postrema

A conditioned taste aversion (CTA) can be produced by pairing a novel tasting solution with a variety of unconditioned stimuli, including ionizing radiation, lithium chloride (LiCl), amphetamine and morphine (6,13). The neural mechanisms underlying the acquisition of a CTA to toxic treatments appear to differ from those underlying taste aversions produced by self-administered compounds (6). In contrast to radiation- and LiCl-induced taste aversions, which require the mediation of the area postrema (AP) (11, 14, 16), AP lesions have no effect on the acquisition of a CTA produced by injection of higher doses of amphetamine (>1.5 mg/kg) (15,16) or morphine (19). Conversely, manipulation of specific catecholaminergic systems disrupts CTA learning following injection of amphetamine, but not that induced by LiCl (10, 17, 20, 21).

Since amphetamine is a dopamine agonist, most research into the neural mechanisms of amphetamine-induced CTA learning has focused on the role of the dopaminergic system in this behavior. However, this research has shown that disruption of dopaminergic activity causes only an attenuation of amphetamine-induced CTA learning rather than its complete elimination (5, 10, 17, 20, 21); as

would be expected if the dopaminergic properties of amphetamine formed the basis for the acquisition of the CTA. Since the observation of an attenuated response, instead of its elimination, may reflect an incomplete disruption of dopaminergic activity, the first experiment in this series was designed to further evaluate the role of dopamine in the acquisition of an amphetamine-induced CTA by using subjects that had been pretreated with the dopaminergic antagonist haloperidol. Haloperidol was selected because it has a high binding capacity for dopamine receptors (1), and should, therefore, block dopaminergic activity at doses that produce few CTA-related side-effects (4).

GENERAL METHOD

Subjects

The subjects were male Crl:CD BR VAF-Plus rats (*Rattus norvegicus*) weighing 300–400 g at the start of the experiment. Rats were quarantined on arrival and screened for evidence of disease before being released from quarantine. They were main-

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ence between the minimum dose needed to produce an attenuation and the dose that produces a maximal effect. Alternatively, it may also reflect the fact that the lowest dose tested was sufficient to produce maximal attenuation of the amphetamine-induced CTA. In the latter instance, the use of even lower doses may be necessary to show dose-dependent haloperidol effects on amphetamine-induced CTA learning. The present data do not allow an evaluation of these alternate hypotheses.

However, it is not clear why, if the amphetamine-induced CTA depends upon the action of the drug at dopaminergic synapses, the disruption of dopaminergic activity produces only an attenuation of CTA learning and not the complete loss of the response. One possibility, suggested by Lorden *et al.* (10), is that the intraventricular injection of 6-hydroxydopamine, which attenuates amphetamine-induced CTA learning, produces depletion of both dopamine and norepinephrine. In contrast, intranigral administration of 6-hydroxydopamine causes depletion only of dopamine and does not affect the acquisition of an amphetamine-induced CTA, suggesting that the combined depletion of both dopamine and norepinephrine is necessary for the attenuation of an amphetamine-induced CTA under these conditions. This hypothesis, however, is not consistent with the observation that treatment with the dopamine antagonists pimozide and haloperidol are effective in attenuating amphetamine-induced CTA learning in a manner similar to that observed following intraventricularly-administered 6-hydroxydopamine, because the effects of these drugs are restricted to the dopaminergic system. It is possible that intranigral injection of 6-hydroxydopamine does not produce the same pattern of dopamine loss as produced by intraventricular injection, such that specific structures that may be important in mediating the CTA response may not have been affected by the treatment (8).

Another possibility may involve the area postrema. Although taste aversions produced by high doses of amphetamine (>1.5 mg/kg) are not affected by AP lesions (15,16), aversions produced by lower doses of amphetamine do seem to be mediated by the AP (15). AP involvement in mediating the CTA response to amphetamine may reflect the operation of two possible mechanisms. First, it may result from activation of dopaminergic receptors located in the AP (9,12). Consistent with this hypothesis are data showing that intracranial injections of amphetamine, unlike LiCl, into the vicinity of the AP will produce a CTA (2,18), and that lesions of the AP can alter the motor responses of rats to injection of amphetamine (3). However, this hypothesis is not consistent with the present results because the haloperidol injection should have blocked all dopaminergic activity, both in the AP as well as centrally.

Alternatively, the AP may be involved as the result of its activation by an endogenous, nondopaminergic, peripheral factor, in a manner similar to that involved in the acquisition of LiCl- and radiation-induced aversions (13). This hypothesis would suggest, therefore, that amphetamine-induced CTA learning involves both a peripheral mediator as well as a central dopaminergic component. It may, therefore, be that the manipulation of dopaminergic synapses by neurotoxins or by use of dopamine antagonists affects only the central mechanisms involved in amphetamine-induced CTA learning, but not the peripheral mechanisms involving the AP. This results in an attenuation of the CTA response rather than its complete disruption following manipulation of the dopaminergic system. If this hypothesis is correct, then it should be possible to produce a complete disruption of amphetamine-induced CTA learning by combining haloperidol treatment with lesions of the AP.

the possibility that amphetamine-induced CTA learning involves both a central component mediated by the dopaminergic system and a peripheral component mediated by the AP.

Procedure

Histologically confirmed lesions were made in the AP of 13 male albino rats. An additional 14 rats served as unoperated controls. The details of the surgical procedures have been published previously (14,15). Briefly, the rats were anesthetized with sodium pentobarbital (35 mg/kg, IP), the AP exposed and cannularized under direct visual control. Following surgery, all animals were given a prophylactic injection of Bicillin (60,000 units), returned to their home cages and allowed to recover from the effects of the surgery for 3-4 weeks before beginning the behavioral testing.

The behavioral procedures were identical to those detailed above except that, because there were no dose effects, only a single dose of haloperidol was used (0.5 mg/kg, IP). The intact rats were divided into two groups ($n=7$ each); one of which was given haloperidol followed 30 min later by amphetamine (3 mg/kg, IP), while the second group was given an equivolume injection of saline followed by amphetamine. All the rats with AP lesions were treated with haloperidol followed by amphetamine. These procedures were similar to those utilized in a previous study of the role of the AP in the acquisition of an amphetamine-induced CTA (15), with the exception that the control rats in that study were not given an injection of isotonic saline prior to the amphetamine injection.

At the conclusion of the experiment, the operated animals were sacrificed with an overdose of pentobarbital (50 mg/rat, IP) and perfused intracardially with isotonic saline and 10% formalin saline. The brains were removed, fixed in formalin saline and 50 μ m sections taken from the brainstem at the level of the AP. Representative sections of the AP and a lesion are presented in Fig. 2. Lesion size was somewhat variable, involving only the AP in some cases, but impinging on the dorsal parts of the nucleus of the solitary tract in others. However, no behavioral differences were observed as a function of lesion size.

Results

There were no significant differences in average fluid intake between the three experimental groups. As shown in Fig. 3, pretreatment with haloperidol in intact animals attenuated the CTA produced by injection of amphetamine, but did not eliminate it. In the rats with AP lesions, however, pretreatment with haloperidol produced a complete disruption of amphetamine-induced CTA learning. This finding contrasts with previous research (15) showing that AP lesions do not, by themselves, produce a significant attenuation of a CTA following injection of 3 mg/kg amphetamine.

Statistical analysis of the data using an analysis of variance followed by orthogonal comparisons (7) indicated that for the intact animals the saline-treated rats showed a significantly reduced test day sucrose intake compared to the haloperidol-treated rats, $F(1,23)=6.67$, $p<0.05$. The comparison between the intact rats and those with AP lesions was also significant, $F(1,23)=29.21$, $p<0.01$, indicating that both groups of intact rats showed a greater test day aversion to the sucrose than did the group with AP lesions.

Discussion

These results clearly indicate that the AP is involved in the acquisition of an amphetamine-induced CTA. As was observed in

EXPERIMENT 2

As indicated above, this experiment was designed to evaluate

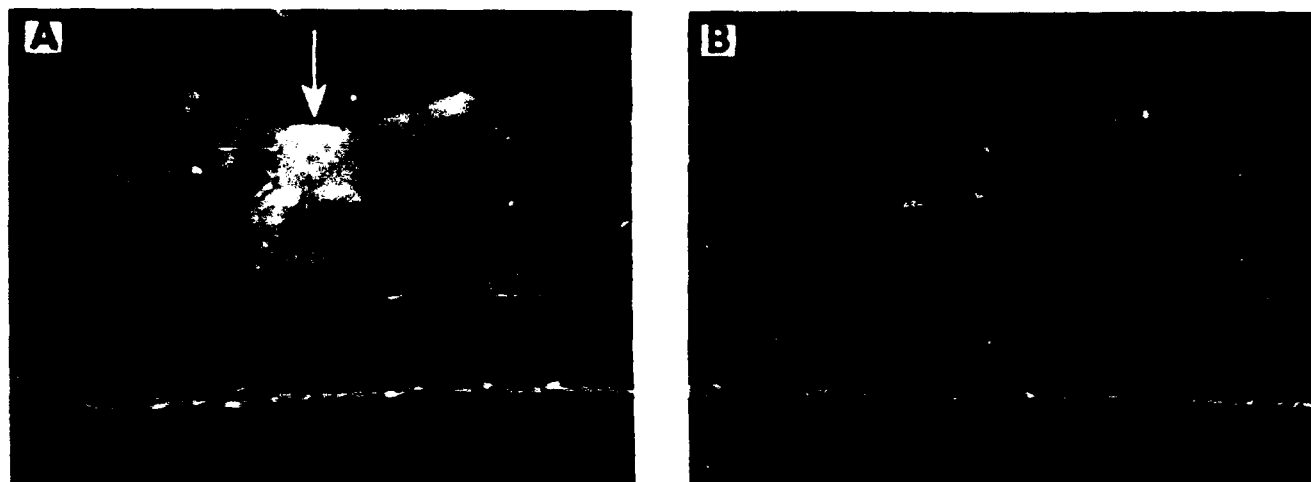


FIG. 2. Photomicrographs of sections of the brainstem of an intact rat showing the area postrema (A, arrow) and a representative lesion (B).

the first experiment of this series, haloperidol pretreatment attenuated the CTA produced by amphetamine, but did not completely block it. Previous research has shown that lesions of the AP do not affect the acquisition of an amphetamine-induced CTA when higher doses of amphetamine are used (15). A complete disruption of the amphetamine-induced CTA was obtained only when the haloperidol was combined with lesions of the AP.

