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ENDOTOXIN: A TWOFOLD EFFECT ON BONE MARROW ULTRASTRUCTURE

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Abstract

Ultrastructural studies of mouse bone marrow reveal that reduction of the marrow-blood barrier precedes the two waves of leukocytosis which follow endotoxin administration. These ultrastructural changes include a decrease in adventitial cover of the marrow sinus wall, an increase in sinus circumference, and a decrease in endothelial cell overlap. The marrow-blood barrier changes which precede the first wave of leukocytosis are transient and are associated with depletion of marrow granulocytes. The changes that precede the second wave of leukocytosis 5 to 6 days later are more sustained and are associated with marked granulocyte hyperplasia. We suggest that endotoxin has two effects on marrow ultrastructure: (1) an early, direct effect on the sinus wall and (2) a later, indirect effect associated with granulocyte proliferation. The late effect may be mediated through endotoxin induced release of colony stimulating factor. Both effects diminish the normal marrow-blood barrier and appear to facilitate cell release from the marrow.

Key Words: Bone marrow, endotoxin, ultrastructure, colony stimulating activity, marrow proliferation, leukocytosis.

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Introduction

It is generally accepted that hemopoiesis in the bone marrow occurs extravascularly and that entry of cells into the circulation requires migration of cells from the hemopoietic cords through the marrow sinus wall into the marrow sinus. [1, 7, 8, 9, 10, 12, 13, 26, 39, 40, 42, 43, 44, 45] Factors controlling this entry of cells are not completely understood, but it is probable that they involve (1) the anatomical organization of the marrow, (2) developmental changes in the surface, cytoplasm, and nucleus of maturing cells, (3) chemical mediators of cell release, and (4) neurovascularly mediated changes in blood flow. [1, 7, 8, 9, 10, 13, 23, 24, 25, 39, 40, 42, 43, 44, 45]

Previous work has indicated that reticulocytosis in erythropoietin treated animals and the early leukocytosis in endotoxin treated animals is associated with an increase in cells in transit through the sinus wall and a decrease in sinus wall adventitial cell cover. [8, 22, 43] In addition, endotoxin induces a rapid increase in colony stimulating factor (CSF), an *in vitro* regulator of granulopoiesis, in serum and tissue. [6, 14, 15, 18, 19, 21, 26, 28, 29, 30, 41, 46] After administration of endotoxin, colony stimulating activity peaks at 6 to 12 h and returns to normal levels by 48 h. [14, 29, 41] Six to eight days after animals have been injected with endotoxin, increases in marrow CFU-C have been demonstrated. [28, 33] Release of marrow granulocytes and a wave of differentiation along the granulocytic pathway have been observed 24 to 48 h after endotoxin administration. [29] In view of the previous observations, we undertook the present study to investigate if granulocytic hyperplasia, a second wave of leukocytosis, and marrow ultrastructural changes could be demonstrated within 6 to 8 days of endotoxin administration. These changes would be analogous to the reticulocytosis, erythroid hyperplasia, and sinus wall ultrastructure findings observed following erythropoietin administration and would suggest that endotoxin induced the production or release of an activity which could act as a leukopoietin *in vivo*.

Materials and Methods

Twenty-five to thirty-five gram, virgin, female, white Swiss-Webster mice (Flow Labs - Dublin, Virginia) were injected intraperitoneally with 25 μg of endotoxin (Sigma Chemical Co., Lipopolysaccharide from Salmonella Typhosa, phenol extract, No. L-3630, lot 77C-0200) dissolved in sterile, pyrogen free isotonic saline (25 μg/ml). Matched controls were injected with sterile, pyrogen free isotonic saline. Cell counts from peripheral blood of endotoxin injected mice and saline injected controls were determined before injection and 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h after injection.

Twenty microliter samples of peripheral blood were drawn into heparinized capillary tubes from a nick in the tail vein of each mouse. Total leukocyte counts were done with a Coulter counter (model ZF, Coulter Electronics, Inc.). [16] Absolute granulocyte counts were calculated from the total leukocyte count and differential counts made from push smears stained with Wright's stain.

