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Bone marrow reconstitution of lethally irradiated canines using autologous bone marrow fractions obtained by counterflow centrifugation-elutriation

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Bone marrow reconstitution of lethally irradiated canines using autologous bone marrow fractions obtained by counterflow centrifugation-elutriation

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SUMMARY. Canine bone marrow fractionated by counterflow centrifugation-elutriation results in three areas of nucleated cell recovery. Fraction 1 accounts for 50% of the total nucleated cells and 25–40% of the total recovered CFU-GM activity. Fraction 2 contains less than 2% of the total nucleated cells and less than 0·2% of the CFU-GM activity. Fraction 3 accounts for approximately 50% of the total nucleated cell recovery and 60–75% of the total recovered CFU-GM activity. Animal survival was not directly correlated with the levels of CFU-GM activity in the transfused fractions. Autologous infusion of these fractions into irradiated canines (9 Gy, 0·1 Gy/min) resulted in distinct survival profiles. Canines receiving autologous fraction-2 cells showed no haematological reconstitution, with death occurring on days 10–11 post-irradiation. Canines receiving autologous fraction-3 cells showed limited myeloid repopulation of both the bone marrow and peripheral blood with a mean survival time for 24 d. Canines receiving autologous fraction-1 cells showed complete haematological reconstitution after 48 d and long-term survival. The data may indicate a separation or enrichment of pluripotential stem cells (fraction 1) from committed myeloid progenitor cells (fraction 3).

The enrichment or isolation of haemopoietic precursors from bone marrow has been described by investigators using various fractionation techniques such as adherence (Burghouts *et al*, 1980), velocity sedimentation (Bol *et al*, 1979; Burghouts *et al*, 1980), continuous or discontinuous buoyant density centrifugation (Bol *et al*, 1979; Richard *et al*, 1964; Wells *et al*, 1977), and light-activated cell sorters (LACS) (Vissner *et al*, 1980). These procedures have all utilized various assay procedures to determine the presence of committed progenitor cells (CFU-GM, BFU_E) or multipotential stem cell activity (CFU_s).

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We have reported previously the use of counterflow centrifugation-elutriation (CCE) in the fractionation of murine, canine, monkey and human bone marrow aspirates (Jemionek *et al*, 1982). Each species displayed a unique profile for fractionated total nuclear cells and associated CFU-GM activity. Although CCE has been used in bone marrow fractionation to isolate cell populations for study of cell to cell interaction in culture (Inoue *et al*, 1981), the relationship of *in vitro* culture assays and *in vivo* animal rescue using the various fractions isolated by CCE has not been reported.

The purpose of this manuscript is twofold. First, to identify that portion of CCE-fractionated canine bone marrow that is capable of marrow repopulation and long-term rescue of a lethally irradiated animal. Second, to attempt a correlation of nucleated cell recovery profile, CFU-GM activity profile, and *in vivo* marrow reconstitution in the various fractions obtained by CCE separation of canine bone marrow.

MATERIAL AND METHODS

Animal preparation. All research was conducted according to the principles enunciated in the 'Guide for the Care and Use of Laboratory Animals,' prepared by the Institute of Laboratory Animal Resources, National Research Council.

Canine. American Kennel Club-registrable male or female beagles (mean body wt 12·2±0·5 kg and approximately 1–2 years of age) were used in the study. Animals were anaesthetized with 4% (w/v) Surital during bone marrow removal and irradiation. Approximately 10 ml of bone marrow aspirate was obtained by multiple (four or five) rib aspirations. The aspirate was placed in a tube containing 1 ml of preservative-free heparin (1000 u/ml). The aspirate was then diluted with 20 ml of 2·5% dextrose–0·45% normal saline and held at 20°C while awaiting fractionation or transfusion.

