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Modification of Immunologic and Hematologic Variables by Method of CO_2 Euthanasia

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Background and Purpose: **The major goal was to determine whether variations in the method of CO_2 euthanasia would induce significant immunologic differences.**

Methods: **Young adult C57BL/6 mice (n = 40) were euthanized, using four regimens: 70% CO_2/30% O_2; 70% CO_2/30% O_2 → 100% CO_2; 100% CO_2-naïve chamber; and 100% CO_2 pre-charged chamber. Time to recumbency and euthanasia and body, liver, lung, spleen, and thymus masses were determined. Blood and spleen were further evaluated for leukocyte, lymphocyte, and thrombocyte counts, erythrocyte characteristics, distribution of lymphocyte subpopulations, spontaneous and mitogen-induced blastogenesis, complement activity, and cytokine production.**

Results: **Time to euthanasia was five- to eightfold longer in mice exposed to 70% CO_2/30% O_2 than that for any other group. There were slight increases in mean erythrocyte volume (MCV) and mean erythrocyte hemoglobin (MCH) for all groups, compared with those for the 100% CO_2 pre-charged group. Circulating cytotoxic T ($CD8^+$) lymphocyte percentages and numbers, and spontaneous blastogenesis of leukocytes in blood and spleen, also were affected by euthanasia method.**

Conclusions: **The method of CO_2 euthanasia can result in significant differences in immunologic/hematologic variables. Thus, consistency in euthanasia procedures may be important in accurate interpretation of research data.**

The American Veterinary Medical Association (1) and the Canadian Council on Animal Care (2) have made recommendations for methods of euthanasia on the basis of several criteria: causing pain and/or suffering to the animal, altering experimental results, inducing hazardous laboratory conditions, or impacting the environment (1, 2). One method suggested for use in minimizing pain and distress in euthanizing rodents has been CO_2 asphyxiation (1, 2). Inhalation of high CO_2 concentrations induces severe hypercapnia leading to complete anesthesia (3). Concentrations as low as 5 to 8% have been reported to induce sedation (4). At slightly higher concentrations, 10 to 12%, there is loss of consciousness (5–7). Subcortical depression resulting in complete anesthesia has been observed at 25 to 30% (5, 7, 8). Carbon dioxide-induced unconsciousness is fairly rapid (9, 10), and death soon results from depression of respiratory centers, cerebral vasodilatation, and circulatory insufficiency (11).

Although euthanasia using pure CO_2 is a fast process and does not expose the animal to exogenous chemicals, it is a central nervous system depressant, increasing breathing rate until the animal is anesthetized (8, 11–13). Hypercapnia is known to affect the cardiovascular (7, 11, 14–16), respiratory (7, 11, 14, 15, 17, 18), endocrine (14, 15), and nervous (14, 15, 19, 20) systems. Euthanasia with CO_2 has been associated with changes in blood pH and $PaCO_2$ (5, 7, 21, 22). Similar changes were noted in cerebrospinal fluid (23–25) and brain tissue (26, 27).

There is some controversy in the area of animal well-being, since CO_2 is consistently used as a noxious stimulus to activate pain receptors (nociceptors) in nasal mucosa (28–30). The gas forms H_2CO_3 in solution, leading to irritation of the nasal passages (11, 31). However, it has been suggested that, in smaller mammals (i.e., rodents), anesthesia is sufficiently quick to bypass any distressful effects (31). In the short period prior to unconsciousness, some investigators reported signs of asphyxia and behavioral excitation, activity indicative of distress (8, 11, 13, 32), whereas others reported no signs of distress (9, 19, 33). These activities, if present, occur while electroencephalogram (EEG) activity is normal or at reduced amplitude (32). One method suggested to alleviate signs of distress involves introducing CO_2 slowly in a non pre-charged chamber. This, however, increases the time to recumbency and time to breathing cessation, compared with that of a pre-charged method (11, 22, 32).

Other investigators have observed that adding O_2 may decrease the negative effects of pure CO_2 on behavior. Adding up to 50% O_2 to a pre-charged chamber still allows rapid loss of consciousness (< 1 minute) (11, 12, 16). Although there appears to be no appreciable relationship between CO_2 concentration and time to recumbency, mean time to either anesthesia or euthanasia increases significantly as the concentration of CO_2 decreases (11). However, some animals exposed to 60 to 80% CO_2 were not euthanized even after 30 minutes (11).

If a CO_2/O_2 mixture is introduced slowly, the time to recumbency is significantly longer than that achieved using chambers pre-charged with the same CO_2/O_2 ratio (11). In contrast, Danneman, et al. reported no significant effect of pre-charging the chamber on time to death (11). These animals became unconscious without signs indicative of asphyxia or suffocation (16, 32, 34, 35). The use of 70% CO_2/30% O_2 has also been reported to decrease the discomfort of hypoxia during euthanasia (34). In one study of use of a 70% CO_2/30% O_2 mixture, there seemed to be signs of distress prior to anesthesia (36). However, this may have been due to the noise of the high rate of airflow

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used to introduce the gas (16). Another report suggests that the frequency and severity of adverse reactions are inversely related to CO_2 concentrations (11). All animals anesthetized with 50% CO_2 experienced at least one adverse reaction (seizures, convulsive chewing, unexpected death, nasal hemorrhage, serosanguinous nasal discharge, excess salvation, or foaming), whereas this was the case for only one rat exposed to 100% CO_2 (11).