A possible explanation for the present observation of AP involvement in the CTA following administration of 3 mg/kg amphetamine may be that, in the untreated animal, the response of the central dopaminergic system to the large dose of amphetamine overshadows a relatively weak response mediated by the AP, leading to the acquisition of a CTA that is apparently not modulated by the destruction of the AP. In contrast, when the activity of the central dopaminergic system has been reduced by treatment with the dopamine antagonist haloperidol, as in the present experiment, or by other means (5, 10, 17, 20, 21), then the

contribution of the AP-mediated mechanisms are expressed in behavior. Thus, the complete elimination of amphetamine-induced CTA learning requires both the disruption of the mechanisms mediated by the dopaminergic system as well as those mediated by the AP, which are independent of the dopaminergic system.

GENERAL DISCUSSION

The present results indicate that the acquisition of a taste aversion following injection of the dopamine agonist amphetamine involves two distinct mechanisms: a central mechanism mediated by the dopaminergic system as well as a peripheral mechanism mediated by the AP. Complete disruption of amphetamine-induced CTA learning requires manipulations that affect both mechanisms simultaneously.

In this regard, amphetamine seems to be somewhat unique. For both radiation- and LiCl-induced aversions, destruction of the AP is sufficient to produce the complete disruption of CTA learning (11, 14, 16). Manipulation of the dopaminergic system by intraventricular injection of neurotoxins (10, 17, 20) or by lesions of the dorsolateral tegmentum (21) have no effect on the acquisition of a LiCl-induced CTA. Similarly, pretreatment with haloperidol (0.1–0.5 mg/kg) has no effect on the acquisition of a radiation-induced CTA (Rabin and Hunt, unpublished results). These findings suggest that, with the exception of amphetamine, a dopamine agonist, the dopaminergic system is not routinely involved in the acquisition of a CTA with these toxins. Therefore, the present results would suggest the hypothesis that the acquisition of a CTA may involve distinct brainstem pathways, depending upon the specific nature of the unconditioned stimulus.

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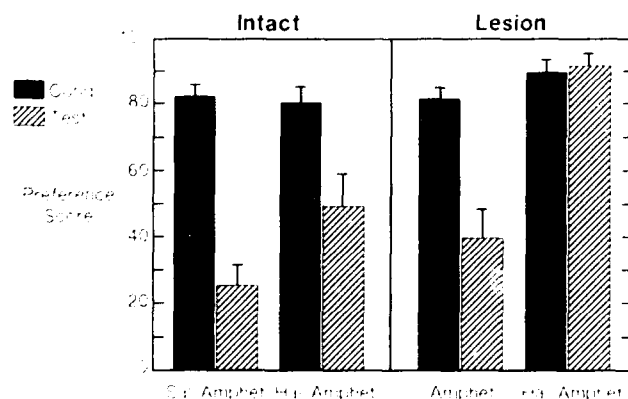


FIG. 3. Effects of haloperidol and area postrema lesions on the acquisition of an amphetamine-induced CTA. Preference scores from the two groups of intact animals are shown in the left-hand panel; from rats with AP lesions, in the right-hand panel. The data for the amphetamine-only group has been recalculated from (15). Error bars indicate the standard error of the mean.

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L-LEUCYL-L-LEUCINE METHYL ESTER TREATMENT OF CANINE MARROW AND PERIPHERAL BLOOD CELLS

INHIBITION OF PROLIFERATIVE RESPONSES WITH MAINTENANCE OF THE CAPACITY FOR AUTOLOGOUS MARROW ENGRAFTMENT¹

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Incubation of canine marrow and peripheral blood mononuclear cells with L-leucyl-L-leucine methyl ester resulted in the inhibition of mitogen- and alloantigen-induced blastogenesis, the elimination of allosensitized CTL and NK activity, and prevented the development of CTL from pCTL. The effects of these incubations were similar to those described in mice and humans. Additionally, in vitro CFU-GM growth from treated canine marrow was reduced, but could be regained when the Leu-Leu-OMe-treated marrow was cocultured with either untreated autologous peripheral blood mononuclear cells or monocyte-enriched PBMC but not with untreated monocyte-depleted PBMC. Six of seven dogs conditioned with 920 cGy total-body irradiation engrafted successfully after receiving autologous marrow that was incubated with Leu-Leu-OMe prior to infusion. These cumulative results indicate that incubation with Leu-Leu-OMe is a feasible method to deplete canine marrows of alloreactive and cytotoxic T cells prior to transplantation.

The success of allogeneic marrow transplantation as treatment for malignant and nonmalignant hematopoietic diseases has been restricted by the serious complications of graft-versus-host disease (1, 2). Experiments in a variety of mammalian marrow transplant models have shown that removal of mature T cells from donor marrow permits engraftment without the development of GVHD (3-6). Based on these and similar observations, studies have been carried out to evaluate the effects of T cell depletion prior to allogeneic marrow transplantation in humans. Most studies have employed marrow treatments with anti-T cell monoclonal antibodies plus complement or with soybean agglutinin followed by E rosette formation and density gradient centrifugation (7-9). In general, removal of T cells has been associated with a marked decrease in both the incidence and severity of GVHD. However, the use of T cell-depleted marrow has also been associated with an increased incidence of marrow graft rejection (10).

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Recently, Thiele and Lipsky have described a dipeptide methyl ester, L-leucyl-L-leucine-methyl ester (Leu-Leu-OMe)* that can eliminate natural killer cells (NK), monocytes (Mø), and precursors of alloantigen-specific cytotoxic T cells (pCTL) from mouse spleen cell suspensions and from both mouse and human peripheral blood. This treatment leaves intact B cells, helper T cells, and murine erythroid and hematopoietic stem cells (11-14). In a murine histoincompatible marrow transplant model (C57BL/6J→(C57BL/6×DBA/2)F₁), treatment of donor marrow and spleen cells with Leu-Leu-OMe resulted in successful donor marrow engraftment and the development of stable long-term hematopoietic chimerism without GVHD (14-16). The use of Leu-Leu-OMe to treat marrow may have advantages over currently used methods. The use of Leu-Leu-OMe is very simple, requiring but a single 15-min incubation. In addition, it appears that marrow incubation with Leu-Leu-OMe results in the elimination of the cells responsible for acute GVHD while at the same time preserving hematopoietic stem cells needed for engraftment and the cells required for immune reconstitution (15, 16).

We and others have used dogs as a large, outbred animal model for us in experimental marrow transplantation (17, 18). The present studies were undertaken to determine whether the incubation of canine marrow and peripheral blood cells with Leu-Leu-OMe would yield alterations of in vitro cellular immune function comparable to those described in human and murine cells and to investigate the effects of marrow incubation with Leu-Leu-OMe on early hematopoietic progenitors and stem cells assayed for both in vitro and in vivo function.

MATERIALS AND METHODS

Dogs. Beagles, hounds, and mixed breed hounds, obtained from commercial vendors in Washington and Virginia or raised at the Fred Hutchinson Cancer Research Center (FHCRC), were dewormed and vaccinated against distemper, hepatitis, leptospirosis, and parvovirus before use in this study. All dogs were at least six months of age and were maintained at the FHCRC canine kennel facilities per guidelines stipulated by the National Academy of Sciences—National Research Council. The research protocol was approved by the Internal Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

Medium. Waymouth's MB752/1 medium (FHCRC media preparation facility), supplemented with 0.1 mM nonessential amino acids and 100 U/ml penicillin and 100 µg/ml streptomycin (all from GIBCO).

* Abbreviations: Leu-Leu-OMe, L-leucyl-L-leucine-methyl ester; Mø, monocytes; B-MLC, bulk MLC; NWA, nylon-wool nonadherent; CFU-C, colony forming unit in culture; PEDS, postendotoxin dog serum; CTAC, canine thyroid adenocarcinoma cell line.

was used for the dilution of heparinized whole blood and marrow for the cell separation procedures. Waymouth's medium supplemented as above with the addition of 10% to 20% heat-inactivated (56°C) normal pooled dog serum (M-NPS/10-20%) was used for the mixed leukocyte culture microassays, bulk MLC (B-MLC), cell-mediated lympholysis, and NK assays.

Cell preparation. Peripheral blood mononuclear cells were obtained by the centrifugation of heparinized venous whole blood (diluted 1:2 with medium) over Ficoll-Hypaque density gradients (Sp. density 1.074) as previously described (19). Bone marrow cells (BMC) for in vitro assays were obtained by syringe aspiration from the humeral head of an anesthetized dog. The marrow was diluted 1:2 with medium and overlaid onto Ficoll-Hypaque density gradients for centrifugation (1000 × g), following which the interface cells were washed once with hemolytic buffer and twice with medium. The PBMC and marrow cells were resuspended into medium for cell counts and viability assessment using the trypan-blue exclusion technique.

Monocyte-enriched cells were obtained by treating PBMC with the anticanine murine monoclonal antibody Dly 6 (20) as follows: 300 × 10⁶ PBMC were incubated for 30 min at room temperature in 30 ml of 1:100 diluted Dly 6 (ascites containing antibody), and then an equal volume of 1:2 diluted rabbit serum complement (Pel Freeze, Rogers, AR) was added for an additional 60 min. The cells were washed once with medium, resuspended in 30 ml of 1:3 diluted rabbit serum complement, and incubated again for 60 min. After washing twice in medium, these cell suspensions contained 61 ± 8% (SEM) viable monocytes, 16 ± 3% lymphocytes, and 23 ± 9% granulocytes, primarily eosinophils.

Monocyte-depleted PBMC were obtained by first passing PBMC over nylon-wool columns as previously described (21), and then transferring 30 × 10⁶ nylon-wool-nonadherent (NWN) cells in 10 ml of medium, containing 5% fetal calf serum, into plastic petri dishes (Falcon No. 3003, Lincoln Park, NJ) for 2 hr incubation at 37°C, 7% CO₂. This depletion technique yielded approximately 95 ± 1% lymphoid cells with greater than 90% viability and less than 3% monocytes as determined by morphologic assessment of Wright-stained cytospin preparations.