Cobalt radiation. Canines placed in Plexiglas restraints were subjected to 9 Gy whole-body radiation (rotated 180° midway through exposure to obtain 4·5 Gy per side) at a dose rate of approximately 0·1 Gy/min using the Atomic Energy of Canada Limited Theratron. All measurements of radiation dose were obtained as midline dose. Immediately after radiation, the animals were given 250–300 ml of 2·5 dextrose–0·45% normal saline containing 1 ml of Multiple Vitamin B Complex (A. J. Buck, Cockeysville, Md).

Cell counts. A Coulter Counter Model ZBI with a Coulter Channelyzer H4 system was used for counting and sizing red blood cells (RBC) and white blood cells (WBC). All RBC (1 : 50 000 dilution) and total nucleated cell counts (TNC) (1 : 500 dilution) were done in triplicate on samples diluted in Isoton, using a 70μ aperture for counting. The TNC counts were obtained by lysing the RBC using Zapoglobin (Coulter Electronics) and counting the nuclei as previously described (Contreras *et al*, 1979). Platelet counts were determined using a haemocytometer. Haematocrit determinations were obtained using microhaematocrit tubes.

Elutriation procedure. Equipment required for bone marrow fractionation is depicted in Fig 1. All tubing lines and the JB6 rotor (Beckman Instruments) were flushed with 250 ml of 50% (v/v) NaClO-distilled water adjusted to pH 7·5 with concentrated HCl, followed by 1 litre of sterile water and finally by 250 ml of sterile 2 g % bovine serum albumin (BSA) Cohn Fraction V (Sigma Corp, St Louis, Mo.) in 0·05 M phosphate-buffered saline (PBS). Formation of the

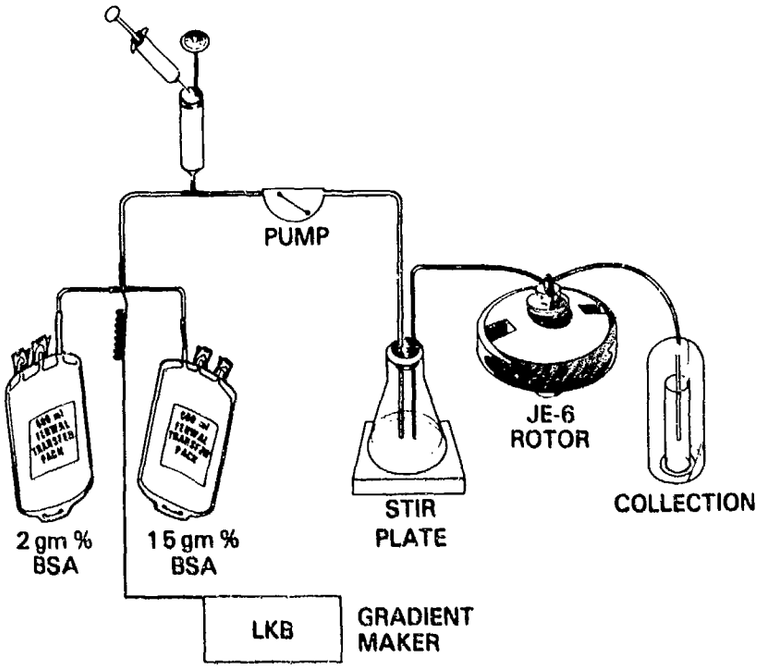

Fig 1. Schematic representation for separation of bone marrow cells by counterflow centrifugation-elutriation using a continuous albumin gradient. Bone marrow samples are entered aseptically into a syringe containing a self-sealing septum. The gradient formation from 2 g% and 15 g% bovine serum albumin media is controlled by an LKB Ultragrad gradient maker.