The aforementioned observations indicate that numerous physiologic systems are affected by variations in the method of euthanasia. However, investigations of potential differences in immunologic responses are few. This is particularly disturbing since animal distress could influence immune function (37–39). The purpose of the study reported here was to provide information that is lacking in this area. Flow cytometric analysis and mitogen-induced blastogenesis were performed on splenic and circulating lymphocyte populations. Hematologic analysis of circulating leukocyte populations also was performed. Finally, the ability of cells to secrete cytokines was assayed. Interleukin 2 (IL-2), tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) were chosen because of their potent ability to recruit, activate, and regulate various immune populations (40), as well as their roles in lymphocyte receptor up-regulation and trafficking (41– 43). These assays should provide a comprehensive view of the effects of different euthanasia methods on immune function.

Materials and Methods

Animals and experimental protocol: Female C57BL/6 mice (n = 40) were purchased (Charles River Breeding Laboratories, Hollister, CA) at 8 weeks of age. Animals were certified to be free of bacterial and parasitic pathogens, including *Brucella bronchiseptica, Corynebacterium freundii 4280, C. kutscheri, Mycoplasma pulmonis, Salmonella spp, S. moniliformis, Helicobacter hepaticus, H. bilis, Helicobacter spp, Klebsiella pneumoniae, K. oxytoca, Pasteurella multocida, P. pneumotropica, Pasteurella spp, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae,* β-streptococci, ectoparasites, endoparasites, enteric protozoa, and a series of common murine viruses. Our facility does not routinely test for microbiological flora in the research animals themselves. However, sentinel animals are housed on each rack of cages and periodically shipped to Rabbit and Rodent Diagnostics, San Diego, CA. Sentinels housed with the mice of this present study were test-negative for mouse hepatitis virus, Sendai virus, pneumonia virus, and *M. pulmonis.*

The animals were maintained in large shoebox cages (7 to 8 mice/cage) under standard conditions. After a two-week acclimatization period, the mice were euthanized by use of four methods of CO_2 exposure. The first group of animals (100 pre-charged) were placed into chambers pre-charged with 100% CO_2. In the second (100 naïve), animals were placed into non-pre-charged chambers and 100% CO_2 was quickly introduced. The third group (70/30 → 100) was placed into a chamber pre-charged with 70% CO_2/30% O_2 until recumbency, then was put into a second chamber pre-charged with 100% CO_2. The last group of animals (70/30) was placed in a chamber pre-charged with 70% CO_2/30% O_2. There was no bedding in any chamber. The study was approved by the Institutional Animal Care and Use Committee.

Behavior parameters and body and organ masses: Body mass was determined immediately prior to euthanasia. Time to recumbency was defined as the interval between time of placement into the chamber and the moment the animals stopped movement for more than 5 seconds. Time to euthanasia was defined as the time to a halt in breathing for at least 10 seconds. Immediately thereafter, the liver, lungs, spleen and thymus were excised and weighed. Organ mass in relation to total body mass was calculated: ROM (relative organ mass) = [mass of organ (g)/mass of body (g)] x 10^3.

Leukocyte counts in blood and spleen: Whole blood was collected in K_2EDTA-containing syringes by use of cardiac puncture at the time of euthanasia. The samples (12 μl) were evaluated, using the ABC Vet Hematology Analyzer (Heska Corp., Waukesha, WI) programmed to analyze murine blood. The measurements included white blood cell (WBC), red blood cell (RBC), and thrombocyte counts, hemoglobin concentration, and hematocrit (percentage of whole blood composed of RBC). Standard formulas based on these measurements were used to calculate the mean corpuscular volume (MCV; mean volume per RBC), mean corpuscular hemoglobin (MCH; mean weight of hemoglobin per RBC), and mean corpuscular hemoglobin concentration (MCHC; concentration of hemoglobin per RBC).

Flow cytometric analysis of spleen and blood lymphocytes: Immunophenotyping of blood and spleen samples was carried out using a FACS-Calibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA) and four-color mixtures of monoclonal antibodies (MAb; Pharmingen, San Diego, CA). This allowed rapid identification and quantification of the various lymphocyte populations and T-cell subsets. The MAb were labeled with fluorescein isothiocyanate, R-phycoerythrin, allophycocyanin, or peridinin chlorophyll protein and were directed against the following markers: CD3 (500A2)-T cell receptor-associated complex present on all mature T cells; CD4 (RM4-5)-T helper/inducer (Th) cells; CD8 (53-6.7)-T cytotoxic (Tc) cells; B220 (RA3-6B2)-B cells; and NK1.1 (PK136)-natural killer (NK) cells. The CD45 vs. side scatter dot plots were used to gate cell lineages based on cytoplasmic complexity. Blasts are generally low on forward and side scatter and have dim CD45, compared with mature cells such as lymphocytes. Lymphocytes generally have the brightest CD45 staining and are low on side scatter (nongranular). This allowed the gating of lymphocytes without including significant monocyte or granulocyte populations**.** Analysis of 10,000 lymphocyte events per tube was performed using CellQuest™ software version 3.1 (Becton Dickinson). To obtain the number of cells for each lymphocyte population, the following formula was used: No. of cells in population/ml = No. of lymphocytes/ml x percentage of population.