Preparation of Leu-Leu-OMe. The Leu-Leu-OMe was synthesized from L-leucyl-L-leucine (Sigma Chemical Co, St Louis, MO) as previously described (11). Qualitative assessment of Leu-Leu-OMe purity was obtained by thin-layer chromatography (TLC) (22). Briefly, 5 μl of 5 × 10⁻³ M solutions of L-leucine methyl ester (Leu-OMe) (dissolved in absolute methanol), L-leucine (Leu), L-leucyl-L-leucine (Leu-Leu) (both dissolved with heat and stirring in absolute methanol containing 0.5 N HCl), and the synthesized Leu-Leu-OMe were applied to pre-coated TLC plates (250 μM, 10 × 20-cm, HPTLC Kieselgel 60 (Merck), Darmstadt, West Germany), and quickly dried under a stream of warm air. The plates were developed for 2.5-hr in an enclosed, equilibrated system containing the following mixture of reagent grade solvents: chloroform, absolute methanol and acetic acid at volume ratios of 19:0.6:12.5, respectively. The migrations of the four compounds were visualized by applying an aerosol spray of 0.2% ninhydrin in ethanol and then placing the plates in a 60°C oven for 30 min. R_f values (the ratio of the distance the compound travels to the distance the solvent front travels) were calculated, in order to assess the resultant migrations, according to the following formula (22):

$$R_f = \frac{\text{distance traveled by compound}}{\text{distance traveled by solvent front}}$$

Leu-Leu-OMe was stored at -20°C in absolute methanol and, based on repeated TLC analysis, was stable for at least three months.

Incubation of PBMC or marrow cells with Leu-Leu-OMe. Equal volumes of PBMC or marrow cell suspensions and Leu-Leu-OMe at the indicated final concentrations were incubated for 15 min at room temperature. Cells for in vitro studies were washed twice and resuspended in medium. Marrow cells used for autologous infusion were incubated at cell concentrations of 20 × 10⁶/ml in Leu-Leu-OMe solu-

tions that contained 0.1 U/ml DNAase (Worthington Enzymes and Biochemicals, Freehold N.J.). After incubation, these cells were washed, counted, and reinfused within 1-3 hr.

Mixed leukocyte culture and mitogen assays. MLCs were established, labeled with [³H]thymidine, harvested, and prepared for liquid scintillation counting as previously described (23) with minor modifications. The 10⁶ Leu-Leu-OMe-treated or untreated responder and 10⁶ irradiated (2300 rads) untreated, stimulator PBMC were cocultured in a final volume of 200 μl M-NPS/20% per well. Mitogen stimulation was assessed by adding either 375 μg/ml PHA (DIFCO, Detroit MI), 200 μg/ml Con A, (Calbiochem, San Diego CA), or 200 μg/ml PWM (GIBCO, Grand Island, NY) to 10⁶ treated or untreated responder cells in a final volume of 200 μl of M-NPS/20%. All cultures were established in triplicate in microtiter plates (Costar No. 3799, Cambridge MA) for 7 days at 37°C, 7% CO₂, in a humidified incubator.

Bulk MLC (B-MLC) and cell-mediated lympholysis assays. Bulk MLCs were established using either untreated or Leu-Leu-OMe-treated PBMC or marrow cells as responders, and untreated, irradiated PBMC as stimulators, to generate CTL for CML assays, as previously described (19), with modifications. CTL were derived from these cultures to form two CML assay groups: (1) responder PBMC or marrow cells treated with Leu-Leu-OMe or MeOH on day 0 prior to mixing with irradiated stimulator PBMC in B-MLC (day 0); and (2) Leu-Leu-OMe or MeOH treatment of 7-day B-MLC generated CTL (day 7). CTLs were mixed at a 50:1 effector:target ratio, with ⁵¹Cr-labeled ⁵¹Na CrO₄ (300-500 μCi/ml, NEN, Wilmington DE) Con A-stimulated PBMC targets. Cultures of PBMC to be used for targets in the CML assays were established on the same day of B-MLC, and stimulated with Con A on day 4 of culture. The 4-hr ⁵¹Cr release assay was performed as previously described (19). The mean spontaneous/maximum ⁵¹Cr release ratio was 16 ± 1% (± SEM), while maximum release/total ⁵¹Cr incorporation was 90 ± 2% for targets used in this series of CML assays.

Proliferation of treated or untreated responder PBMC or marrow cells in 7-day B-MLC was measured by distributing 200-μl aliquots from each B-MLC flask into triplicate microculture wells and labeling for 7 hr with [³H]thymidine. Cell harvest and liquid scintillation counting were performed as described for MLC (23).

Natural killer cell (NK) assays. Leu-Leu-OMe or MeOH treated or untreated PBMC and marrow cells were assayed for NK activity against a ⁵¹Cr-labeled canine thyroid adenocarcinoma cell line (CTAC) in a 18-hr assay with the percentage of ⁵¹Cr release calculated as previously described (24).

In vitro marrow cultures for CFU-GM growth. Leu-Leu-OMe and MeOH treated and untreated marrow cells were tested for in vitro hematopoietic progenitor growth using an agar-based colony formation assay that utilizes postendotoxin dog serum (PEDS) as the source of colony-stimulating factor (25). Growth of granulocyte/macrophage colonies (CFU-GM) was assayed in cultures containing 10⁵ or 3 × 10⁵ treated or untreated marrow cells only, and in cultures in which marrow cells were cocultured with either 10⁶ autologous PBMC, Mφ-enriched or Mφ-depleted PBMC, or Leu-Leu-OMe treated PBMC (incubated with 1000 μM Leu-Leu-OMe). Cultures were incubated in triplicate for 10 days in a 37°C, 7% CO₂ humidified incubator and CFU-GM colonies were enumerated as previously described (25).

Autologous marrow transplantation. Marrow for autologous transplantation was obtained by a vacuum pump aspiration procedure (26), processed over a Ficoll-Hypaque density gradient and incubated with Leu-Leu-OMe. All recipients were conditioned with 920 cGy total-body irradiation delivered as a single exposure from two opposing ⁶⁰Co sources at 7.0 cGy/min (26). The recipients were infused with treated autologous marrow within 3 hr of TBI. Supportive care pre- and posttransplantation with antibiotics, i.v. fluids, and whole-blood transfusions, was given as previously described (27). In addition, recipients were given oral antibiotics (Neomycin sulfate and polymyxin B sulfate) daily for five days before TBI and posttransplant until the granulocyte count reached 500/mm³.

RESULTS

Thin-layer chromatographic analysis of synthesized Leu-Leu-OMe. Three batches of Leu-Leu-OMe were synthesized for use in the *in vitro* studies and autologous transplant experiments described. Consistent R_f values were obtained for each batch of Leu-Leu-OMe and for the three control compounds tested. Within the Leu-Leu-OMe, there was a secondary spot that migrated with a R_f value equal to that observed for the L-Leucyl-L-leucine. The consistency of the R_f values indicated that constant yield and purity was achieved with minimal batch-to-batch variations.

Viability of PBMC and marrow cells after incubation with Leu-Leu-OMe. The viability of treated PBMC and marrow cells was assessed within 1 hr after incubation with Leu-Leu-OMe. There was no significant difference in viabilities observed in cells treated with either Leu-Leu-OMe (up to 1000 μ M), 0.5% MeOH in PBS, or PBS only. No time-course studies were done to assess the viability of PBMC and marrow cells more than 1 hr after incubation with Leu-Leu-OMe.

Elimination of alloantigen responsiveness and mitogen-induced lymphocyte blastogenesis by incubation of PBMC with Leu-Leu-OMe. PBMC were treated at either 2×10^6 or 20×10^6 / ml with Leu-Leu-OMe and tested in micro-MLC, and blastogenesis assays (Fig. 1). In all assays there was a Leu-Leu-OMe dose-dependent reduction in the proliferative response such that virtually no blastogenesis was observed after incubation with 1000 μ M Leu-Leu-OMe.

Treatment of 20×10^6 responder PBMC or marrow cells before bulk MLC resulted in similar Leu-Leu-OMe dose-dependent reductions in proliferative response (data not shown). The marrow cells gave a lower baseline level of [^{3} H]thymidine incorporation and were more sensitive than the PBMC to treatment with Leu-Leu-OMe (data not shown). Treatment of PBMC marrow cells with 0.5% MeOH in PBS had no effect on the alloproliferative response in B-MLC (data not shown).

Elimination of the generation of antigen-specific cytotoxic T lymphocytes by incubation of PBMC and marrow cells with Leu-Leu-OMe. The effect of Leu-Leu-OMe on the generation of antigen-specific cytotoxic T cells was measured by incubating responder PBMC and marrow cells with Leu-Leu-OMe (day 0 treatment), and then testing these cells in a standard CML assay after 7 days of culture in B-MLC. Treatment with 1000 μ M Leu-Leu-OMe eliminated the generation of cytolytic activity against ^{51} Cr-labeled alloantigen-specific Con A-stimulated PBMC (Fig. 2). These results indicated that the generation of CTL from precursors is sensitive to incubation with Leu-Leu-OMe. The effect of Leu-Leu-OMe incubation on CTL already generated in 7-day bulk MLC (day 7 treatment) was also evaluated. This treatment eliminated antigen-specific cytolytic activity (Fig. 2). Treatment with 0.5% MeOH, either on day 0 or day 7, did not interfere with either the development of CTL or the specific cytotoxicity of targets assayed on day 7 (data not shown).

Elimination of NK activity after incubation of PBMC and marrow cells with Leu-Leu-OMe. Marked diminution of NK activity, in a dose-dependent manner, was seen after incubation of PBMC or marrow cells with Leu-Leu-OMe, and NK activity was essentially eliminated after treatment with 1000 μ M Leu-Leu-OMe (Fig. 3).

In vitro CFU-GM colony growth reduction by Leu-Leu-OMe

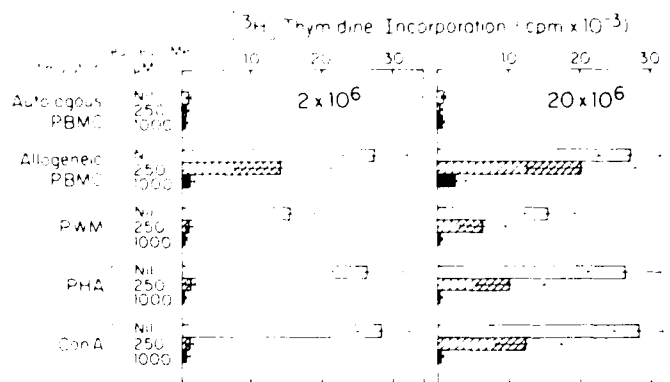


FIGURE 1. Effect of Leu-Leu-OMe on alloantigen- and mitogen-induced lymphocyte blastogenesis. Data for [^{3} H]thymidine incorporation are mean $\text{cpm} \pm \text{SEM}$ from multiple assays in which PBMC were treated at concentration of 2×10^6 or 20×10^6 cells/ml.