continuous gradient from 2 g% BSA in 0·05 M PBS (refractive index 1·3390) and 15 g% BSA in 0·05 M PBS (refractive index 1·3613) was regulated by an LKB Ultragrad gradient maker using a linear mode of 0–100 on a 1 h time scale. The bone marrow aspirate was diluted to a final volume of approximately 150 ml with 2 g % BSA in PBS (RBC count required to be below $4{\cdot}5 \times 10^8$ RBC per ml) filtered through nylon mesh and entered aseptically into a syringe containing a self-sealing rubber septum with a filtered air vent to ensure sterile conditions. With a rotor speed set at 2020 ± 10 rpm (determined by stroboscopic monitoring), the temperature of the centrifuge well set at 15°C, and the flow rate adjusted to 8·0 ml/min, the bone marrow sample was introduced into the CCE system via a three-way valve, which was repositioned for 2 g% BSA after completion of sample entry. As soon as the diluted sample began to enter the rotor, fractions were collected aseptically for 5 min intervals. The LKB gradient maker was initiated at the start of fraction 5. An aliquot was removed from each sample for cell counts and refractive index. The fractions were then centrifuged at 400 *g* and the cell pellet resuspended in 10 ml of 2·5% dextrose–0·45% normal saline.

Modified elutriation procedures. To ensure that the cells recovered in the later tube numbers of 9–12 were not altered by differences of time in the separation chamber or exposure to higher albumin concentrations relative to the cells recovered in the early tube numbers 1–5, the following modification to the elutriation procedure was also conducted. At the start of tube 5, the albumin gradient was not activated. At the start of tube 6, the rotor was turned off

and the cells were collected in subsequent tubes as the chamber cleared of cells. The cells collected in tubes 6–9 were then combined to form a modified fraction-3 collection.

Dextran sedimentation of RBC. To remove RBC from the early recovered cells of fraction 1, the cells recovered in tubes 1–5 were centrifuged at 400 *g* and resuspended in 50 ml of dextrose–saline. The RBC were sedimented at unit gravity using a 1:10 ratio of 6% dextran T500 (Pharmacia, Uppsala, Sweden) to cell suspension. After 45 min the nucleated cell population was decanted and diluted to 75 ml with dextrose–saline.

Marrow retransfusion. 2 h after irradiation, those animals selected for marrow recovery received either the whole marrow aspirate or pooled fractions obtained by CCE. The cells were returned intravenously (i.v.) via the cephalic vein.

Animal maintenance. For the first 3–4 d after irradiation, all food was withheld from the animals in order to minimize irritation to the gastrointestinal tract. For the subsequent 3–4 d, the animals received dry food moistened in warm water. By day 7, the animals were receiving normal dry dog food. At all times, water was freely available to the animals. Following irradiation and until the WBC count recovered to above 1000 WBC/mm^3, the animals received on a daily basis 250 ml of 1:1 ratio (v/v) of lactated Ringer's solution–2·5% dextrose–0·45% normal saline (i.v.) and antibiotics (250 mg ampicillin and 20 mg gentamycin) subcutaneously. In addition, beginning on day 1 post-radiation and on every alternating day until the WBC count recovered to above 1000 WBC/mm^3, 1×10^6 units of penicillin G benzathine suspension was administered intramuscularly.

Platelet concentrates obtained by plateletpheresis of 16 kg beagles were collected using the IBM 2997 cell separator. Approximately 8×10^{10} platelets in a 150 ml collection were transfused on days 7, 10, 14 and 17 post-radiation. Before transfusion, the platelet concentrate was irradiated with 50 Gy from a cobalt source.

Cell culture techniques. The double-layer soft agar culture technique was used to assay CFU-GM activity using the procedure described previously (MacVittie & Porvaznik, 1978). The source of colony-stimulating activity (CSA) in the canine model was plasma harvested from a dog injected 4 h previously with 50 μg of endotoxin (MacVittie & Walker, 1980).

Statistical analysis. All data are reported as the mean ± standard error of the mean.

RESULTS

Elutriation profile of canine marrow

In the canine model, the average nucleated cell recovery after CCE was 88 ± 2%. The profile of nucleated cell recovery per fraction obtained by CCE using a continuous albumin gradient is shown in Fig 2. Two major peaks of nucleated cell recovery are obtained by CCE as previously described. The fractionation profile may be thus subdivided into three major fractions. The first fraction comprises tubes 1–5 and is collected during sample entry into the separation chamber and before the start of the albumin gradient. Approximately 50% of the total nucleated count and approximately one third of the total CFU-GM activity are recovered in this fraction (Table I). Aside from the RBC, the major nucleated cell population recovered in Fraction 1 is composed primarily of normoblasts (14–20%) and lymphocytes (19–54%).

