Blood Flow to Bone Marrow During Development of Anemia or Polycythemia in the Rat

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We applied the radioactive microsphere method to follow the magnitude and time course (0 to 96 hours) of blood flow changes during development and recovery from anemia in awake rats. Blood flow was also monitored during a 96-hour period after polycythemia was induced by erythropoietin administration. The possible influence of innervation was also examined. After a blood loss of approximately 50% (hypovolemia), blood flow to the femoral marrow tripled within 12 hours and remained elevated for the entire 96-hour period. The relative increase in blood flow to the femoral bone was even greater. Similar findings were obtained in rats with phenylhydrazine (PHZ) hemolytic anemia (normovolemia). Denervation had no detectable effect on the increased blood flow to either marrow or bone. The augmented blood flow during hemolytic anemia was accompanied by a doubling of the oxygen consumption rate by the marrow, while the glucose uptake was not detectably altered. Erythropoietin supplements (3 × 1,000 IU/kg, SC, 6-hour intervals) increased blood flow to the marrow by approximately 25% after 48 hours, and at 72 hours the blood flow had reached a value twice that obtained under control conditions. These results indicate that blood flow to bone marrow is highly variable and hormonally and/or locally regulated. This may have practical consequences for marrow transplantation technology and for administration of drug therapy to patients with insufficient bone marrow hematopoiesis.

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was denervated. The sciatic nerve on one side was exposed at the neck, filled with heparin, exteriorized adjacent to the mean arterial blood pressure. The other end of this catheter was accessed to the catheters was ensured. The exact positions of the catheters were verified by dissection postmortem.

In each of the 32 rats in groups C and D (see below), one hind leg was denervated. The sciatic nerve on one side was exposed just below the inguinal ligament and cut. The contralateral hind leg served as control.

All 155 rats were given ampicillin (15 mg/kg body weight, intramuscularly [IM]). Rats in groups A to F (see below) were returned to their cages to recover. Weight loss during the first 24 hours was at most 2.2% of initial body weight. Weight reduction per 24 hours was smaller during the remaining experimental period. Values for leukocytes, arterial blood gases, and arterial pH all remained within the normal range during the experimental period in all 155 rats.

Blood Flow Measurements

The microsphere method described by Heymann et al.9 was adopted to the present study as detailed by Iversen et al.5 Evidence for the validity and reliability of our version of this method has recently been presented.10 Briefly, microspheres (diameter, 16 μm; New England Nuclear, Boston, MA) labeled with either 111In, 51Cr, 198Au, or 152Eu and stored in 0.9% NaCl containing 0.2% Tween 80, were ultrasonicated for 2 minutes. Shortly after and immediately before an infusion, the microspheres were vigorously mixed for 1 minute with a vortexer. The microspheres were then transferred to a 10-mL syringe and placed on the infusion pump (Harvard Instruments, Cambridge, MA). The infusion system was designed to ensure adequate mixing of the microspheres during the infusion period. This was verified in a previous study where we found that less than 0.5% of the microspheres were deposited as pairs or clusters.9 This latter result stems from microscopic examinations of skeletal muscles from a rabbit that had received 6.5 x 10⁶ microspheres. Each infusion contained approximately 3.0 x 10⁷ microspheres labeled with only one nuclide (randomly chosen). The infusions each lasted 30 seconds at a constant rate of 3 mL/min. The reference catheter in the brachial artery was connected to a similar pump. Withdrawal of blood through this catheter, at a rate of 1 mL/min, started 30 seconds before the infusion and was stopped 1 minute after the infusion was ended. The volume deficit of 0.5 mL (volume in reference sample minus the volume infused) was immediately corrected with 0.9% NaCl (intraarterially). The 143 unrestrained rats in groups A to F (see below) were all standing quietly during the periods of tracer infusions.