Spontaneous and mitogen-induced blastogenesis: These assays have been described in detail (44, 45). To quantify baseline proliferation of leukocytes, 50-μl aliquots of whole blood or spleen cells (2 x 10^6/ml) were diluted with supplemented RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) and dispensed into wells of microculture plates. One microcurie of thymidine ([^{3}H]TdR; specific activity = 46 Ci/μmol; ICN Biochemicals, Costa Mesa, CA) was immediately added, and the plates were incubated for 3 hours at 37°C. In addition, spleen leukocytes (2 x 10^6/ml in medium) were dispensed into microtitration plates with phytohemagglutinin (PHA), concanavalin A (conA), lipopolysaccharide (LPS), or no mitogen. All mitogens were purchased (Sigma Chemical Co., St. Louis, MO) and were pretitrated for maximal response. The cells were incubated for 48 hours and pulse-labeled with [^{3}H]TdR (1 μCi/50 μl/well) for the final 4 hours. In both assays, cells were harvested, using a multiple sample har-

vester, and the incorporated radioactivity was quantified by use of a beta-scintillation counter. Response of spleen cells to the mitogens is expressed as stimulation index (SI): SI = (disintegrations per minute [dpm] with mitogen–dpm without mitogen)/ dpm without mitogen.

Complement activity: This assay was a modification of the 'EZ Complement' procedure (Diamedix Corp., Miami, FL). Whole blood, collected in capillary tubes without anti-coagulant, was centrifuged, and 40 μl of serum/mouse was diluted 1:5 with 160 μl of RMPI 1640 medium. Aliquots (100 μl) of each diluted sample were dispensed into duplicate wells of microtitration plates; reference control serum (Diamedix) and wells for spontaneous lysis, medium only, and blank controls were included. Sheep RBC sensitized with antibodies to sheep erythrocytes (100 μl; Diamedix) were added to all wells except the blanks, the plates were incubated for 1 hour at room temperature, then were centrifuged for 5 minutes. The supernatants were transferred to new microtitration plates, and the optical density was read at 415-nm wavelength. The test results were converted to CH_{50} values: CH_{50} of test sample = percentage of reference serum OD x CH_{50} titer of reference serum.

Assays for plasma TGF-β1 and IL-2 and TNF-α in spleen cell supernatants: These tests were performed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine™, R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. The assay for TGF-β1 in plasma quantified the total amount (latent plus active forms) of the cytokine. Prior to testing for IL-2 and TNF-α, spleen cells were incubated with PHA for 48 hours as described previously for mitogen-induced blastogenesis, but without [^{3}H]TdR; supernatants were aspirated and centrifuged. Plasma and supernatant samples from mice in the appropriate groups were pooled (3 to 5 samples/group). The activities of TGF-β1, IL-2 and TNF-α in the test samples were interpolated from the appropriate standard curve. Sensitivities of the assays were 0.05 ng/ml (TGF-β1), 3 pg/ml (IL-2), and 5.1 pg/ml (TNF-α).

Statistical analysis: The results were analyzed by use of one-way analysis of variance (ANOVA) and Tukey's pairwise multiple comparison test. A P-value < 0.05 was selected to indicate significance, whereas $P < 0.1$ indicated a trend. These analyses were performed by use of SigmaStat™ software, version 2.03 (SPSS Inc., Chicago, IL).

Results

Time to recumbency and euthanasia: Table 1 indicates that time to euthanasia was significantly prolonged (approx. five- to eightfold longer) in mice exposed to the 70% CO_2/30% O_2 environment, compared with all other groups. In contrast, there was no difference among groups with respect to duration of time for recumbency.

Body and organ masses: As indicated (Table 2), there were no significant differences in total body mass or any organ mass among the various groups. There was a trend in mean values for spleen mass and normalized spleen mass to be lower in animals of the 70% CO_2/30% O_2 group, compared with those for animals of the pre-charged 100% CO_2 group ($P < 0.10$). However, this may have been due to the abnormally large spleen found in one of the animals of the 100% CO_2 pre-charged group. If the results from this animal are deleted, there is no longer a trend (data not shown). Because values for this animal were not significantly different in any of the other measured parameters, its data were not removed from the analysis.

Table 1. Time (seconds) to recumbency and euthanasia

	Group (mean ± SEM)[a]			
Parameter	70/30	70/30→100	100 Naïve	100 Pre-charged
Recumbency	11.48 ± 1.28	12.19 ± 0.93	11.31 ± 2.16	8.96 ± 0.89
Euthanasia	281.38 ± 16.61[b]	60.52 ± 3.76	47.93 ± 5.26	40.47 ± 2.06

[a] *See* Materials and Methods for code.
[b] $P < 0.001$ compared to all other groups.
ANOVA: $P < 0.001$ for time to euthanasia.