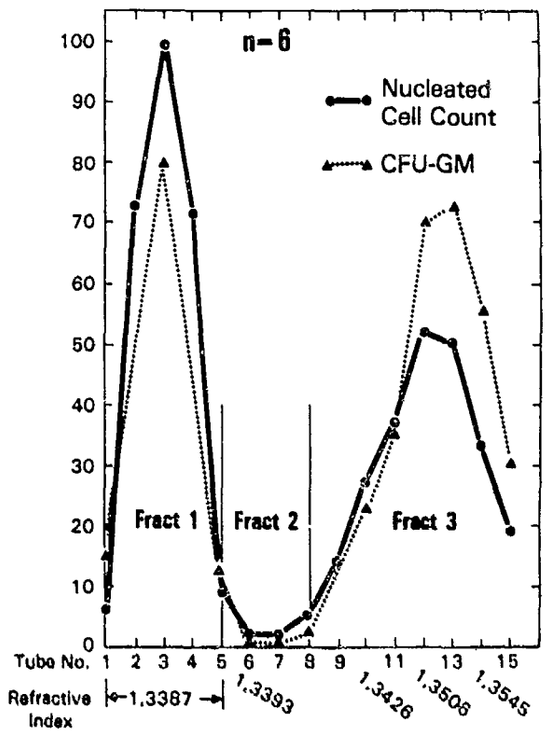

Fig 2. Recovery profile of per cent maximal nucleated cell count or CFU-GM activity versus tube number following bone marrow fractionation by counterflow centrifugation elutriation. Fraction numbers indicate pooled tube samples used for transfusion of marrow cells back to irradiated animals.

The second fraction accounts for less than 2% (Table I) of the total nucleated cells and less than 0·2% of the total CFU-GM activity. The major cell population in this fraction appears to be lymphocytes (38–55%), myeloblasts (1–15%), and neutrophils (6–19%). The third fraction accounts for approximately 45–50% to the total recovered nucleated cell count and 60–75% of the total recovered CFU-GM activity. The predominant cells in this fraction are

Table I. Analysis of cell count and cell culture activity in three major fractions obtained by counterflow centrifugation-elutriation from canine bone marrow

	Initial sample	Fraction 1	Fraction 2	Fraction 3
Mean nuclear cell count $\times 10^8$	7·14 ±0·73	3·69 ±0·34	0·13 ±0·03	3·23 ±0·41
Mean CFU-GM activity $\times 10^5$	6·50 ±0·67	2·26 ±0·44	0·06 ±0·01	4·95 ±0·83
Mean CFU-GM per 10^5 nucleated cells plated for	98 ±8	62 ±8	6 ±4	158 ±21

Data presented as mean ± standard error of mean.

cells of the granulocytic series: myelocytes (3–15%), metamyelocytes (3–10%), bands (10–25%) and segmented neutrophils (41–58%).

Radiation recovery and exposure

Canines subjected to 9 Gy total-body irradiation that receive an autologous bone marrow infusion 2 h post-radiation exposure can be expected to recover a normal haematological profile (Fig 3). Three positive control animals (3.4 ± 2 kg) received a transplant of whole bone marrow containing $5{\cdot}32 \pm 2{\cdot}22 \times 10^8$ nucleated cells, which contained a CFU-GM population of $7{\cdot}06 \pm 2{\cdot}74 \times 10^5$ cells (133 ± 10 CFU-GM per 10^5 nucleated cells plated). The profile of platelets and WBC in the peripheral blood indicates a nadir in WBC count on day 8. By day 16, the WBC count increases above 1000 WBC/mm^3 and returns to near normal levels by day 52. The platelet count, on the other hand, remains depressed between days 12 and 44 and returns to normal levels (> 100 000/mm^3) between days 48 and 55. The animal suffered no haemorrhagic episodes even though the animal was supported between days 7 and 17 with four platelet transfusions.