Organ blood flow (F, mL/kg/100 g x min) was calculated according to the equation: F, = F x Q/Q, where F, is the reference sample flow (1 mL/min), Q, is the number of microspheres deposited per 100 g tissue, and Q is the number of microspheres in the reference sample. The lowest number of microspheres deposited in any sample was 572.11

Assessment of Glucose Uptake

The deoxyglucose method was used to determine the uptake of glucose from plasma into the marrow cells from the femurs of group F rats (see below).12 We gave 0.74 MBq (20 μCi) of [3H]-deoxyglucose (specific activity, 3.8 GBq/mg, 103 mCi/mg; Amersham, Buckinghamshire, England) as an intraarterial bolus injection. The [3H]-deoxyglucose was then allowed to circulate 30 minutes before the rats were killed. All marrow cells from one femur in each rat were then removed as described below, and the radioactivity determined. The value for glucose uptake for all marrow cells sampled from one femur was expressed as a percentage of the injected dose of [3H]-deoxyglucose. This technique thus yielded one estimate of the glucose uptake into the marrow in each of the 36 rats, at six time points, six of the rats serving as preintervention controls. In four separate rats, we perfused the whole body postmortem and then determined the marrow glucose uptake. The values for glucose uptake in these rats were similar to those in the six preintervention control rats, indicating that the measured values represent true uptake of glucose by the marrow cells.

Determination of Oxygen Consumption Rate

Oxygen consumption rate in group G rats (see below), six test rats, and six control rats was estimated from the changes in oxygen tension in an incubated marrow cell suspension as described by Opdahl et al.13 Immediately after each rat was killed, all marrow cells were removed from one femur as described below. All marrow cells were then suspended in Fisher's medium (Gibco, Paisley, Scotland) containing 0.5% bovine albumin (Sigma, St Louis, MO) and adjusted to pH 7.35 with 10 mmol/L HEPES buffer (Sigma). One milliliter of this suspension was enclosed in a chamber. The suspension was kept at 37°C and constantly stirred, its oxygen tension being measured continuously with a polarographic electrode (MSE, UK). In this way, one measurement could be obtained from each rat. The values for oxygen consumption rate were expressed as mmol O₂/min.

Analyses of Blood Variables

Hemoglobin (Hb), hematocrit (Hct), and reticulocytes were measured in duplicate with standard procedures. The values for reticulocytes were corrected according to the formula: Corrected reticulocytes (%) = actual reticulocytes (%) x (actual Hct/control Hct). When several measurements of either Hb, Hct, or reticulocytes were made from one rat, the person performing the measurements was ignorant about the order in which the samples had been collected.

Levels of erythropoietin were determined in plasma from groups A and E rats with a commercial kit (Medac, Hamburg, Germany) based on an enzyme-linked immunosorbent assay (ELISA) procedure as outlined by Goto et al.14

Experimental Protocol

Group A (bleeding and hypovolemic anemia). Forty-eight hours after surgery, one batch of microspheres was infused into 30 rats (control infusion). Hypovolemic anemia was then induced by bleeding through the intracardial catheter at a rate of 2 mL/min with a withdrawal pump (Harvard Instruments). The blood loss was made to be approximately 1.5% of the body weight and was typically between 5 and 7 mL. Microspheres were then infused into 10 rats 5 minutes, 24 hours, and 48 hours after the control infusion, into another 10 rats after 6, 17, and 96 hours, and finally into another 10 rats after 12, 72, and 84 hours. Into five of these latter rats, we also infused 0.19 MBq (5 μCi) [57Fe]-citrate (specific activity, 1.3 GBq/mg, 36 mCi/mg, IP; New England Nuclear, Dreieich, Germany) 5 hours before the bleeding. Five other rats (sham anemic) were bled in a similar manner as described above, but the blood was immediately reinfused. These five rats also received 0.19 MBq [57Fe]-citrate 5 hours before the reinfusion. Microspheres were
infused just before bleeding (control infusion) and then after 5 minutes, 24 hours, and 96 hours.

Group B (hemolysis and normovolemic anemia). Microspheres were infused into 20 rats 48 hours after surgery. The hemolyzing agent phenylhydrazine (Phz; 50 mg/kg body weight, IP) was then administered immediately. Microspheres were then infused into 10 of the rats 12 and 48 hours after the control infusion and into the remaining 10 rats after 36, 72, and 96 hours.

Group C (derennervation and hemolysis). Twenty rats with one hind leg denervated were each given one batch of microspheres 96 hours after surgery. Normovolemic anemia was induced as described for group B and microspheres were administered into 10 of the rats 48 and 96 hours after the control infusion. The remaining 10 rats received microspheres after 12, 36, 72, and 72 hours.