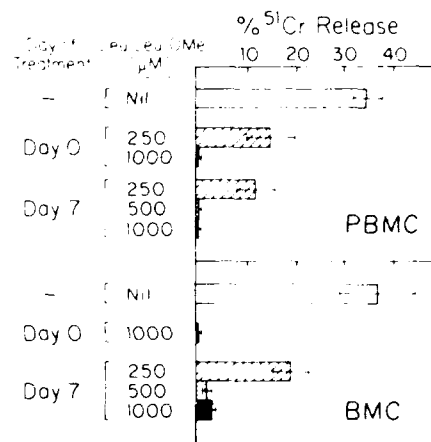


FIGURE 2. CML assays illustrating the effect of Leu-Leu-OMe treatment on p-CTL (day 0) or on 7-day B-MLC generated CTL (day 7), derived either from PBMC or BMC. The ordinate indicates mean percentage of ^{51}Cr release $\pm \text{SEM}$ using specific alloantigen-sensitizing ^{51}Cr -labeled Con A-stimulated PBMC as targets.

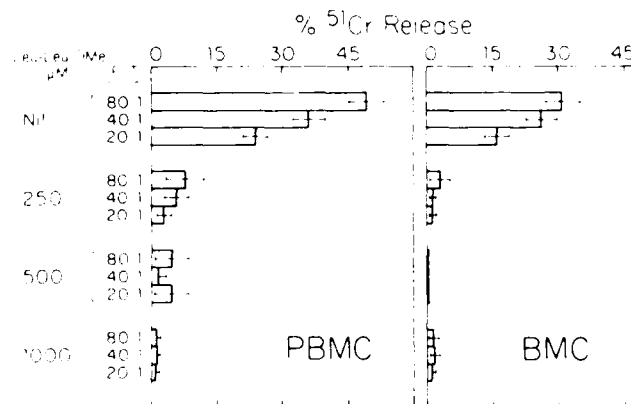


FIGURE 3. Leu-Leu-OMe treatment of PBMC and BMC eliminates NK cytotoxic activity. The ordinate indicates mean percentage of ^{51}Cr release $\pm \text{SEM}$ from CTAC at three different effector:target (E:T) ratios. For this series of assays, the mean spontaneous/maximum ^{51}Cr release ratio was $23 \pm 1\%$ ($\pm \text{SEM}$) and the maximum release/total ^{51}Cr incorporation ratio was $65 \pm 7\%$.

treatment of marrow cells. There was a marked reduction in CFU-GM colony growth obtained from marrow cells incubated with varying concentrations of Leu-Leu-OMe (Table 1). Minimal CFU-GM growth was observed with 10^5 Leu-Leu-OMe treated marrow cells per plate were cultured. There was some recovery of CFU-GM growth when the cell number was increased to 3×10^5 treated marrow cells per plate, but only to a level that was approximately 25% of that observed with 3×10^5 untreated marrow cells. Addition of either untreated, autologous PBMC or Mø-enriched autologous PBMC increased CFU-GM growth, but not to the levels observed with untreated marrow cells in similar cocultures. The addition of Mø-depleted or Leu-Leu-OMe treated autologous PBMC to treated marrow cells did not augment growth.

The effect of incubating marrow cells with Leu-Leu-OMe on autologous marrow engraftment. Six of the seven dogs given Leu-Leu-OMe treated autologous marrow engrafted and did so with kinetics similar to those seen in recipients of untreated autologous marrow (Table 2 and Fig. 4). Platelet counts returned to normal levels between 20 to 30 days posttransplant. Dog C521 did not survive past day 20 posttransplant. After Leu-Leu-OMe incubation, the marrow cells from this dog were clumped and had only 20% viability, resulting in the infusion of a very low marrow cell dose. The marrow from C580 was incubated with the same concentration of Leu-Leu-OMe as

C521, but the treated marrow cells of C580 did not clump and this dog survived with rapid engraftment. Dog BB8701 had to be euthanized on day 19 posttransplant due to an accidental, severe, foot injury that failed to respond to treatment, but did show evidence of engraftment as indicated by a rise in WBC and the marrow cellularity at autopsy.

DISCUSSION

Thiele and Lipsky have demonstrated that exposure of mouse spleen cells or human peripheral blood leukocytes to Leu-Leu-OMe depletes these populations of monocytes, NK cells, and cytotoxic lymphocytes at both the precursor and effector stages of differentiation, without apparently affecting other cell populations (11-13). They further showed that incubation of mixtures of murine marrow and spleen cells with Leu-Leu-OMe did not interfere with engraftment and could, in certain circumstances, prevent GVHD (14-16). In the studies presented here, we found that canine peripheral blood and marrow cells behave similarly following exposure to Leu-Leu-OMe with the elimination of functional monocytes, NK cells, and alloantigen-sensitized CTL, and inhibited the development of CTL from pCTL. Further, we found that incubation of marrow cells with Leu-Leu-OMe (even at very high doses) did not inhibit autologous engraftment in recipients conditioned with 920 cGy TBI. These studies also demonstrated that the treatment of canine

TABLE 1. In vitro CFU-GM colony growth from marrow cells incubated with varying concentrations of Leu-Leu-OMe

Leu-Leu-OMe (μ M)	CFU-GM colonies obtained from CFU-C cultures ^a									
	10 ⁵ BMC cocultures ^b					3 $\times$ 10 ⁵ BMC cocultures ^c				
	No coculture	10 ⁵ PBMC	10 ⁵ Mø enriched	10 ⁵ Mø depleted	10 ⁵ Leu-Leu-OMe treated PBMC	No coculture	10 ⁵ PBMC	10 ⁵ Mø enriched	10 ⁵ Mø depleted	10 ⁵ Leu-Leu-OMe treated PBMC
Nil	66 $\pm$ 13	84 $\pm$ 9	96 $\pm$ 15	76 $\pm$ 23	78 $\pm$ 13	207 $\pm$ 15	255 $\pm$ 18	348 $\pm$ 9	288 $\pm$ 7	252 $\pm$ 17
250	0.4 $\pm$ 0.2	64 $\pm$ 32				55 $\pm$ 31	178 $\pm$ 63			
500	0	14 $\pm$ 8				3 $\pm$ 2	51 $\pm$ 24			
1000	1.2 $\pm$ 0.5	10 $\pm$ 3	29 $\pm$ 11	2 $\pm$ 1	2 $\pm$ 0.6	8 $\pm$ 3	42 $\pm$ 3	74 $\pm$ 29	0.3 $\pm$ 0.3	3 $\pm$ 1
2000	2 $\pm$ 1	5 $\pm$ 2				2 $\pm$ 0.8	13 $\pm$ 5			
4000	0.7 $\pm$ 0.6	11 $\pm$ 5				0.9 $\pm$ 0.5	16 $\pm$ 9			

^a Represented are mean values $\pm$ SEM, obtained from multiple experiments in which triplicate CFU-GM cultures were established for each parameter tested.

^b All cocultures employed autologous cells. The limited yield of Mø prevented coculture with all concentrations of Leu-Leu-OMe tested. There was no CFU-GM growth when untreated PBMC (between 10^5 to 6×10^5 /ml) were cultured without marrow cells.

^c The concentration of marrow cells treated was constant at 20×10^6 /ml.

TABLE 2. Recipients conditioned with 920 cGy TBI and receiving Leu-Leu-OMe treated autologous marrow^a

Dog ID	[Leu-Leu-OMe] ^b (μ M)	No. viable treated BMC ^c infused (10^6 /kg)	Survival (days post- BMT)	Cause of death	Marrow cellularity
C563	1000	1.4	152	Sodium pentothal ^d	Normocellularity (3 cell lines)
BB8701	1000	0.7	19	Sodium pentothal ^d	75% of Normocellularity (3 cell lines)
C622	1000	1.41	>124	Still living	Normocellularity (3 cell lines)
C521	2000	0.052	20	Pneumonia; sepsis	Focal hematopoiesis
C580	2000	1.67	173	Sodium pentothal ^d	Normocellularity (3 cell lines)
B950	4000	1.3	114	Sodium pentothal ^d	Normocellularity (3 cell lines)
C662	4000	1.5	112	Sodium pentothal ^d	Normocellularity (3 cell lines)

^a Autologous marrow aspiration, BMC treatment, and infusion on same day as 920 cGy TBI to recipient (BMT: bone marrow transplant).

^b 20×10^6 BMC/ml treated with Leu-Leu-OMe.

^c Viability of bone marrow cells infused determined by trypan-blue stain exclusion technique.

^d Sodium pentothal injection for euthanasia at end of study.

^e The Leu-Leu-OMe treated autologous marrow was frozen and stored at -80°C for one week prior to reinfusion. The procedure for marrow cryopreservation was as described (26).

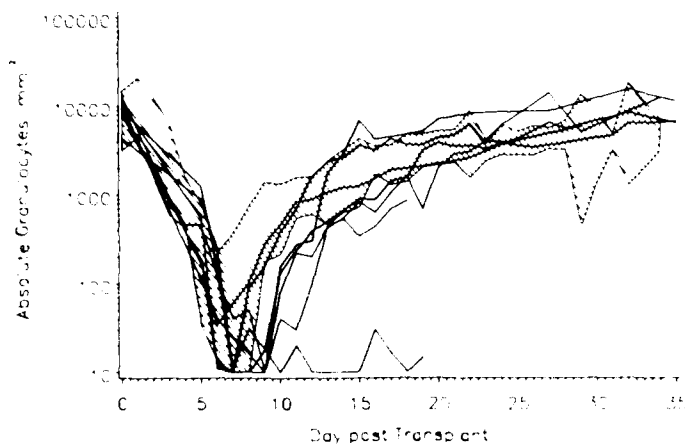


FIGURE 4. Hematologic recovery in recipients conditioned with 920 cGy TBI and infused with Leu-Leu-OMe treated autologous marrow. The ordinate indicates the absolute granulocyte counts obtained daily posttransplant until death or recovery to the normal range. The shaded area represents the normal range (mean $\pm$ SD) of recovery observed in 16 dogs conditioned with 920 cGy TBI and given untreated autologous marrow (28).

marrow cells with Leu-Leu-OMe, in a concentration-dependent fashion, could reduce or eliminate *in vitro* CFU-GM growth. This reduction could be partially reversed with the addition of unfractionated autologous PBMC or M ϕ -enriched PBMC but not with M ϕ -depleted PBMC or autologous PBMC treated *in vitro* with Leu-Leu-OMe. Our assay employs postendotoxin-treated dog serum as a source of colony-stimulating factor. Reversal of the effects of Leu-Leu-OMe on CFU-GM growth by monocytes demonstrated the monocyte dependence of this CFU-GM assay system and suggests that PEDS does not act directly as a single factor on the granulocyte/macrophage progenitors.