Table 2. Total body and organ masses

	Group (mean ± SEM)[a]			
Body and organs	70/30	70/30→100	100 Naïve	100 Pre-charged
Body (g)	22.27 ± 0.57	22.92 ± 0.24	22.93 ± 0.40	22.53 ± 0.53
Liver (mg)	1,217 ± 57	1,206 ± 24	1,179 ± 26	1,199 ± 52
Lung (mg)	166 ± 10	158 ± 4	162 ± 6	157 ± 4
Spleen (mg)	76.3 ± 5.3[c]	91.4 ± 2.6	83.5 ± 2.0	113 ± 21.4
Thymus (mg)	82.6 ± 3.8	83.9 ± 4.0	73.4 ± 3.5	80.6 ± 4.0
ROM[b]				
Liver	54.85 ± 1.62	52.68 ± 1.28	51.48 ± 1.16	52.61 ± 1.46
Lung	7.44 ± 0.28	6.89 ± 0.20	7.11 ± 0.35	6.92 ± 0.19
Spleen	3.41 ± 0.20[c]	3.99 ± 0.10	3.64 ± 0.08	4.90 ± 0.83
Thymus	3.71 ± 0.15	3.66 ± 0.17	3.22 ± 0.17	3.56 ± 0.13

[a] *See* Materials and Methods for code.
[b] Relative Organ Mass (ROM) = [mass of organ (g)/mass of body (g)] x 10^3.
[c] $P < 0.10$, compared with 100 Pre-charged.
ANOVA: $P < 0.10$ for ROM – spleen.

Erythrocytes and thrombocytes: Table 3 shows the characteristics of RBC from peripheral blood. There were no significant differences in total RBC counts. However, there was a trend for higher RBC numbers and hematocrit values in animals of the 70% CO_2/30% O_2 group, compared with those of the 100% pre-charged group ($P < 0.10$). Significant increases were found in MCV for all groups, compared with that for animals of the 100% pre-charged group; a tendency toward significance was observed between the 70% CO_2/30% O_2 and 100% naïve groups. Similarly, increases were found in MCH for animals of the 70% CO_2/30% O_2 and 70% CO_2/30% O_2 → 100% CO_2 groups, compared with those of the 100% CO_2 pre-charged group ($P < 0.01$ and $P < 0.05$, respectively). Differences were not found in MCHC or in the number of platelets (PLT).

Percentages and numbers of leukocyte populations in blood: Percentages of lymphocyte populations in blood are shown in Table 4. There were significant decreases in the proportion of $CD3^+$ T cells in animals of the 70% CO_2/30% O_2 group, compared with those of the 100% pre-charged group ($P < 0.05$). In the mice subjected to 70% CO_2/30% O_2, cytotoxic T cell ($CD8^+$) percentages were decreased significantly ($P < 0.01$), compared with values for all other groups, and B cell ($CD19^+$) percentages were increased ($P < 0.05$) relative to values for animals of the 100% CO_2 pre-charged group. The percentage of NK cells (NK1.1+) in animals of the 100% CO_2 naïve group was significantly lower than that in animals of the 100% CO_2 pre-charged group ($P < 0.05$). The proportion of helper T cells ($CD4^+$) was not affected by method of euthanasia.

Phenotype counts for the peripheral blood are presented in Table 5. The WBC count was fairly constant for three of the groups, the only exception being the 100% naïve group, which had significantly higher numbers ($P < 0.05$ vs. all other groups). With respect to lymphocytes in the 100% CO_2 naïve group, significantly lower numbers of T ($CD3^+$), helper/inducer T ($CD4^+$), and cytotoxic T ($CD8^+$) cells were found compared with those in animals of the 70% CO_2/30% O_2 group ($P < 0.05$). The mice of the

Table 3. Red blood cell (RBC) characteristics and platelet (PLT) counts in peripheral blood

Parameters[b]	Groups (mean ± SEM)[a] 70/30	70/30→100	100 Naïve	100 Pre-charged
RBC x10^9/ml	9.99 ± 0.10	9.64 ± 0.13	9.85 ± 0.15	9.52 ± 0.34
HGB (g/dl)	14.98 ± 0.15[c]	14.37 ± 0.15	14.60 ± 0.19	13.95 ± 0.45
HCT (%)	46.70 ± 0.55[c]	44.67 ± 0.58	45.37 ± 0.70	43.36 ± 1.40
MCV (fl)	46.80 ± 0.13[d,e]	46.56 ± 0.18[d]	46.14 ± 0.14[d]	45.40 ± 0.22
MCH (pg)	14.99 ± 0.05[d]	14.93 ± 0.07[d]	14.86 ± 0.07	14.67 ± 0.07
MCHC (g/dl)	32.10 ± 0.10	32.22 ± 0.13	32.23 ± 0.18	32.21 ± 0.09
PLT x 10^6/ml	462 ± 97	511 ± 91	470 ± 120	540 ± 107

[a] *See* Materials and Methods for code.
[b] HGB = hemoglobin; HCT = hematocrit (% of whole blood composed of RBC); MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.
[c] $P < 0.10$, compared with 100 Pre-charged.
[d] $P < 0.05$, compared with 100 Pre-charged.
[e] $P < 0.10$, compared with 100 Naïve.
ANOVA: $P < 0.001$ for MCV; $P < 0.01$ for MCH.

Table 4. Percentages of lymphocyte subpopulations in peripheral blood

Lymphocytes	Groups (mean ± SEM)[a] 70/30	70/30→100	100 Naïve	100 Pre-charged
T (CD3$^+$)	41.03 ± 2.00[b]	46.74 ± 2.31	49.59 ± 3.52	49.72 ± 1.60
Th (CD4$^+$)	26.33 ± 1.44	28.9 ± 1.86	29.56 ± 2.54	29.40 ± 1.11
Tc (CD8$^+$)	11.85 ± 0.87[c]	16.32 ± 0.46	18.48 ± 1.20	18.44 ± 0.71
B (CD19$^+$)	50.80 ± 2.31[b]	44.95 ± 2.37	44.29 ± 4.07	38.74 ± 2.77
NK (NK1.1$^+$)	8.17 ± 0.82	8.31 ± 0.76	6.12 ± 1.09[b]	11.54 ± 1.92

[a] See Materials and Methods for code.
[b] $P < 0.05$, compared with 100 Pre-charged.
[c] $P < 0.01$, compared with all other groups.
ANOVA: $P < 0.05$ for T (CD3$^+$), NK (NK1.1$^+$), and B (CD19$^+$) cells; $P < 0.001$ for Tc (CD8$^+$) cells.