Group D (derennervation, bleeding and plasma transfusion; normovolemic anemia). From each of 12 rats, we removed approximately 6 mL plasma 2 weeks before surgery. The plasma was stored at -70°C until use. These 12 rats had one hind leg denervated and were each given one batch of microspheres 96 hours after surgery. The rats were then bled as described for group A rats. Immediately following the bleeding, the blood loss was replaced with an equal amount of autologous plasma. Microspheres were then given to six of the rats 3, 6, and 12 hours after the control infusion. The other six rats received microspheres after 24 hours.

Group E (polycythemia). Twenty rats were all given one infusion of microspheres 48 hours after surgery. Recombinant human erythropoietin (rHuEPO, Integrated Genetics, Boston, MA 1,000 IU/kg body weight, SC) was then administered three times at 6-hour intervals. Ten rats received microspheres 6, 24, and 72 hours after the control infusion. The remaining 10 rats were given microspheres after 12, 48, and 96 hours.

Group F (glucose uptake and hemolysis; normovolemic anemia). Forty-eight hours after surgery, six control rats received one intraarterial bolus injection of 1H-deoxyglucose. In another 30 rats, normovolemic anemia was induced as described for group B. Deoxyglucose was injected either 5 minutes, or 12, 24, 48, or 96 hours after injection of Phz, with six rats in each group. The rats were killed 30 minutes after the deoxyglucose injection. The activity of 1H in plasma was then virtually zero.

Group G (oxygen consumption rate and hemolysis; normovolemic anemia). Forty-eight hours after the injection of ampicilline, six rats were killed, and the marrow cells within one femur from each rat were analyzed with respect to the oxygen consumption rate. Hemolytic anemia was induced as described for group B rats in six other rats 24 hours before the oxygen consumption rate was determined.

Preparation of Samples

Experiments were terminated by giving rats a lethal dose of pentobarbital.

One femur was removed from rats belonging to groups B, E, F, and G and both femurs from group A rats. Both tibiae were removed from rats in groups C and D. The bone was cleared of all visible soft tissue by dissection. The femurs and the tibiae were then weighed before the marrow was flushed out. Marrow cells were retrieved as completely as possible from the bones by flushing the marrow cavity with 0.9% NaCl and by scraping the cavity wall. Flushing was performed several times with a total amount of 60 mL saline. In addition, one femur from each of the group A rats was flushed twice with 20 mL 1% collagenase in saline (37°C; Sigma type 2). Between these flushes, the collagenase-containing femurs were incubated for 5 minutes at 37°C. Finally, all bones, except from group G rats, were flushed once with 20 mL aceton and dried with air before they were reweighed.

From 11 specimens of decalcified bone taken from group A rats, histological preparations (20-μm cross-sections) were made and stained with hematoxylin and eosin.

One nutritional artery was removed from both tibiae in four rats in group E. These arteries were prepared for fluorescence histochemical demonstration of catecholamines according to the method of Furness and Costa.16

Assessment of Radioactivity

Gamma-emitting nuclides within the bones and marrow samples from groups A to E were counted in an Auto-Gamma 5220 (Packard, Massachusetts). Cross-over between the various γ-emitting nuclides was corrected for by stripping, neglecting the minute (<0.5%) overlap of the lower energy nuclides into the higher ones. Bones from the rats in group B were counted before and after they had been dissolved in 2 mL ammonium hydroxide (Soluene-350, Packard). The count rates were identical and thus there was no self-absorption in the bone.

Bone and marrow samples from group F rats were also dissolved in 2 mL ammonium hydroxide. Then, 2 mL scintillation liquid (Hionic Fluor, Packard) was added. The 1H activity was then recorded in a β-counter (Tri Carb 460, Packard). The degree of quenching of the 1H-nuclide (usually ~17%) was determined with an internal (3H) standard.

The smallest number of counts above a low background for any sample was 6,900, from either a γ- or a β-emitting nuclide.

Statistics

Values are expressed as medians with their 95% confidence intervals, determined with a nonparametric method. If the median of a control group was not included in the 95% confidence interval of the test group, and vice versa, then the two groups of data were considered significantly different at the 5% level. In the figures, the median symbols sometimes cover one or both ends of the confidence bars.