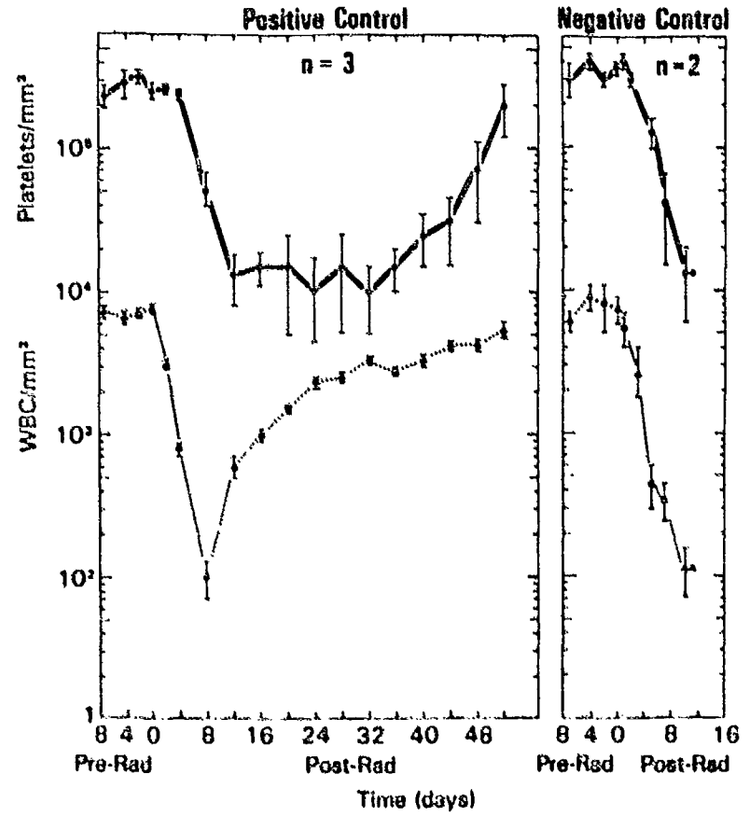

Fig 3. Post-irradiation recovery profile of WBC and platelets in irradiated animals which received autologous bone marrow cell transfusion (positive control) versus no bone marrow cell transfusion (negative control).

In contrast, the two negative control animals (12 ± 1 kg), which received no bone marrow infusion after irradiation, succumbed to sepsis or haemorrhagic episodes between days 10 and 11 even with antibiotic and platelet transfusions. The profile of peripheral blood WBC and platelet count (Fig 3b) shows both a platelet and a WBC nadir on day 10 or 11 at the time of death. No evidence of WBC recovery in the bone marrow or peripheral blood was evident.

Irradiated canines that received either fraction 1, 2 or 3 of CCE-fractionated bone marrow reveal quite dissimilar profiles of peripheral blood recovery and survival rates (Fig 4). Canines (12 ± 1 kg) that received fraction-2 cells were transfused with $0{\cdot}13 \pm 0{\cdot}03 \times 10^8$ nucleated cells that contained $6 \pm 1 \times 10^3$ CFU-GM cells (6 ± 4 CFU-GM per 10^5 nucleated cells plated). The peripheral blood profile of fraction-2 recipient animals is shown in Fig 4 and is identical to the negative control animals. Both the platelet and WBC count declined rapidly until the death of the animal between days 8 and 11.