100% CO_2 pre-charged group had significantly higher cytotoxic T cell counts, compared with mice of the 70% CO_2/30% O_2 group, and higher NK cell numbers, compared with those in mice of the 70% O_2/30% O_2 and 100% CO_2 naïve groups; a trend toward increased NK cell numbers was noted in comparison with values for animals of the 70% CO_2/30% O_2 → 100% CO_2 group. There were no significant differences found in total lymphocyte and total T, helper/inducer T- or B-cell counts.

Percentages and numbers of leukocyte populations in the spleen: Tables 6 and 7 indicate the splenic population distributions and cell counts, respectively. There were no significant differences among the groups in total T, helper/inducer T, B, and NK cell percentages. In contrast, cytotoxic T (CD8$^+$) cell percentages were increased in animals of the 70% CO_2/30% O_2 and 100% CO_2 naïve groups, compared with those in animals of the 100% CO_2 pre-charged group ($P < 0.05$ for both). Similarly, there were no differences in most of the phenotype cell counts; WBC, total T, helper/inducer T, B, and NK cell counts were all unaffected by the euthanasia method. However, cytotoxic T (CD8$^+$) cell counts in animals of the 100% CO_2 naïve group were significantly ($P < 0.05$) increased, and a slight trend for an increase in the number of lymphocytes was observed, compared with counts in animals of the 100% CO_2 pre-charged group.

Spontaneous and mitogen-induced blastogenesis: Results of assays for spontaneous and mitogen-induced blastogenesis are shown in Table 8. Mean dpm values obtained in the spontaneous blastogenesis assay for blood and spleen were significantly higher for animals of the 100% pre-charged group, compared with values for all other groups ($P < 0.01$ and $P < 0.05$, respectively). However, there were no differences among the groups for mitogen-induced blastogenesis of splenocytes.

Complement activity and cytokines: Complement and cytokine results are presented in Table 9. There were no significant differences found in CH_{50} and plasma TGF-β1 values. After stimulating splenocytes with PHA for 48 hours, there were no significant differences among the groups in the activities of IL-2 or TNF-α in the supernatant.

Table 5. Peripheral blood leukocyte and lymphocyte subpopulation counts

Leukocytes (counts/ml) x 10^{-6}	Groups (mean ± SEM)[a] 70/30	70/30→100	100 Naïve	100 Pre-charged
WBC[b]	4.45 ± 0.66	4.84 ± 0.80	7.57 ± 0.58[c]	5.01 ± 0.44
Lymphocytes	2.58 ± 0.55	2.67 ± 0.37	3.59 ± 0.63	2.80 ± 0.32
T (CD3$^+$)	1.58 ± 0.18	1.96 ± 0.20	2.45 ± 0.25[d]	2.13 ± 0.15
Th (CD4$^+$)	0.99 ± 0.09	1.21 ± 0.13	1.45 ± 0.15[d]	1.26 ± 0.09
Tc (CD8$^+$)	0.48 ± 0.08	0.69 ± 0.08	0.92 ± 0.11[e]	0.79 ± 0.06[d]
B (CD19$^+$)	2.14 ± 0.48	1.95 ± 0.28	2.44 ± 0.54	1.78 ± 0.27
NK (NK1.1$^+$)	0.31 ± 0.04	0.33 ± 0.02	0.27 ± 0.02	0.45 ± 0.05[f]

[a] *See* Materials and Methods for code.
[b] WBC = white blood cell count.
[c] $P < 0.05$, compared with all other groups.
[d] $P < 0.05$, compared with 70/30.
[e] $P < 0.01$, compared with 70/30.
[f] $P < 0.05$, compared with 70/30 and 100 Naïve; $P < 0.10$, compared with 70/30 →100.
ANOVA: $P < 0.05$ for WBC and T (CD3$^+$); $P < 0.10$ for Th (CD4$^+$); $P < 0.01$ for Tc (CD8$^+$); $P < 0.01$ for NK (NK1.1$^+$).

Table 6. Percentages of spleen lymphocytes

Lymphocytes	Groups (mean ± SEM)[a] 70/30	70/30 → 100	100 Naïve	100 Pre-charged
T (CD3$^+$)	29.27 ± 11.25	28.27 ± 0.72	30.10 ± 0.68	28.30 ± 0.95
Th (CD4$^+$)	17.82 ± 0.86	16.84 ± 0.37	17.67 ± 0.67	16.84 ± 0.51
Tc (CD8$^+$)	10.10 ± 0.57[b]	9.06 ± 0.35	10.45 ± 0.45[b]	8.36 ± 0.41
B (CD19$^+$)	29.27 ± 1.25	28.27 ± 0.72	30.10 ± 0.68	28.30 ± 0.95
NK (NK1.1$^+$)	0.94 ± 0.12	1.02 ± 0.11	0.89 ± 0.13	1.03 ± 0.07

[a] *See* Materials and Methods for code.
[b] $P < 0.05$, compared with 100 Pre-charged.
ANOVA: $P < 0.05$ for Tc (CD8$^+$) cells.