RESULTS

Validation Studies

Group A. Compared with the five sham-operated rats, the uptake of 59Fe into the marrow was approximately 60% higher in the anemic rats 96 hours after bleeding. No appreciable activity of 59Fe was detected in any of the flushed bones, indicating that the removal of marrow by flushing the bones had been complete. No significant differences were found in blood flow to bone between those femurs that had been flushed with collagenase and those that had not.

Histological preparations from the interface between the bone and the marrow cavity showed only occasional and small fragments of marrow. Microscopic examination of the marrow content of 10 rats showed only red and no yellow marrow.

Group E. The arteries supplying denervated tibiae were only faintly stained with glyoxylic acid, in marked contrast to the brightly stained arteries from the contralateral innervated bones. Thus, catecholamine stores had been depleted, indicating that denervation had been fairly complete.
Hypovolemic Anemia

Group A. Approximately 12 hours after bleeding, both Hb and Hct were reduced by approximately 50% (Fig 1). During the remaining experimental period, Hb and Hct remained fairly constant. The number of reticulocytes was markedly elevated approximately 48 hours after bleeding (Fig 1).

Ninety-six hours after bleeding, the plasma concentration of erythropoietin had increased markedly to 140 (135 to 155) mU/mL, as compared with the control value of 7 (5.5 to 8.5) mU/mL.

Values for mean arterial blood pressure and heart rate are presented in Table 1. During bleeding, the mean arterial blood pressure decreased, from approximately 95 mm Hg to approximately 60 mm Hg. The rats then remained hypotensive throughout the observation period. A concomitant increase in heart rate from approximately 450 to 530 bpm persisted for approximately 24 hours after bleeding. The heart rate then gradually decreased.

Blood flow to the marrow had increased substantially by 6 hours after the bleeding, and it remained elevated approximately three times above the control level for the entire 96-hour period (Fig 2). A surprising increase \((P < .05)\) in blood flow to the bone was even more pronounced in relative terms, but showed a more transient course (Fig 2).

Normovolemic Anemia

Group B. Data for Hb, Hct, and reticulocytes were similar to those presented for group A (Fig 1), but with a slower development of the anemia.

No major changes in either mean arterial blood pressure or heart rate were detected (Table 1).

The temporal changes in blood flow to the marrow and bone following Phz injection are depicted in Fig 3. In these rats, blood flow to the marrow increased more slowly, thus reflecting the slow development of anemia. However, marrow blood flow reached a higher peak value than in group A rats (50 v 35 mL/100 g x min).

Group C. The blood flow pattern for both the marrow and bone was similar in the innervated and denervated hind leg (Fig 4); consequently, denervation had no detectable effect on the initiation and maintenance of a high level of blood flow to marrow and bone. The tibial bones showed a more marked hyperemia than the femoral bones in group B rats (Fig 3 v Fig 4); however, no direct comparison has been performed of the blood flow rates to these bones.

Group D. Blood flow increased rapidly to marrow and bone in both the innervated and denervated hind leg (Fig 4). Thus, the sympathetic vasomotor activity did not play any appreciable role for the rapid induction of the hyperemic response to the acute normovolemic anemia. Neither mean arterial blood pressures nor heart rates were significantly different from the values obtained during control conditions in rats of groups C and D.

Injection of rHuEPO

Group E. Hb, Hct, and reticulocytes all increased within 72 hours after rHuEPO injections (Fig 1), indicating an

Fig 1. Time course of changes in Hb, Hct, and corrected reticulocyte counts for 30 rats in group A (bleeding, hypovolemic anemia), 20 rats in group B (Phz-hemolysis, normovolemic anemia), and 20 rats in group E (rHuEPO, polycythemia). The arrows denote the time point when bleeding (group A), Phz injection (group B), or injection of rHuEPO (group E) was initiated. Data points corresponding to the arrows are preintervention control values. Median values with their 95% confidence intervals are shown.
Table 1. Hemodynamic Characteristics of Experimental Rats During Control and Experimental Phases