Marrows were treated at two different cell concentrations in anticipation that this procedure might be useful for allogeneic marrow transplantation in large animals and possibly in man. Reduction or elimination of various cells involved either in response to alloantigen stimulation (MLC, pCTL, CTL) or NK function could be accomplished by treating PBMC or marrow cells at 20×10^6 /ml with 1000 μ M Leu-Leu-OMe. The murine models using H2-disparate P $\rightarrow$ F $_1$ marrow donor/recipient pairing showed that Leu-Leu-OMe treatment could prevent acute GVHD. The resultant chimeras developed normal cellular immune responses and showed donor and host-specific immunologic tolerance (16). The cell concentration treated in those studies was 2×10^6 /ml, and the highest concentration of Leu-Leu-OMe used was 250 μ M. Cell concentration directly influenced the concentration of Leu-Leu-OMe required to achieve a given effect. Thus, in the present study when the cell concentration was increased tenfold, the concentration of Leu-Leu-OMe had to be increased approximately fourfold to achieve the same inhibition of cellular immune functions. The potential problem of larger cell numbers and expanded marrow volumes needed for the transplantation of larger animals can be circumvented by increasing the concentration of Leu-Leu-OMe.

Data in the canine model suggest that Leu-Leu-OMe should be explored further as a possible substitute for other currently used methods of marrow T cell depletion. It needs to be determined whether treatment of marrow with Leu-Leu-OMe will

leave behind a population of cells that facilitate engraftment and recovery of immunity while removing cytotoxic T cells that may comprise the major population of cells involved in the development of acute GVHD.

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Stimulation of Canine Hematopoiesis by Recombinant Human Granulocyte-Macrophage Colony-stimulating Factor

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Abstract. The effect of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) on canine hematopoiesis was evaluated. rhGM-CSF stimulated granulocyte-macrophage colony formation of canine marrow depleted of accessory cells up to tenfold. Stimulation of colony formation was abrogated by anti-rhGM-CSF antiserum or heat inactivation. rhGM-CSF also stimulated *in vivo* canine hematopoiesis both when given as continuous *i.v.* infusion and as intermittent *s.c.* injections. Neutrophil, monocyte, and lymphocyte counts were increased three- to eightfold above controls, whereas values for eosinophils, reticulocytes, and hematocrits were not changed. Bone marrow histology after 2 weeks of treatment with rhGM-CSF showed hypercellularity with myeloid hyperplasia and left-shifted granulocytopenia. After discontinuation of rhGM-CSF, peripheral leukocyte counts returned to control level within 3–7 days. Platelet counts decreased rapidly after starting rhGM-CSF, to 5000–15,000 platelets/mm³, and increased within 24 h after stopping rhGM-CSF treatment, whereas marrow histology after 2 weeks of rhGM-CSF application showed the normal number and morphology of megakaryocytes.

Key words: GM-CSF — Canine hematopoiesis

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a T-cell-derived lymphokine with a broad range of biological activities. It stimulates the proliferation of neutrophil, macrophage, and eosinophil colonies and, in the presence of erythropoietin, supports the proliferation of erythroid and megakaryocytic progenitors [1–3]. GM-CSF also represents an important functional activator of granulocytes and monocytes [2, 3]. Human GM-CSF in its native form is a variably glycosylated protein with a molecular weight of 15,000–31,000 daltons [3]. The cloning of the gene encoding human GM-CSF [4–8] and the expression of this recombinant protein in yeast [9], mammalian cells [10], and *Escherichia coli* [11] made it possible to study the biological activities of this hormone *in vitro* and *in vivo*.

This study describes stimulation of canine granulocyte-macrophage colony-forming unit (CFU-GM) colony formation and increase of dog peripheral blood neutrophils,

monocytes, and lymphocytes by recombinant human GM-CSF (rhGM-CSF).

Materials and methods

Experimental animals. Twelve dogs of both sexes and various breeds (beagles, foxhounds, or mongrels), 6–14 months of age, were treated with rhGM-CSF or were used as a source of marrow for *in vitro* culture assays. Animals were either raised at the Fred Hutchinson Cancer Research Center (FHCRC) or purchased from commercial US Department of Agriculture licensed dealers. Dogs were quarantined on arrival for a minimum of 2 months before being released for use. All dogs were dewormed and vaccinated for rabies, distemper, leptospirosis, hepatitis, and parvovirus. They were housed in an American Association for Accreditation of Laboratory Animal Care accredited facility in standard indoor runs, and provided commercial dog chow and chlorinated tap water *ad libitum*. Animal holding areas were maintained at 70° ± 2°F with 50% ± 10% relative humidity using at least 15 air changes/h of 100% conditioned fresh air. The dogs were on a 12-h light dark full spectrum lighting cycle with no twilight. The protocol for this study was approved by the internal animal use committee of the FHCRC.

rhGM-CSF. The rhGM-CSF used in this study was prepared and provided by Immunex Corporation (Seattle, Washington). Human rhGM-CSF cDNA was isolated from a library constructed by using mRNA from the human T-cell line HUT-102 [4], inserted into and expressed in yeast, and purified to homogeneity by reversed-phase high-performance liquid chromatography [5]. The purified rhGM-CSF had a specific activity of ~5 × 10⁵ colony-forming units/mg of protein. It consisted of three molecular weight species of 15,500, 16,800, and 19,500 daltons, due to differences in glycosylation. The three species were present in approximately equal proportions. Endotoxin contamination was < 1 ng/mg of protein as measured by the *Limulus* amoebocyte lysate assay.

Rabbit antiserum to rhGM-CSF. Rabbit antiserum to rhGM-CSF was provided by Immunex Corporation and was prepared by immunizing rabbits with purified rhGM-CSF. The animals were bled 10 days following the last immunization. Serum dilution of 1:100 was effective in neutralizing 30 ng/ml of rhGM-CSF in the human bone marrow proliferation assay.

Treatment of dogs with rhGM-CSF. Dogs were given rhGM-CSF either *s.c.* three times a day, or by continuous *i.v.* infusion, using a central venous catheter and a portable infusion pump (Infumed 200, Med Fusion Systems Incorporated, Norcross, Georgia). Central venous catheters were inserted under general anesthesia through the external jugular vein 7 days before starting the experiment to allow for healing of the wound. The catheter was connected to an Infumed 200 portable infusion pump, which was placed in a jacket fitted around the trunk of the dog. The reservoir of the pump was filled daily with 10, 30, or 90 µg/kg rhGM-CSF in 50 ml 0.9% NaCl

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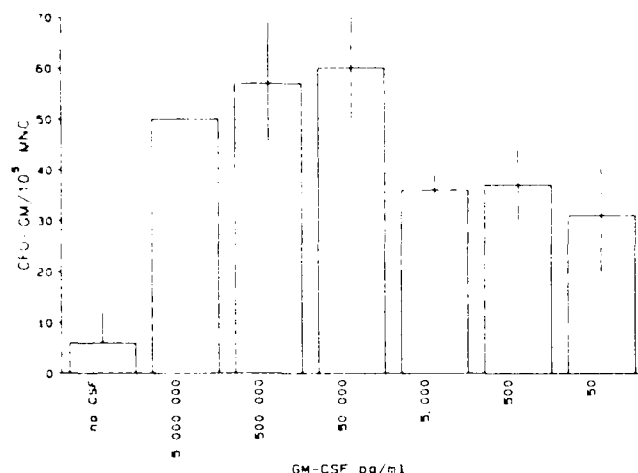


Fig. 1. Effect of rhGM-CSF on canine CFU-GM colony formation. MNC (3×10^4 plate) depleted of monocytes and T-lymphocytes were cultured for 10 days in agar medium without and with addition of rhGM-CSF at decreasing concentrations. Culture were plated in triplicate. Data represent mean values $\pm$ 1 SD of two separate experiments.

containing 1% normal dog serum. The infusion rate was 2.5 ml/h at an accuracy of $\pm$ 5%. Control dogs received either human serum albumin or heat-inactivated rhGM-CSF (100°C, 30 min).

Marrow aspiration and biopsy. For marrow aspiration dogs were anesthetized by i.v. injection of Fentanyl (Innovar-Vet) at 0.03 mg/kg. The collection site was aseptically cleansed with a povidone-iodine scrub and alcohol rinse. Bone marrow was obtained from the humeral condyle via needle aspiration using a sterile 20-ml syringe and 14-gauge disposable needle. Bone marrow was collected (10 ml) from each dog at 3-week intervals. Marrow biopsies were obtained from the humeral condyle using Jamshidi bone marrow needles (American Pharmaseal Company, Valencia, California).