Table 7. Leukocyte and lymphocyte subpopulation counts in spleen

Leukocytes (counts/ml) x 10^{-6}	Groups (mean ± SEM)[a] 70/30	70/30 → 100	100 Naïve	100 Pre-charged
WBC[b]	38.15 ± 4.32	47.19 ± 5.15	42.10 ± 4.66	40.32 ± 4.06
Lymphocytes	31.31 ± 1.57	32.94 ± 1.81	35.05 ± 1.41[c]	28.71 ± 1.60
T (CD3$^+$)	12.21 ± 0.85	13.60 ± 0.78	13.59 ± 0.87	11.49 ± 0.87
Th (CD4$^+$)	7.43 ± 0.60	7.29 ± 0.52	7.96 ± 0.49	6.84 ± 0.51
Tc (CD8$^+$)	4.22 ± 0.37	3.91 ± 0.29	4.76 ± 0.39[d]	3.39 ± 0.27
B (CD19$^+$)	28.90 ± 1.51	30.36 ± 1.59	31.26 ± 1.93	28.39 ± 1.57
NK (NK1.1$^+$)	0.40 ± 0.06	0.46 ± 0.07	0.41 ± 0.08	0.41 ± 0.04

[a] *See* Materials and Methods for code.
[b] WBC = white blood cell count.
[c] $P < 0.10$, compared with 100 Pre-charged.
[d] $P < 0.05$, compared with 100 Pre-charged.
ANOVA: $P < 0.10$ for lymphocytes and Tc (CD8$^+$) cells.

Discussion

Results of the study reported here indicate that euthanasia in mice exposed to 70% CO_2/30% O_2 was achieved after a mean time interval of approximately 5 minutes, significantly longer than that for any of the other groups (approx. ≤ 1 minute). There were no significant differences in time to recumbency for any of the methods. In both groups involving the 70/30 CO_2/O_2 mixture, this period was consistently longer than in the groups inhaling 100% CO_2. However, if data for the two 70/30 groups were combined and compared with those for the two 100% CO_2 groups, using a Student's *t*-test, there was still only a trend ($P < 0.06$, data not shown). Overall, these results are consistent with the literature (11). It has been reported that euthanasia of rats by placing them in a chamber and slowly adding 70% CO_2/30% O_2 caused death only after 9 minutes. Pre-charging the chamber with the same mixture decreased this time to 5 minutes (16).

Table 8. Spontaneous and mitogen-induced blastogenesis

Parameter	Groups (mean ± SEM)[a] 0/30	70/30 → 100	100 Naïve	100 Pre-charged
Blood				
Spontaneous[b]	14.30 ± 2.27[d]	12.07 ± 1.78[d]	7.18 ± 0.94[d]	28.01 ± 3.75
Spleen				
Spontaneous[b]	10.69 ± 2.19[e]	9.18 ± 0.62[e]	8.76 ± 0.79[e]	20.1 ± 3.58
ConA – SI[c]	256 ± 99	180 ± 44	229 ± 45	336 ± 123
PHA – SI[c]	206 ± 74	148 ± 35	176 ± 45	239 ± 77
LPS – SI[c]	49 ± 116	306 ± 52	475 ± 97	406 ± 121

[a] *See* Materials and Methods for code.
[b] Spontaneous blastogenesis (dpm/50 μl of whole blood and dpm/50 μl of spleen cells adjusted to 2 x 10^6/ml).
[c] SI = stimulation index = (dpm with mitogen – dpm without mitogen)/dpm with mitogen.
Mitogens:
ConA, concanavalin A; PHA, phytohemagglutinin; LPS, lipopolysaccharide.
[d] $P < 0.01$, compared with 100 Pre-charged.
[e] $P < 0.05$, compared with 100 Pre-charged.
ANOVA: $P < 0.001$ for spontaneous blastogenesis in blood; $P < 0.01$ for spontaneous blastogenesis in spleen.

Prolongation of the dying process may have an impact on certain types of studies. For example, normal or reduced EEG activity continues longer in animals exposed to a slow rate increase in 100% CO_2 or a 66% CO_2/33% O_2 mixture, compared with that in animals placed into a chamber pre-charged with 100% CO_2. A similar pattern of response was seen in electrocardiogram (ECG) activity (32).

As expected, we found no differences in total body, liver, lung, thymus or spleen mass for any of the euthanasia conditions and gross changes were not observed. However, it is possible that histopathologic changes were present in one or more of the organs. There are reports of CO_2 concentration-dependent histologic changes after euthanasia. Edema and hemorrhage of the lungs have been reported in rats euthanized with 50 to 100% CO_2 (11). However, the severity of lung lesions was inversely correlated to CO_2 concentration (11).

There were few significant differences in measures characterizing circulating erythrocytes. The MCHC and PLT count were unaffected by carbon dioxide-induced euthanasia. There were, at best, trends for increases in total hemoglobin concentration and hematocrit in animals of the 70%/30% group, compared with those in animals of the 100% pre-charged group. It is interesting that animals of the 100% pre-charged group had lower, although not significantly, values for these parameters, compared with those of all of the other groups. The MCV and MCH were also consistently lower in animals of the 100% pre-charged group in relation to the other three groups. However, mean values for animals of all four of the groups were as expected for healthy mice, based on reference values accompanying the ABC Vet Hematology Analyzer. The biological mechanisms underlying the observed differences may reflect changes in extracellular and/or intracellular pH. Carbon dioxide anesthesia is known to cause acidosis which, in turn, has been documented to affect erythrocyte parameters (46).