<table>
<thead>
<tr>
<th>Group</th>
<th>Before One Infusion</th>
<th>After One Infusion</th>
<th>Before Last Infusion</th>
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<tbody>
<tr>
<td></td>
<td>MABP (mm Hg)</td>
<td>HR (bpm)</td>
<td>MABP (mm Hg)</td>
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<tr>
<td>E</td>
<td>90-105</td>
<td>425-446</td>
<td>93-104</td>
<td>435-452</td>
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Mean arterial blood pressure (MABP, mm Hg) and heart rate (HR, bpm) obtained before and after the first and last infusion of microspheres, respectively, in groups A, B, C, D, and E. Values are the 95% confidence intervals of the medians. Data obtained before the first infusion represent baseline values.

increased erythropoietic activity. A concomitant hyperemia was observed both in the marrow and in the bone (Fig 5). However, the blood flow increase was not significant until 48 hours after the first rHuEPO injection, and not marked before the 72-hour sampling. The plasma concentration of erythropoietin increased from 6.0 (4.5 to 7.5) mU/mL under control conditions to 9.5 (8.5 to 11.5) mU/mL at 48 hours, further to 55 (35 to 85) mU/mL at 72 hours, and finally to 145 (130 to 155) mU/mL at 96 hours.

Marrow Uptake of Glucose

Group F. Glucose uptake remained at control levels throughout the experimental period (P > .05), measurements being made just before or either 5 minutes, or 12, 24, 48, or 96 hours after PHz injection. The median uptake of glucose into the retrieved marrow cells from one femur was approximately 2.5 x 10^{-5} % of the injected dose (36 rats). Thus, the induced hemolytic, normovolemic anemia was not accompanied by a detectable change in marrow metabolic activity as measured by glucose uptake.

Marrow Oxygen Consumption Rate

Group G. The oxygen consumption rate of femoral cells (see group F above) doubled (P < .05) from 15.3 (12.4 to 18.6) to 32.4 (30.8 to 36.7) nmol O_2/min (six rats) during transition from the control to the anemic condition 24 hours after induction of hemolysis. The enhanced erythropoietic activity within the marrow thus appeared to be accompanied by an increase in both blood flow and oxygen consumption rate.

DISCUSSION

Blood Flow to Marrow and Bone During Stimulated Erythropoiesis

We found that bone and bone marrow blood flow increased markedly in rats after induction of both a hypovolemic (bleeding) and a normovolemic (hemolytic) anemia and after polycythemia elicited by rHuEPO injections. The increase in blood flow occurred early after bleeding, being significant after 6 hours, when the Hb was still only slightly below normal. The start of the increased blood flow was intermediate in time under the hemolytic regimen, but late after the rHuEPO injections. This succession in time was reflected by the three reticulocyte curves, in that their half-maximum values were reached approximately 24 hours after the half-maxima of the respective marrow blood flow curves.

To our knowledge, this is the first report on changes in bone marrow blood flow occurring during perturbations of
MARROW BLOOD FLOW DURING ERYTHROPOIESIS

steady-state hematopoiesis, with serial measurements obtained from one animal. Comparisons with previous data based on single measurements during steady-state, mostly on patients, are therefore of limited value.

Michelsen showed that blood flow to a denervated, perfused femur of a rabbit with Phz hemolytic anemia and accelerated erythropoiesis was approximately 2.5 times higher than the mean control values. Chen et al confirmed with the microsphere method that an increased marrow blood flow was present after 3 days and persisted for about 1 week after one large (25 mg/kg body weight, IP) dose of Phz to rabbits.

The mechanism behind the increased blood flow is unknown. Bone marrow contains sympathetic, noradrenergic, and vasoconstrictor nerves. In theory, the blood flow regulation is therefore possibly both hormonally/locally and neurally. However, a rapidly induced normovolemic, hemorrhagic anemia (group D, bleeding and plasma infusion) and a more slowly developing hemolytic anemia (group B) were accompanied by a rapidly and slowly occurring blood flow increase, respectively, that were not detectably different in the innervated and the denervated tibiae. This indicates that the augmented marrow and bone blood perfusion was not dependent on an intact innervation of bone and marrow in any of our experimental models. This conclusion is supported by our finding of a seemingly constant blood flow during the first 5 minutes after rapid bleeding (Fig 2), since a nervous regulatory mechanism would presumably have been apparent within this time period.

