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Effects of Cervical Dislocation on Colony-Forming Cells in Murine Marrow Cultures[1]

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Bone marrow obtained in a conventional manner after killing mice by cervical dislocation demonstrates increased absolute numbers of GM-CFC when compared to that from living anesthetized mice. The anesthetic agent does not account for the observed difference. Interpretation of all studies of murine marrow culture must take this observation into account, and extrapolation of the results of such studies to living human marrow should be made with care. Further studies of the mechanisms by which cervical dislocation increases the numbers of GM-CFC may help to elucidate certain mechanisms of commitment of the pluripotential hemopoietic stem cell.

Key words: cervical dislocation – granulocytes – macrophages – colony forming cells

Murine bone marrow for *in vitro* study of granulocyte-macrophage colony-forming cell counts (GM-CFC) is conventionally obtained after killing the animals by cervical dislocation or with ether (1). The femur or tibia is immediately removed and cells are flushed out from the marrow cavity.

Cervical dislocation has usually been used because of its simplicity and efficency. It has always been presumed that cells obtained in this manner are essentially normal. However, cervical dislocation causes sudden cessation of cardiorespiratory function associated with cervical spinal cord transection, airway obstruction and laceration of the great vessels. The effects of this acute insult upon bone marrow function do not appear to have been studied. We therefore compared GM-CFC counts in cultures of bone marrow obtained from living, anesthetized mice with those from mice killed in the conventional manner, by cervical dislocation.

MATERIALS AND METHODS

Syngenic female $C_{57}B1$ mice (Jackson Labs, Bar Harbor, Maine) were used in all experiments.

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Marrow was obtained and cultured from three groups of animals:

Group A: Pentobarbital Sodium (Diabutal, Diamond Labs) was administered intraperitoneally in a dose of 30–40 mg/kg to each mouse, which was sufficient to prevent all but minimal responses to painful stimuli, but to permit regular respiration. Between 10–20 minutes after the injection, the skin was prepared with 70% alcohol and the left femur removed in a sterile fashion. All animals continued to have regular respirations for 1 minute after femur removal, at which time they were sacrificed.

Group B: All animals received Pentobarbital in a similar fashion to Group A. After 10–20 minutes of anesthesia, cervical dislocation was performed and the femurs removed.

Group C: All animals were killed by cervical dislocation and the femurs removed within 5 minutes.

Marrow cells were obtained from all groups as follows: The femur was first washed superficially with CMRL-1066 medium (GIBCO, Long Island, New York). The marrow cavity was entered with a 25 gauge needle and cells flushed out with the same medium. Single cell suspensions were obtained by repeated aspiration and mixing of cells suspended in medium, using a 19 gauge needle and plastic syringe.

The cells were cultured at a final concentration of 75,000/ml, suspended in a 1.8% methylcellulose containing CMRL-1066 medium supplemented with vitamins, pyruvate and amino acids, with 16% fetal calf serum, 0.1 ml of conditioned medium (CM) prepared from mouse L-cells was added to each 1 ml (final) culture in 35 × 10 mm Petri dishes (Falcon Plastics). This 10% concentration of CM was above that required to produce maximal responses in all cultures. GM-CFC were counted on day 7, following incubation at 37°C in 10% CO_2 and 100% humidity.

All marrow collections and preparations were performed by a single person using the same batches of medium, fetal calf serum and CM. Cultures were performed in duplicate, coded and counted in a blind fashion by one observer. All cell groups containing 50 cells were counted as colonies.

RESULTS

Post-mortem examination of the necks of animals killed by cervical dislocation revealed cervical spine damage, high spinal cord transection, severance of the trachea and extensive hemorrhage from rupture of the great vessels in the neck and mediastinum. These changes were consistently seen in all animals. During actual removal of the femur, active bleeding was encountered in all live animals, but not in those killed by cervical dislocation.

Table 1 compares GM-CFC formation in the experimental groups. Group A

TABLE 1

Comparison of GM-CFC formation in marrow obtained from living versus dead† mice

Status at time of femur removal	Group	No.	Pentobarbital*	Cervical dislocation	Colony counts**
Living	A	6	+	–	48.5 ± 7.48
Dead	B	6	+	+	92.1 ± 12.87
	C	6	–	+	92.9 ± 7.57

Student '*t*' test: A versus B: $P < 0.01$
A versus C: $P < 0.005$
A versus B + C: $P < 0.005$

*Pentobarbital dose = 30–40 mg/kg

**Mean values ± s.e. mean of replicate cultures of 75,000 bone marrow cells in 1.0 cm^3, counted on day 7.

†All mice killed by cervical dislocation

(living) had a significantly lower count than groups B and C (killed by CD) on day 7 ($P < 0.005$). There was no significant difference between groups B (cervical dislocation after pentobarbital) and C (cervical dislocation alone). No significant changes in GM-CFC counts occurred when serum obtained from heart blood of killed mice was added to the culture (Table 2).