Circulating WBC numbers appeared to be fairly constant across three of the four groups; the animals of the 100% naïve group had a significant increase in this measure, compared with values for all other groups. However, total lymphocyte counts were unaffected by the euthanasia methods tested. These findings indicate that the relative leukocytosis in animals of the 100% CO_2 naïve group was due to an increase in granulocytes (neutrophils, eosinophils, and basophils) and/or monocytes, rather than lymphocytes.

Table 9. Complement activity and cytokine production

Parameter	Groups (mean ± SEM)[a] 70/30	70/30 → 100	100 Naïve	100 Pre-charged
Serum CH_{50}[b]	330 ± 88	233 ± 31	461 ± 241	270 ± 54
Plasma TGF-β1 (pg/ml)[c]	3640 ± 135	3678 ± 585	2905 ± 480	2897 ± 427
Spleen supernatant				
IL-2 (pg/ml)[d]	210 ± 28	184 ± 28	225 ± 57	166 ± 57
TNF-α (pg/ml)[d]	195 ± 52	185 ± 27	243 ± 31	201 ± 39

[a] *See* Materials and Methods for code.
[b] CH_{50} of test sample = % of reference serum OD x CH_{50} titer of reference serum.
[c] TGF-β1 = transforming growth factor-β1 (latent plus active forms).
[d] IL-2 = interleukin 2; TNF-α = tumor necrosis factor-α. Spleen cells were activated with PHA for 48 hours before testing for the cytokines in the supernatant.

There were some significant increases in circulating T-cell and helper T-cell counts in animals of the 100% CO_2 naïve, compared with those of the 70% CO_2/30% O_2 group. Because these populations were not significantly different, compared with the 100% pre-charged populations, these results are difficult to interpret. However, though not always significant, the animals euthanized with 100% CO_2 (naïve or pre-charged) appeared to have greater T-cell counts than did animals euthanized with 70% CO_2/30% O_2. This is especially true for the cytotoxic T-cell population. Although circulating T-lymphocyte percentages were low in mice of the 70% CO_2/30% O_2 group, compared with those in mice of the 100% CO_2 pre-charged group, only the cytotoxic (not the helper/inducer) T-cell percentages were decreased significantly. These findings were confirmed by a similar pattern of changes in cell counts: circulating cytotoxic T-cell numbers were decreased in mice of the 70% CO_2/30% O_2 group, whereas helper/inducer T-cell counts remained constant, compared with values in mice of the 100% CO_2 pre-charged group. Although there is little in the literature concerning effects of variations in CO_2 euthanasia on any of the lymphocyte populations, a decrease in cytotoxic T cells is consistent with reported decreases in cell-mediated lympholysis in mixed lymphocyte reactions. Euthanasia by use of a slow increase in CO_2 to 100% reduced the lytic activity of alloreactive cytotoxic T cells, compared with that by use of decapitation (10).

Circulating B-cell percentages were high in the 70% CO_2/30% O_2 animals. However, because B-cell counts were unaffected by the euthanasia method, this change in percentage was likely due to a downward shift in another population (i.e., cytotoxic T or NK cells or granulocytes). The NK cell percentage in blood was slightly decreased mice of all groups, compared with that in mice of the 100% CO_2 pre-charged group. However, only the decrease in mice of the 100% naïve group was significant. Unlike what was seen in the B-cell population, these proportional changes were confirmed by the NK cell counts, indicating that the population was indeed affected by the euthanasia method.

There were fewer significant differences in the splenic leukocyte populations than what was observed in the blood. Differences were not found among the groups in WBC, T ($CD3^+$), helper/inducer T ($CD4^+$), B ($CD19^+$), or NK ($NK1.1^+$) cell percentages or counts. However, there was a tendency for percentages and numbers of splenic lymphocytes to be high in mice of the 100% CO_2 naïve group, a finding that likely was due to the cytotoxic T ($CD8^+$) cells. The cytotoxic T-cell percentages in mice of the 100 % CO_2 naïve and 70% CO_2/30% O_2 groups were higher than those in mice of the 100% CO_2 pre-charged group.

There are at least two possible explanations for the slight changes in lymphocyte subpopulation distributions. The distribution shifts may be due to differences in phenotype-specific apoptotic activity. It is well known that lymphocyte subpopulations can exhibit various degrees of apoptosis, depending upon the apoptosis-inducing agent used, level of cell activation, and status of the host, as well as other factors (47). However, the maximal time between animal euthanasia and phenotypic analysis was only two hours. Thus, if apoptosis were indeed occurring, it seems unlikely these changes would greatly affect flow cytometric analysis over the time course of this study. However, because we did not perform an apoptotic assay on these cells, this possibility cannot be excluded.

It is far more likely that the slower methods of euthanasia may cause cytotoxic T cells to migrate out of the blood into the spleen. Hemodynamic homing mechanisms may be a factor. These mechanisms are those that involve blood flow rate through a specific organ. Changes in splenic blood flow rate could occur with changes in plasma pH or partial CO_2 pressure (48). All of these conditions are known to be associated with CO_2-induced euthanasia (5, 7, 11, 21, 22). However, these mechanisms may not fully account for the observed differences in cytotoxic T cells in mice of the 100% CO_2 naïve group, since time to euthanasia for these mice was similar to that in mice euthanized with either 70% CO_2/30% O_2 → 100% CO_2 or pre-charged 100% CO_2.