Erythropoietin might be a regulator of blood flow to bone marrow directly or indirectly. This suggestion has been made by earlier investigators for bone marrow and for spleen blood flow. When the increased marrow blood flow was similar in the bled and in the rHuEPO-injected groups of rats (A and E), 96 hours after the start of erythropoietic perturbations, we found similar and markedly greater than normal plasma concentrations of erythropoietin in the two groups. It could be that increased metabolism triggered by the enhanced erythropoiesis might lead to a larger blood flow, as is the case in, for example, skeletal muscle, putative chemical mediators here being K⁺, H⁺, phosphates, or endothelium-derived relaxing factors. Perhaps some more specific mediator, such as a cytokine or an intermediary of heme metabolism, is released in the marrow when erythropoiesis is accelerated. In any case, we observed a doubling of oxygen consumption rate in erythropoietically stimulated marrow, which indicates a higher metabolic rate. We cannot explain the lack of any detectable increase in the measured glucose uptake by the marrow, but it could mean that the erythroid cells compete with the myeloid cells for glucose as an energy substrate, so that the total glucose uptake remains approximately the same during increased

Fig 4. Blood flow to the tibial marrow and bone with intact and cut nerves. Results are from group C rats (hemolytic normovolemia), and group D rats (bleeding and plasma transfusion, normovolemic anemia).

Blood flow

ml/100g·min

Fig 5. Blood flow to the marrow and bone in group E rats given three rHuEPO injections (1,000 IU/kg, SC, 6-hour intervals). Symbols as in Fig 2.
metabolism is more prevalent in myelopoietic than in erythropoiesis.\textsuperscript{24} However, this hypothesis also rests on the assumption that anaerobic metabolism is more prevalent in myelopoietic cells.

We found concomitant changes in blood flow to marrow and to bone, the only exception being the late phase of hypovolemic anemia, when marrow blood flow stayed high, while bone blood flow had declined. The parallel blood flow increase could be due to the fact that the nutritional artery looses its muscular tunica media shortly after penetration to the marrow cavity.\textsuperscript{25} Branches given off thereafter to either the haversian canals of the bone or to the marrow would receive increased blood supply if the total blood flow is enhanced by dilatation of the muscular parts of the vessels.

Why the increase in blood flow to the bone after bleeding was transient is not easily explained. Chen et al\textsuperscript{6} did not detect any significant increase in cortical blood flow at all in anemic rabbits, and Michelsen's results led him to postulate that the blood flow increase to the marrow was at least partly due to decreased venous resistance.\textsuperscript{3} New approaches therefore appear necessary to clarify this confusing picture. The microsphere method allowed accurate determination of blood flow to the bone marrow. This method does not give information as to which vessels contribute to any changes in vascular resistance. Furthermore, it does not give any information about events within the microcirculation, except for their contribution to overall vascular resistance.

The Microsphere Method

The validity and reliability of the microsphere method for estimation of cardiac output and organ blood flows in conscious rats have been firmly established.\textsuperscript{26-29} However, there are conflicting data concerning hemodynamic stability after infusion of high numbers of microspheres. While Tsuchiya et al\textsuperscript{30} reported a significant decrease in cardiac output and an increase in heart rate when the number of infused microspheres exceeded $1 \times 10^6$, Stanek et al\textsuperscript{11} observed a significant decrease in heart rate only when as many as $1.4 \times 10^6$ microspheres had accumulated within the rat. In the present study, we infused at most a total dose of $1.2 \times 10^6$ microspheres into a single rat (divided over several infusions). No changes in either arterial blood gases, heart rate, or mean arterial blood pressure were observed following any infusion. We therefore conclude that the infusion of microspheres did not interfere with the circulation in these rats.

Possible Clinical Implications

In bone marrow transplantation, for marrow failure, and in the future also probably as a remedy for inborn errors of metabolism, as many as possible of the intravenously introduced stem cells should "home to fertile soil" in the marrow. Here, pretreatment of the patient to secure a high blood flow to the bone marrow during the transplantation may be worthwhile. On the other hand, during chemotherapy of neoplasms without suspected marrow deposits, a spare blood flow would be desirable. Some recombinant cytokines (colony-stimulating factors) have had unexpected granulocyte-mobilizing effects, which might depend on blood flow changes in the marrow, since earlier experiments suggested a relationship between the rate of bone marrow perfusion and the rate of cell release.\textsuperscript{32} To clarify these and other issues, the present work should be continued; for example, the possible effects of recombinant cytokines on bone marrow blood flow should be investigated.

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