In contrast, animals (12 ± 2 kg) that received fraction-3 cells or modified fraction-3 cells ($3{\cdot}23 \pm 0{\cdot}41 \times 10^8$ nucleated cells containing $4{\cdot}95 \pm 0{\cdot}83 \times 10^5$ CFU-GM based on 158 ± 21 CFU-GM per 10^5 nucleated cells plated) resulted in a significantly increased survival time relative to the negative control and a 20–25% recovery of WBC count but no recovery of the

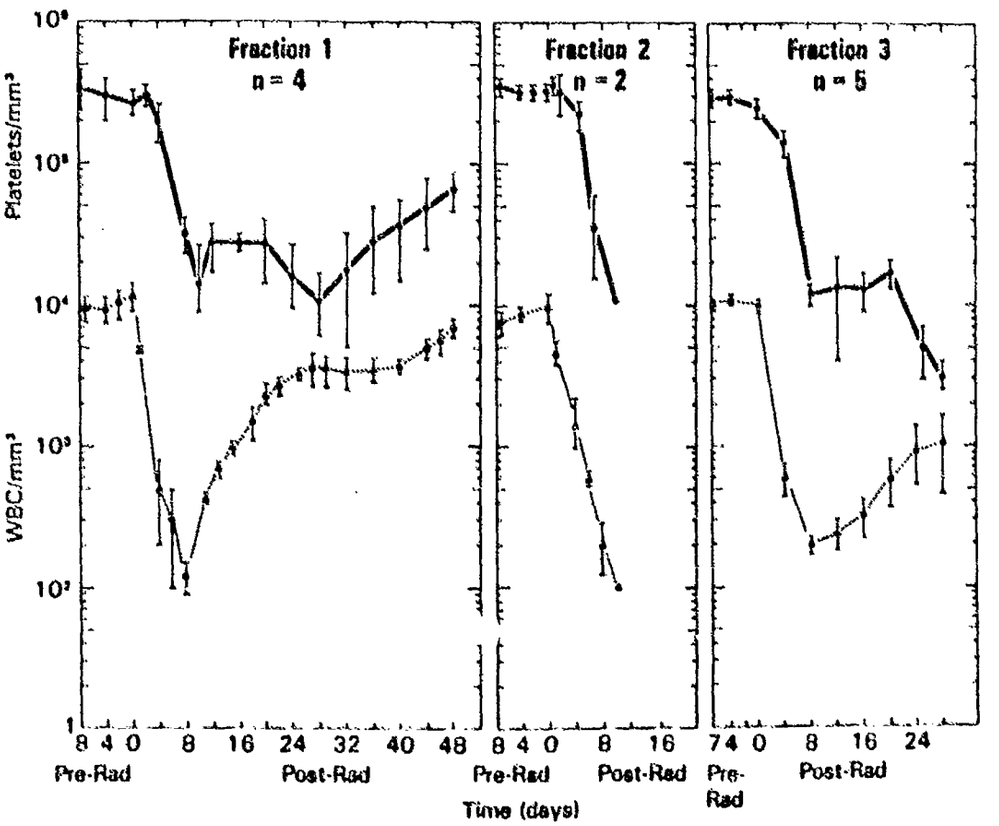

Fig 4. Post-irradiation recovery profile of WBC and platelets in irradiated animals which received pooled sample fractions as indicated in Fig 2.

platelets or long-term survival. Of the five dogs transfused with fraction-3 cells or modified fraction 3 cells, two died between days 13 and 16 of sepsis, one died on day 12 of thrombocytopenia and gastrointestinal haemorrhage, another died on day 27 of gastrointestinal haemorrhage, and one was killed on day 43 because of pancytopenia. As seen in the peripheral blood profile for fraction-3-transfused bone marrow (Fig 4), there was no recovery in the platelet count. However, the WBC count increased slowly from the nadir on day 8 but remained substantially depressed relative to positive control animals, with an average survival time of 22 d.

Those canines (11 ± 2 kg) that received fraction-1 cells or fraction-1-dextran-sedimented cells showed a peripheral blood profile (Fig 4) similar to the positive control animals. The fraction-1 recipient dogs received $3{\cdot}69 \pm 0{\cdot}34 \times 10^8$ nucleated cells containing $2{\cdot}26 \pm 0{\cdot}44 \times 10^5$ CFU-GM based on 62 ± 8 CFU-GM per 10^5 nucleated cells plated. The fraction-1 recipient dogs showed complete recovery of both marrow and peripheral blood profiles after 48 d. All fraction-1 recipient canines and positive control animals have survived beyond 9 months and still display a normal haematopoietic profile.