It is also possible that carbon dioxide euthanasia may have some effects on cytokine production. This, in turn, could affect endothelial cell receptors that allow lymphocytes to bind to and migrate through immune tissues (43, 49). Cytokines, such as interleukins, tumor necrosis factors, and interferons, can influence lymphocyte homing by increasing homing receptor levels on endothelial cells (41–43). In the data presented here, plasma TGF-β1 activity remained unchanged. Similarly, the ability of stimulated splenocytes to produce IL-2 and TNF-α was not affected by the euthanasia method. It is interesting, however, that plasma TGF-β1 activity and splenic IL-2 production were higher (although not significantly so) in all groups, compared with those measures in mice of the 100% pre-charged group. This suggests the possibility that these cytokines may play a limited role in the noted euthanasia-induced changes in cytotoxic T-cell trafficking. A more sensitive assay may be able to distinguish these differences. Additionally, the lack of significant changes in the cytokines assayed here does not rule out an affect of euthanasia method on other cytokines.

Finally, because of the reported distressful nature of CO_2 euthanasia, hormonal effects on trafficking must be considered. The response to CO_2 inhalation has been reported to be similar to a generalized stress response (3). Carbon dioxide-induced anesthesia has been reported to cause an opioid-mediated (50), or a non opioid-mediated (21), stress-induced antinociceptive (pain-ameliorating) response. Removing the pituitary gland prior to exposure to CO_2 blocked the antinociceptive effect (21), suggesting a hormonal rather than an opioid mediated stress-induced response (51, 52). Hormones implicated in lymphocyte trafficking and chemotaxis include corticosterone, prolactin, and catecholamines epinephrine and norepinephrine (53–57).

The literature suggests that there are few, if any, CO_2 anesthesia-induced changes in circulating hormone concentrations. Compared with values during decapitation alone, many circulating hormone concentrations (i.e., luteinizing hormone, follicle-stimulating hormone, and prolactin) in female rats were not significantly affected by pre-anesthetizing with 50% CO_2/ 50% O_2 for 1 minute before decapitation (12). However, this was not the always case for circulating corticosterone values. In one report (12), pre-anesthetizing the animals decreased this measure, perhaps indicating a decrease in the handling stress sometimes associated with decapitation. In another report (58), glucocorticoid concentrations were unaffected by CO_2 administration. Exposure to CO_2 has also been reported to result in changes in plasma catecholamine (epinephrine and norepinephrine) values (58, 59). It is not known at this time whether the reported changes in one or more of these hormones could account, at least partly, for the immunologic differences observed in this study.

There were decreases in peripheral blood and splenic spontaneous blastogenesis for all groups, compared with changes in mice of the 100% pre-charged group. This suggests carbon dioxide-induced euthanasia has some limited effect on the basal proliferation of leukocytes. It is also possible that apoptosis played a role in this change in proliferative activity. Cells damaged in the euthanasia process (e.g., via a change in circulating pH) may possibly undergo programmed cell death. Because the cell counts for the spontaneous blastogenesis assays were taken immediately after euthanasia, potentially before cell death occurred, apoptotic cells may have been included in the assay. However, because apoptosis was not specifically assayed, this remains an open question.

In contrast, differences were not found among any of the groups for splenocyte mitogen-induced blastogenesis. It is interesting that the response to both T-cell mitogens (PHA and conA) was decreased, although nonsignificantly, in all groups, compared with the response in mice of the 100% pre-charged groups. By use of a more sensitive assay, these parameters could potentially reach significance. However, literature suggests that conA-stimulated proliferative activity in cells taken from the inguinal lymph nodes of mice euthanized with a slow increase in CO_2 is not significantly different from that of decapitated animals (10).

Some aspects of liver function appear to be affected by method of euthanasia. For example, liver glycogen, pyruvate, and ATP contents are reduced in animals placed into a chamber pre-charged with 100% CO_2, then decapitated. However, other metabolic factors, such as G1P, G6P, AMP, and ADP concentrations, were not significantly affected by this treatment (60). Similarly, differences in complement (CH_{50}) activity were not associated with any of the euthanasia methods presented here.

In conclusion, although differences in immune parameters across euthanasia methods were few, they may be critical in accurate interpretation of data. Changes in cytotoxic T-cell (and possibly NK cell) trafficking could confound results specifically targeting this process. Similarly, changes in spontaneous blastogenesis activity (i.e., increased DNA synthesis) could indicate heightened immune readiness due simply to anesthesia. Because the cultures were not phenotyped after this assay, the cell types involved in this enhanced readiness are not known. However, because the response to T and B cell-specific mitogens were not significantly affected by the euthanasia method, it is unlikely there were effects on the antigenic function of these populations.

There were considerable differences in the duration required for euthanasia. Depending on the size of the experiment, this could become an important issue from the practical point of view. Similarly, other lymphoid tissues not yet examined may be exquis-

itely sensitive to carbon dioxide. Because the changes were small in all measured immune categories, other issues must be considered. The availability and cost of various CO_2/O_2 mixtures, facilities, time to death, possible distress, and other factors may override the importance of minimizing small immunologic differences, as long as the same euthanasia method is used throughout the study.

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