Depletion of monocytes and T-lymphocytes from marrow. Bone marrow was diluted 1:3 with RPMI-1640 medium (M.A. Bioproducts, Walkersville, Maryland) and separated by density gradient centrifugation over Ficoll-Hypaque (density 1.077 g/ml) at 1000 g for 30 min. Cells at the interface were washed twice in RPMI-1640 medium and resuspended at 7×10^6 mononuclear marrow cells (MNC) per ml in RPMI-1640 medium containing 10% fetal calf serum (FCS, GIBCO, Grand Island, New York). To remove monocytes, 15 ml of this cell suspension were incubated for 1 h at 37°C in a humid atmosphere of 10% CO₂ in air in the presence of 200 mg carbonyl iron. Following incubation, the cells containing carbonyl iron were removed by a strong magnetic field. The remaining monocyte-depleted cells were then depleted of T-lymphocytes by direct panning using anti-Thy 1 monoclonal antibody (mAb) F3-20-7 [12]. Protein A-Sepharose-purified mAb F3-20-7 was diluted with phosphate-buffered saline (PBS, pH 7.2) to a final concentration of 50 μ g/ml. Six ml of mAb containing PBS were pipetted into 75-cm² coated-neck flasks (Corning, Corning, New York) and incubated for 30 min at 4°C. Flasks were then gently washed five times with calcium- and magnesium-free PBS. Marrow cells were suspended in calcium- and magnesium-free Hanks' solution (GIBCO) containing 20 μ g HEPES (GIBCO) and 2% FCS at a concentration of 1×10^6 MNC/ml. Up to 15×10^6 MNC were incubated in each flask at 4°C for 1 h. Unattached cells were removed and panning was repeated two more times. After depletion, marrow contained $< 2\%$ monocytes as measured by morphology. The efficiency of T-lymphocyte depletion was determined by incubating marrow cells with mAb F3-20-7 at saturation for 30 min at 4°C. The cells were then washed and incubated with a 1:20 dilution of fluorescein isothiocyanate-conjugated F(ab)₂ fragments of goat antimouse IgG (Cigo, Burlingame, California). After further washing, cells were analyzed using a fluorescence-activated cell sorter (FACS 440, Becton Dickinson FACS Systems,

Mountainview, California). Less than 2% Thy-1 positive cells were detected.

CFU-GM assay. CFU-GM assays were carried out as described [13, 14]. MNC separated over a Ficoll-Hypaque gradient and depleted of monocytes and T-lymphocytes as described above were cultured at 0.03×10^6 plate for 10 days at 37°C in a humidified 10% CO₂ incubator in 35-mm plastic petri dishes containing 2 ml of agar medium. The agar medium consisted of an equal volume mixture of 0.6% (wt/vol) Bacto-Agar (Difco, Detroit, Michigan) and double-strength Dulbecco's modified Eagle's medium (GIBCO) containing 40% (vol/vol) heat-inactivated FCS. Postendotoxin dog serum or rhGM-CSF was added to the petri dishes (0.1 ml/plate) prior to the agar-medium mixture containing the bone marrow cells.

Results

Stimulation of canine CFU-GM colony formation by rhGM-CSF

Recombinant human GM-CSF was added to the CFU-GM cultures at different concentrations. Culture plates without any addition of growth factor served as negative control and plates with 10% postendotoxin dog serum as positive control. In two separate experiments, an up to tenfold stimulation of canine CFU-GM colony formation by rhGM-CSF was observed compared to the negative control (Fig. 1). In a separate experiment, rhGM-CSF was either incubated for 30 min with anti-rhGM-CSF antiserum (diluted 1:100) or heat-inactivated at 100°C for 30 min before adding the growth factor to the culture plates. The unmanipulated rhGM-CSF stimulated CFU-GM colony formation up to fourfold compared to the negative control. Recombinant human GM-CSF treated with either anti-rhGM-CSF antiserum or heat inactivation did not show any stimulatory activity above the colony growth in the negative control (Table 1). Anti-rhGM-CSF antiserum by itself did not have any effect on CFU-GM colony growth (data not shown). These results are consistent with the notion that rhGM-CSF specifically interacts with canine CFU-GM.

Stimulation of hematopoiesis in dogs by continuous i.v. infusion of rhGM-CSF

Three dogs were treated with 10, 30, or 90 μ g rhGM-CSF per kg/day by continuous i.v. infusion for 14 days. Recombinant human GM-CSF was well tolerated and no systemic toxicity was observed over the dose range studied. Dogs showed increased neutrophil counts within 24 h of starting the infusion (Fig. 2a). By day 14 neutrophil counts reached levels that were three to six times higher than the preinfusion counts and those of two control dogs receiving either continuous i.v. infusion of 10 μ g of human serum albumin per kg/day for 14 days (range indicated by two horizontal lines) or 30 μ g heat-inactivated rhGM-CSF per kg/day. The marrows in rhGM-CSF-treated dogs on day 14 were hypercellular with myeloid hyperplasia and left-shifted granulocytopenia. After discontinuation of rhGM-CSF, the neutrophil counts returned to control levels within 3–7 days. Whereas neutrophil counts increased at all three dosages of rhGM-CSF to about the same extent, monocyte and lymphocyte counts were markedly increased (eight- and two- to fourfold, respectively) only at the two higher rhGM-CSF dosages (Fig. 2b and c). Eosinophil and reticulocyte counts and hemato-

Table 1. Stimulation of canine CFU-GM colony formation by rhGM-CSF

	CFU-GM 10^4 MNC								10% Peds
	No GM-CSF	5×10^{-7} g/ml GM-CSF	5×10^{-6} g/ml GM-CSF	5×10^{-5} g/ml GM-CSF	5×10^{-4} g/ml GM-CSF	5×10^{-3} g/ml GM-CSF	5×10^{-2} g/ml GM-CSF	5×10^{-1} g/ml GM-CSF	
GM-CSF	19 $\pm$ 4	23 $\pm$ 3	34 $\pm$ 1	34 $\pm$ 3	69 $\pm$ 7	81 $\pm$ 10	68 $\pm$ 17	40 $\pm$ 3	372 $\pm$ 76
Rabbit α GM-CSF plus GM-CSF	19 $\pm$ 4	11 $\pm$ 3	20 $\pm$ 6	15 $\pm$ 6	11 $\pm$ 6	5 $\pm$ 3	6 $\pm$ 0	13 $\pm$ 4	—
Heat-inactivated GM-CSF (100°C, 30 min)	19 $\pm$ 4	3 $\pm$ 1	13 $\pm$ 4	11 $\pm$ 3	17 $\pm$ 8	8 $\pm$ 4	5 $\pm$ 3	6 $\pm$ 4	—

MNC (3×10^4 plate) depleted of monocytes and T-lymphocytes were cultured for 10 days in agar medium without and with rhGM-CSF at decreasing concentrations. Cultures were plated in triplicate. Colony numbers represent mean value $\pm$ 1 SD. Peds, postendotoxin dog serum; α GM-CSF, anti GM-CSF antiserum.

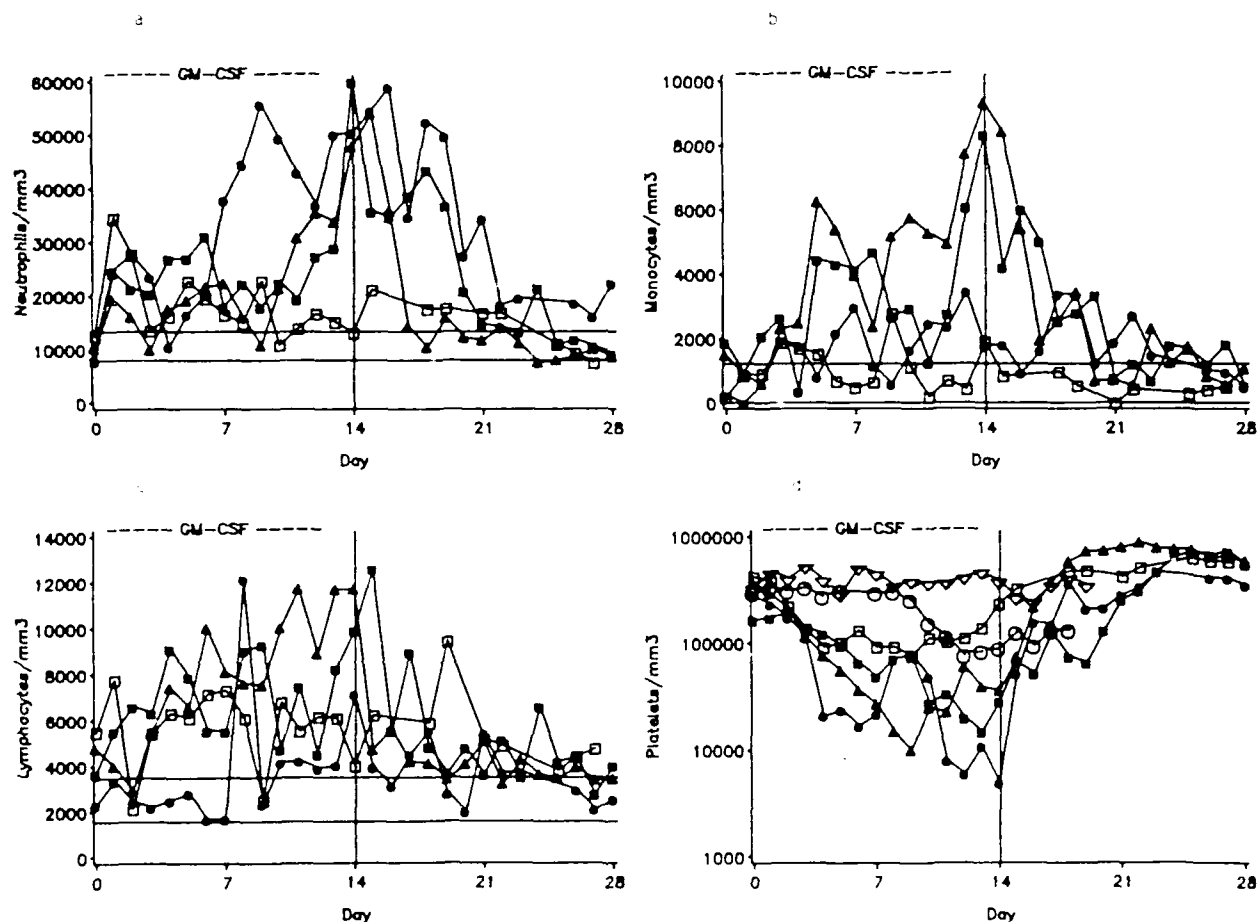


Fig. 2. Effect of continuous (14 day) i.v. infusion of rhGM-CSF on peripheral blood neutrophils (a), monocytes (b), lymphocytes (c), and platelets (d) of four normal dogs. Recombinant human GM-CSF was given at 10 (●), 30 (■), or 90 (▲) μ g/kg/day. A control dog received 30 μ g heat-inactivated (100°C, 30 min) rhGM-CSF per kg/day (□). The two horizontal lines indicate the range of the peripheral blood counts of a control dog given continuous i.v. infusion of 10 μ g human serum albumin per kg/day for 14 days. The open triangles in d represent the platelet counts of a control dog receiving continuous i.v. saline infusion for 14 days, the open circles represent the platelet counts of a control dog receiving a continuous i.v. infusion of 10 μ g human serum albumin per kg/day for 14 days.

crits during rhGM-CSF infusion were similar to the pre-infusion levels and the levels in the control dogs receiving human serum albumin or heat-inactivated rhGM-CSF (data not shown).