DISCUSSION

The results of our experimentation are summarized in Table II and the following conclusions deduced from the data.

First, even though fraction 3 or modified fraction 3 contain approximately 50% of the total nucleated cell count and 60–75% of the total CFU-GM activity of the fractionated canine bone marrow, this fraction is unable to restore completely haemopoiesis or achieve long-term survival of lethally irradiated animals. This fraction, however, is capable of partial restoration (approximately 10% of normal WBC count) in both the peripheral WBC and bone marrow population and a mean survival time of 22 d. This may indicate the presence of an enriched committed myeloid progenitor cell population in this fraction. Support for this supposition was based on five animals; one survived to day 43 and was killed when the haematocrit fell to 8 while maintaining a WBC count of 800 per mm^3. The other dog survived to day 28 with a

Table II. Summary of animal survival after irradiation and transfusion of fractionated canine bone marrow

	No. of dogs	No. of survivors	Mean survival
Positive control	3	3/3	Longer than 9 months
Negative control	2	0/2	Days 10 or 11
Fraction 1 or fraction 1 dextran-sedimented cells	4	4/4	Longer than 9 months
Fraction 2	2	0/2	Day 10 (days 8, 11)
Fraction 3 or modified fraction 3 cells	5	0/5	Day 22 (range days 13–43)

sustained peripheral WBC count of 1400 per mm^3. This is in marked contrast to the negative control and fraction-2 animals that died on days 8–11. The lack of complete marrow recovery for fraction-3 transfused cells is not due to the longer isolation time or presence of elevated albumin levels (Inoue *et al*, 1981) relative to fraction-1 cells, since the use of modified fraction-3 collected cells (abbreviated run time and no increased albumin exposure) failed to rescue the animals.

Second, fraction-2 cells, which contain few nucleated cells and virtually no CFU-GM activity, are unable to rescue lethally irradiated animals. Determining whether this is due to the low cell number or the total lack of progenitor cells will require further investigation.

Third, fraction-1 cells or fraction-1 dextran-sedimented cells are capable of long-term rescue of lethally irradiated animals and complete restoration of both bone marrow and peripheral blood profiles. This fraction therefore appears to contain at least the pluripotential progenitor cell population.

It should be noted that the survival of the animal was not related to the level of CFU-GM activity in the respective fractions. Fraction 1 cells contain only 25–40% of the total recovered CFU-GM activity and is capable of complete haemopoietic recovery, whereas fraction-3 cells account for 60–75% of the total recovered CFU-GM activity but does not permit animal survival.

The benefit of CCE in the study of haematopoiesis and bone marrow progenitor cell isolation is twofold. First, a large number of nucleated bone marrow cells may be fractionated within a reasonable time with minimal sample manipulation. Second, approximately 90% of the initial canine nucleated cell count can be reproducibly recovered following fractionation by CCE. The rapidity of sample fractionation, with minimal sample manipulation and efficient cell recovery of large numbers of cells, makes CCE an ideal first step in progenitor cell isolation and purification. The time required for cell separation of up to 9×10^8 TNC using CCE was approximately 1·5 h.

Our laboratory has fabricated two enlarged separation chambers of 13·3 and 28·3 ml capacity for cell isolation (Jemionek *et al*, 1980a, b). The 13·3 ml chamber has been found capable of fractionating at least 5×10^9 nucleated cells from canine marrow with a similar fractionation profile as obtained with the Beckman 4·3 ml chamber. The capability of one of these chambers to fractionate large numbers of human marrow cells ($> 5 \times 10^9$ nucleated cells) has not been attempted. If human marrow can be shown to be completely identical to the canine marrow in separation cell profile (Jemionek *et al*, 1982), with the possible separation of pluripotential progenitor cells from committed progenitor cells, the horizons for both basic haematological and clinical research will be greatly expanded.

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