DISCUSSION

Cells obtained for murine marrow culture after killing the animal by cervical dislocation have always been considered to approximate the 'normal' marrow. In the present study however, GM-CFC counts in marrow from mice killed by cervical dislocation were significantly higher than those from living anesthetized animals. Since the latter group probably represents more closely the resting physiological state of the marrow, it appears that events associated with cervical dislocation significantly alter the marrow stem cell pool.

There is no information in the literature describing the effects of sudden death on the bone marrow. Schubert (2) described the post-mortem histopathological appearances of 'shock marrow' in 109 patients who died in shock of various causes. He noted platelet aggregates (100%), fibrin networks (33%), small areas of necrosis (28%), microthrombi (25%), and great numbers of nucleated cells and their precursors in the sinusoids (20%). However, he did not comment upon the possible pathophysiological correlations in the living.

Since maximal CSF stimulation was provided in all cultures, cervical dislocation appeared to cause an absolute increase in the numbers of GM-CFC. This increase must arise either from division of pre-existing GM-CFC or by commitment of pluripotential hemopoietic stem cells (PHSC). The latter possibility is of special interest because very little is

TABLE 2

Effect of serum from killed mice on GM-CFC formation in marrow from living and dead mice*

Status at time of femur removal	Group	Pento-barbital**	Cervical dislocation	Without killed mouse serum		With killed mouse serum		Student *'t'* test (*P*)
				n	Colony counts	*n*	Colony† counts	
Living	A	+	–	4	59.7 ± 4.05	2	61.5 ± 7.50	<0.80
	B	+	+	4	89.5 ± 6.09	3	94.6 ± 3.50	<0.50
Dead	C	–	+	4	95.86 ± 7.07	4	109.5 ± 1.38	<0.30

*Pooled serum obtained from intracardiac samples 5–10 minutes after cervical dislocation in groups B and C.

**Pentobarbital dose 30–40 mg/kg

†Mean values ± s.e. mean of colonies with > 50 cells, in replicate cultures of 75,000 bone marrow cells in 1.0 cm^3, counted on day 7.

presently known about the factors which control commitment of the PHSC (3).

Cervical dislocation causes sudden death by a multiplicity of factors, including spinal cord transection with respiratory paralysis, airway obstruction and massive hemorrhage. An increase in GM-CFC was noted as early as 40–60 seconds after death; several different mechanisms could be responsible.

Serum obtained from the heart blood of recently killed mice did not significantly increase GM-CFC numbers in any of the cultures, when added in a 10% concentration, suggesting that serum factors may not be responsible for the observed phenomenon. Studies on the effects of chronic hypoxia on guinea pig bone marrow (4, 5) suggested that, if anything, the absolute count of morphologically recognizable granulocyte precursors decreased in number after prolonged exposure to low oxygen tensions. This suggests that hypoxia may not have caused the cervical dislocation-mediated effects. Since complete spinal cord transection occurs during cervical dislocation, potential effects mediated by changes at neurotransmitter release sites need to be considered. Little is known about the innervation of the bone marrow, and even less about the significance, if any, of these nerves (6, 7). One plausible explanation of these data in relation to prior observations may relate to the extensive hemodynamic changes that undoubtedly arise from the sudden disruption of the mediastinal vessels and consequent hemorrhage. These changes must be reflected in the sinusoids of the marrow, and may have a role in causing changes in the 'hemopoietic inductive microenvironment', changes that are thought to regulate PHSC commitment (1). Murine marrow has always been noted to contain higher absolute numbers of GM-CFC in cultures than human bone marrow. Our data suggest that this may relate less to species differences than to the fact that the latter is always obtained from living subjects.

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REFERENCES

1. Metcalf D, Hemopoietic colonies. *Recent Results in Cancer Research,* Vol 61 pp 5–35 Springer-Verlag New York, 1977.
2. Schubert G E, Pathologisch-anatomische Befunde im Knochenmark des Menschen nach Schockgustanden. *Klin Wschr* **53:** 481–488, 1975.
3. Van Bekkum D W, The appearance of the multipotential hemopoietic stem cell. *Experimental Hematology Today* (Baum S J & Ledney G D eds) pp 3–10 Springer-Verlag New York, 1977.
4. Yoffey J M, Jeffreys R V, Osmond D G, Turner M S, Tahsin S C & Niven P A R, Studies on hypoxia. VI. Changes in lymphocytes and transitional cells in the marrow during the intensification of primary hypoxia and rebound. *Ann N Y Acad Sci* **149:** 179–192, 1968.
5. Rickard K A, Rencricca N J, Shadduck R K, Monette F C, Howard D E, Garrity M & Stohlmann F Jr, Myeloid stem cell kinetics during erythropoietic stress. *Brit J Haemat* **20:** 537–547, 1971.
6. Miller M L & McCuskey R S, Innervation of bone marrow in the rabbit. *Scand J Haemat* **10:** 17–23, 1973.
7. Calvo W, The innervation of the bone marrow in laboratory animals. *Amer J Anat* **123:** 315–328, 1968.

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