The three dogs receiving active rhGM-CSF showed declines in platelet counts with nadirs ranging from 5000 to 15,000 mm^3 (Fig. 2d). After discontinuing the infusion of rhGM-CSF, platelet counts increased rapidly above pre-

infusion levels. By comparison, the platelet counts of a saline control remained virtually unchanged, whereas the counts of a human serum albumin control decreased after 10 days of infusion to about 100,000. The control dog receiving heat-inactivated rhGM-CSF also had a decrease of platelets to about 100,000 mm^3 , but this decrease occurred 4 days after rhGM-CSF infusion. Bone marrow histology on day 14 of rhGM-CSF infusion showed normal number and morphol-

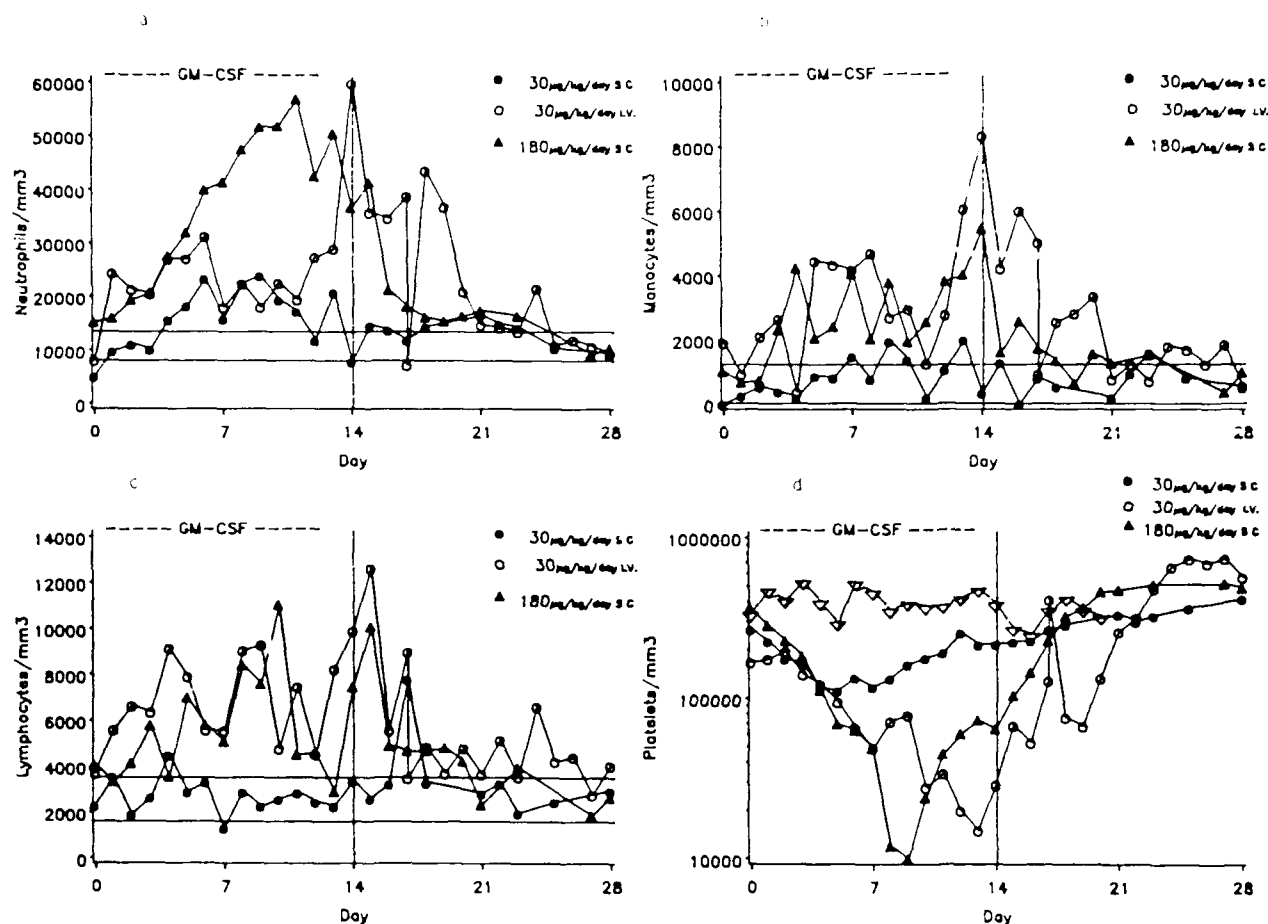


Fig. 3. Effect of intermittent (three times a day) s.c. injection (14 days) of rhGM-CSF on peripheral blood neutrophils (a), monocytes (b), lymphocytes (c), and platelets (d) of two normal dogs. Recombinant human GM-CSF was given at 30 (●) or 180 (▲) µg/kg/day. For comparison, the results seen in a dog receiving 30 µg/kg/day rhGM-CSF by continuous i.v. infusion are shown (○). The two horizontal lines indicate the range of the peripheral blood counts of a control dog given continuous i.v. infusion of 10 µg human serum albumin per kg/day for 14 days. The open triangles in d represent the platelet counts of a control dog receiving continuous i.v. saline infusion for 14 days.

ogy of megakaryocytes. Preliminary results of platelet survival studies using ^{51}Cr -labeled autologous platelets suggest that platelet survival is decreased to 50% of normal in the first week after starting rhGM-CSF infusion, compared to normal survival times in dogs given saline or human serum albumin.

Stimulation of hematopoiesis in dogs by intermittent s.c. injection of rhGM-CSF

One dog was treated with 30 µg rhGM-CSF per kg/day and two dogs received 180 µg rhGM-CSF per kg/day by intermittent s.c. injections three times a day for 14 days. The s.c. administration of rhGM-CSF was well tolerated and no systemic toxicity was observed over the dose range studied. Recombinant human GM-CSF at a dose of 30 µg/kg/day s.c. increased the neutrophil counts twofold compared to the preinfusion counts and those of a control dog receiving 10 µg human serum albumin per kg/day by continuous i.v. infusion for 14 days (range indicated by two horizontal lines) (Fig. 3a). Monocyte and lymphocyte counts were not increased (Fig. 3b and c). An s.c. dose of 180 µg rhGM-CSF per kg/day increased the number of neutrophils fivefold above

control (Fig. 3a), and monocyte and lymphocyte counts were increased fivefold and fourfold, respectively (Fig. 3b and c; data from the second dog were similar and are not shown). Compared to results obtained by continuous i.v. infusion of 30 µg rhGM-CSF per kg/day, the intermittent s.c. injection of the same amount of rhGM-CSF seemed to be less effective in stimulating peripheral blood counts (Fig. 3a-c).

Platelet counts decreased to only about 100,000 mm^{-3} when 30 µg/kg/day of rhGM-CSF was given s.c. (Fig. 3d); with a dose of 180 µg/kg/day, platelets declined to 10,000 mm^{-3} , similar to what was seen with continuous i.v. infusion of 30 µg/kg/day.

Discussion

This study shows that rhGM-CSF stimulates canine hematopoiesis *in vitro* as well as *in vivo*. Recombinant human GM-CSF increased CFU-GM colony formation of canine marrow depleted of accessory cells, suggesting that rhGM-CSF acts directly on canine CFU-GM. Stimulation of canine CFU-GM by rhGM-CSF seems to be specific because neutralization with rabbit antiserum directed against rhGM-CSF, as well as heat inactivation, abrogated the stimulatory ca-

capacity of the growth factor. Endotoxin as a possible cause of colony stimulation is unlikely because the GM-CSF used was endotoxin free.

Infusion of rhGM-CSF into dogs resulted first in a fast, but transient, elevation of neutrophil counts beginning 24 h after starting infusion. This initial increase was most likely due to demargination or release of mature cells from the marrow [15]. It was followed by a gradual and sustained increase in circulating neutrophils during the 2-week treatment period consistent with increased production of neutrophils in response to rhGM-CSF. This assumption was confirmed by the finding of increased bone marrow cellularity with myeloid hyperplasia and left-shifted granulocytopoiesis on day 14 of treatment with the factor. After discontinuation of rhGM-CSF, the neutrophils returned to baseline level within 3–7 days, indicating that the effect of rhGM-CSF is only transient. At higher dosages rhGM-CSF stimulated lymphoid progenitor cells. Because lymphoid cells have been reported to lack receptors for rhGM-CSF [16], this stimulation is likely to be mediated through accessory cells. Peripheral monocyte counts were also increased by rhGM-CSF whereas no elevations of eosinophil, reticulocyte, and platelet counts were seen. rhGM-CSF also stimulated hematopoiesis when given by intermittent s.c. injection, although s.c. doses needed to obtain given elevation in neutrophil levels were higher than i.v. doses.

The stimulatory activity of rhGM-CSF in dogs showed similarities with but also differences from that in nonhuman primates [17] and in mice [18]. Leukocyte counts both in dogs and monkeys reached levels up to six times above controls, mainly due to increases in neutrophils. However, eosinophil counts remained unchanged in dogs, but were increased up to 30-fold in monkeys. After rhGM-CSF infusion was stopped, peripheral leukocyte counts in dogs returned to control level within 3–7 days, similar to results in monkeys. Mice injected i.p. with recombinant murine GM-CSF developed only a twofold increase in blood neutrophils [18]. In contrast, a 10- to 100-fold rise of neutrophils and eosinophils and an up to 15-fold increase of macrophage counts in the peritoneal cavity was seen. Neutrophils and monocytes were also increased in liver, lung, and spleen, whereas the number of myeloid cells in the bone marrow remained unchanged. The effect of GM-CSF on murine hematopoiesis seems to be different from the one observed in monkeys and dogs. This may be due to the i.p. route of administration.

Recombinant human GM-CSF increases peripheral blood neutrophils, eosinophils, and monocytes in cytopenic patients with myelodysplastic syndrome [15, 19], aplastic anemia [19], acquired immunodeficiency syndrome (AIDS) [20], and primary or secondary bone marrow failure [21]. In some of these patients an improvement of reticulocyte and platelet counts was also observed [15, 19, 21], although this was not a consistent finding. Recombinant human GM-CSF also shortens the period of neutropenia after chemotherapy-induced myelosuppression [22] and after autologous marrow transplantation in nonhuman primates [23, 24] and in man [25, 26].

With both continuous i.v. as well as intermittent s.c. application of rhGM-CSF, we observed a rapid drop of platelet counts in dogs. Because bone marrow histology showed normal numbers and morphology of megakaryocytes and be-

cause platelets increased after discontinuing rhGM-CSF infusion rapidly above preinfusion levels, it seems unlikely that the thrombocytopenia was caused by a deficient platelet production in the marrow. Preliminary experiments showed a decreased survival of ^{51}Cr -labeled autologous platelets following infusion of rhGM-CSF. The cause of this complication is currently unclear.

The effect of rhGM-CSF on platelet counts in man is unclear and may depend on the clinical situation, but severe thrombocytopenia seems to be an exceptional complication. Vadhan-Raj et al. reported that three of eight patients with myelodysplastic syndrome treated with rhGM-CSF showed increases in platelet counts, whereas two patients had transient decreases [15]. Anun et al. did not observe any effect on platelet counts when treating patients with aplastic anemia or myelodysplastic syndrome with rhGM-CSF [19]. Similarly, Groopman et al. did not see any changes in platelet counts in leukopenic AIDS patients receiving rhGM-CSF [20]. In contrast, Antman et al. reported that in patients with sarcoma and normal peripheral leukocyte and platelet counts, platelet counts decreased during treatment with rhGM-CSF [22]. A patient treated with rhGM-CSF because of poor engraftment after autologous marrow transplantation for nonHodgkin's lymphoma developed diffuse thrombocytopenic bleeding during the infusion of rhGM-CSF that stopped when rhGM-CSF infusion was discontinued (S. Slichter, personal communication). Brandt et al. described no consistent effect of rhGM-CSF on platelet counts in patients with breast cancer or myeloma treated with high-dose combination chemotherapy and autologous bone marrow transplantation support [25]. Nemunaitis et al. observed in a retrospective comparison accelerated recovery of platelet counts after autologous marrow transplantation for lymphoid malignancies when rhGM-CSF was given [26]. Accelerated platelet recovery with rhGM-CSF was also described in two reports on monkeys transplanted with autologous marrow [23, 24].

In summary, we have shown that rhGM-CSF cross-reacts with canine hematopoietic progenitor cells. It stimulates *in vitro* CFU-GM colony formation specifically through direct stimulation of canine hematopoietic progenitor cells. Recombinant human GM-CSF also stimulates *in vivo* canine hematopoiesis both when given as continuous i.v. infusion and as intermittent s.c. injections.

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Letter to the Editor

Response to: 'Are solitons responsible for energy transfer in oriented DNA?'

Experiments by Arroyo *et al.* (1986) indicated that free radical yields in oriented DNA exposed to neutrons depend on the orientation of the DNA molecules relative to the direction of the incident radiation flux. This effect could be explained provided a large asymmetry in the transport of energy existed in the oriented DNA sample (Miller *et al.* 1988).

Quantitative calculations assumed an asymmetry of a thousand for the ratio of parallel to perpendicular transport. Although, the carrier of this excess energy was not identified, Monte Carlo calculations predicted an average energy migration distance of the order of 50 to 150 nm. Baverstock and Cundall (1989) have recently suggested that the carrier could be a soliton-like state since these quasi particles have been shown to exist in other polymeric systems and are known to transfer energy without loss over large distances (Heeger *et al.* 1988). There is no direct experimental evidence supporting this view; nevertheless, it is an attractive hypothesis since singlet and triplet exciton migration within the DNA is probably limited to at most 10-30 base pairs due to the effects of diagonal disorder and their short lifetime.

Several experiments support the latter contention. We note that the fluorescence of double stranded DNA at 77K is considerably red-shifted and broadened as compared to that observed in the corresponding mixtures of mononucleotides (Eisinger and Lamola 1971) and is ascribed to exciplex formation between neighboring stacked bases. Since exciplexes have lower energy than individual bases their energy cannot be transferred to other bases; hence, the excited singlet state is trapped. Exciplex formation is a rapid process, estimated to be the order of 10^{12} s^{-1} (Birks 1970), and thus any singlet transfer of energy must occur prior to relaxation. The maximum singlet transfer distance, d_s , can be estimated from the relationship, $d_s^2 = 2Fa^2t_T$, where F is the nearest neighbour Förster transfer rate, presumably the order of 10^{13} s^{-1} if transfer is to occur before exciplex formation, a is the neighbour base pair stacking distance; 0.34 nm in B-DNA and t_T is the trapping time, the order of the exciplex formation time $\sim 10^{-12} \text{ s}$; hence, $d_s \sim 1.5 \text{ nm}$. Transfer distances this short are consistent with reported data on thymine phosphorescence (I_p) at 77K as a function of adenine-thymine (A-T) content, which demonstrated that the linear dependence of I_p^{-1} on n^{-1} (n the number of base pairs) could only be reconciled with singlet before relaxation transfer because only excitations trapped on A-T sites give rise to the thymine triplet; whereas, singlet states on guanine-cytosine (G-C) sites are rapidly quenched (Guéron and Shulman 1968).

The probability of energy transfer in the DNA triplet state has been discussed by Eisinger and Lamola (1971), Guéron and Shulman (1968) and others. For our purpose we note here that the most definitive experimental evidence for triplet migration is the quenching of DNA phosphorescence by metal ions (Isenberg *et al.* 1967). From such experiments it has been inferred that the number of bases

quenched by Ni^{2+} , Co^{2+} , and Mn^{2+} ions are 17, 14 and 10, respectively. These values correspond to transfer distances of approximately 1.5, 1.2, and 0.8 nm, respectively, and are somewhat larger than those calculated theoretically by Förster's equation (Pope and Swenberg 1982). For this reason the data are currently taken to be indicative of triplet base to base migration as opposed to a single Förster transfer. These experimental results and other (see Hélène 1973) strongly support our contention that excitation migration in DNA of heterogeneous base composition is short range; this is in contrast to the very efficient energy migration for triplet states in both polyadenylic acid and in adenosine aggregates (Hélène 1973).

Charge transport through the stacked DNA bases, estimated by Van Lith *et al.* (1986) to be of the order of 9 nm, or along the phosphate-sugar backbone is also not sufficient to explain transport over distances large enough to account for the enhancement in the $\text{TH}\cdot$ radical formation at 77K for parallel irradiation configuration relative to that observed in the perpendicular configuration. Experimental data do support the possibility of long-range charge transport within the structured hydration layer of DNA. Based on radiation-induced conductivity of wet DNA samples, Van Lith *et al.* (1986) concluded that excess electrons in hydrated DNA have a mobility comparable to highly mobile conduction-band electrons in pure ice ($25 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$) and a considerably longer lifetime, which implies migration distances in excess of 100 nm. Furthermore, by using the narrow-band polaron model (Roberts *et al.* 1980) to extrapolate data on proton mobilities in ice (Keinst and Warman 1983) to lower temperature, we estimate that proton mobility in the hydration layer of DNA should be between 0.1 and $1 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at 77K. To achieve a root mean square displacement of 100 nm, protons with mobility in this range would require lifetimes between 4 and 40 ns. Since lifetimes of this magnitude are consistent with existing data on trapping rates for electrons and protons in ice (de Haas *et al.* 1983) proton migration in the hydration layer of DNA is a mechanism to explain the orientation dependence on $\text{TH}\cdot$ yields that is worthy of further investigation.

An alternative mechanism that we are considering is the transport of vibrational and rotational energy along DNA chains by solitary wave motion. These collective states have been invoked to explain the hydrogen-deuterium exchange reaction of double helical-polynucleotides (Nakanishi and Tsuboi 1978, Teitelbaum and Englander 1975). It may be difficult to reconcile this model with recent observations (Benight *et al.* 1988) of base-pair specificity in exchange rates and activation energies; however, these data are consistent with a spatially-extended exchanging state. As a first approximation one might consider the configuration formed by the partial unwinding of the DNA helix over a few base pairs as a solitary excitation. Estimates of their lowest energy (E_0), size (L_0) and velocity (v) have been inferred by Yomosa (1984) by comparison of a model Hamiltonian calculation with the temperature dependence of the equilibrium constant for hydrogen-deuterium exchange. His results, which includes hydrogen base pair interactions and stacking energies, give $E_0 = 0.35 \text{ eV}$, an energy sufficient for thymine anion protonation, $L_0 = 10$ base pairs which demonstrates the non-local character of this excited mode and $v = 8.3 \times 10^3 \text{ cm/s}$, a value less than the velocity of sound in DNA (Hakim *et al.* 1984). In our model for the formation of $\text{TH}\cdot$ radicals in the parallel irradiation configuration (Miller *et al.* 1988), the radiation-induced thermal spike has a lifetime of approximately 1 ns, a value considerably shorter than the measured open state

lifetime of 10^{-7} s reported by Guéron *et al.* (1987). A 1 ns lifetime gives a migration distance of 83 nm for the solitary wave; a distance consistent with that calculated by Monte Carlo simulation of the pattern of energy deposition events. Although Yamosa's model of open states does not include the DNA backbone bending energy (Ramstein and Lavery 1988), we feel that it does provide reasonable estimates for the inferred parameters quoted above.

We also note that the proton transport mechanism discussed above can be viewed as solitary wave motion in a hydrogen-bonded network (Pnevmatikos 1988). Hence, as Baverstock and Cundall (1989) suggest, the soliton concept may provide new insight into energy and charge transport that are important for understanding DNA damage by ionizing radiation. However, more conventional theories like the narrow-band polaron model may be adequate to explain the unusual observations reported by Arroyo *et al.* (1986). We fully agree with Baverstock and Cundall that ample experimental data exist to question the conventional wisdom that radiation damage in living matter is localized at the initial site of energy deposition. Understanding the extent to which solitary wave motion contributes to delocalization will require further research with carefully selected model systems.

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