Preparation of Marrow for Electron Microscopy

Animals were sacrificed 2, 4, 8, 24, 48, 72, 96, 120, 144, 160, and 192 h after endotoxin injection. Mice were anesthetized with sodium pentobarbital, 0.065 mg/gm, intraperitoneally. Mice were perfused with 30 ml of Earle's balanced salt solution (flow rate 3 ml/min) at room temperature via a 22-guage teflon catheter introduced into the thoracic aorta. Perfusate was drained from the animal by severing the portal vein and was clear by the end of the perfusion. The mouse was then fixed by perfusion with 100 ml (flow rate 3 ml/min) of Karnovsky's fixative [20] diluted 1:3 in 0.1 M sodium phosphate buffer pH 7.4 to a final osmolality of 430 milliosmoles. 0.5% dimethylsulfoxide (DMSO) was added to the fixative and enhanced preservation of marrow structure. When the perfusion was complete, both femurs were dissected from hind limbs and placed in undiluted fixative. The bony cortex of each femur was carefully removed from an area one-third to one-half the diameter of the diaphysis by shaving with new number 11 scalpel blades, and the femur was placed in fresh undiluted fixative overnight. The fixed marrow was then gently dissected from the bone, floated into the fixative, and cut into one millimeter discs. The marrow was subsequently prepared for transmission electron microscopy by dehydration in a graded alcohol series, displacement of alcohol with propylene oxide and embedding in araldite resin (Durcupan-ACM, Fluka AG, Switzerland). [9] Thick sections were cut with glass knives on an A/O Ultracut ultramicrotome and were stained with toluidine blue. [2] Thin sections were cut by DuPont diamond knives, and were stained as previously described. [9] The preparations were examined and photographed with a Philips 201 electron microscope.

Quantitation of Ultrastructural Changes

In order to quantify the ultrastructural differences between control and endotoxin treated animals, we measured several features of bone marrow ultrastructure: (1) sinus circumference, (2) length of endothelial cell junction overlap, (3) mean endothelial cell thickness, (4) the number of leukocytes in transit through the sinus wall, (5) adventitial cell cover, and (6) the myeloid-erythroid (ME) ratio. Bone marrow from 6 animals was studied by electron microscopy for each experimental group and measurements were made of at least 10 sinuses per animal from randomly selected micrographs.

The sinus wall circumference was determined by measuring the total perimeter of the endothelial layer along its abluminal surface in electron micrographs of 8,000 to 20,000 total magnification. Endothelial cell, adventitial cell, and endothelial cell junction lengths were measured with an electronic map measure (Panasonic model JE-8210U). Endothelial cell thickness was measured with a Vernier caliper. Adventitial cell cover, the ratio of adventitial to endothelial cell lengths, was expressed as a percentage. Incidence of cells in transit was expressed as number per millimeter of sinus wall.

Cell counts of at least 250 cells obtained from randomly selected electron micrographs of hemopoietic cords were used to calculate the ME ratio of each animal. Since mouse marrow contains little fat, this ratio is comparable from group to group and reflects changes in numbers of granulocytes.

Analysis of variance was done by a Scheffé multiple comparison procedure. [34]

Results

It can be seen in Table 1 that neutrophil counts in endotoxin treated mice showed a marked decrease within 2 h of injection and a threefold rise at 12 h when compared to controls ($p<0.05$). A twofold rise in neutrophil count in these endotoxin treated animals could be demonstrated at 144 h ($p<0.05$) as well.

To investigate whether these changes in neutrophil counts could be correlated with a progression of anatomical changes in marrow sinuses and hemopoietic cords, we studied the marrow in our experimental groups by transmission electron microscopy over a period of 8 days. The control mice (Fig. 1) demonstrated the ultrastructural features of normal marrow. The endothelial cell wall of the sinus was usually devoid of cells in transit and openings in the sinus wall unassociated with cells in migration were absent. Adventitial cell processes covered about one half of the abluminal surface of the sinus and processes extending into hemopoietic cords were readily apparent. Hemopoietic cords contained both erythroid cells and mature granulocytes.

Two hours after endotoxin injection (Fig. 2) there were granulocytes in migration and a reduction of adventitial cell cover to about one third of the abluminal surface. Adventitial cell processes extending into the cords were not as apparent and mature granulocytes were scarce in the hemopoietic cords although granulocytes could be seen clustering around the sinus.

By 120 h (Fig. 3), in addition to an obvious reduction of adventitial cover, hemopoietic cords again contained mature granulocytes.

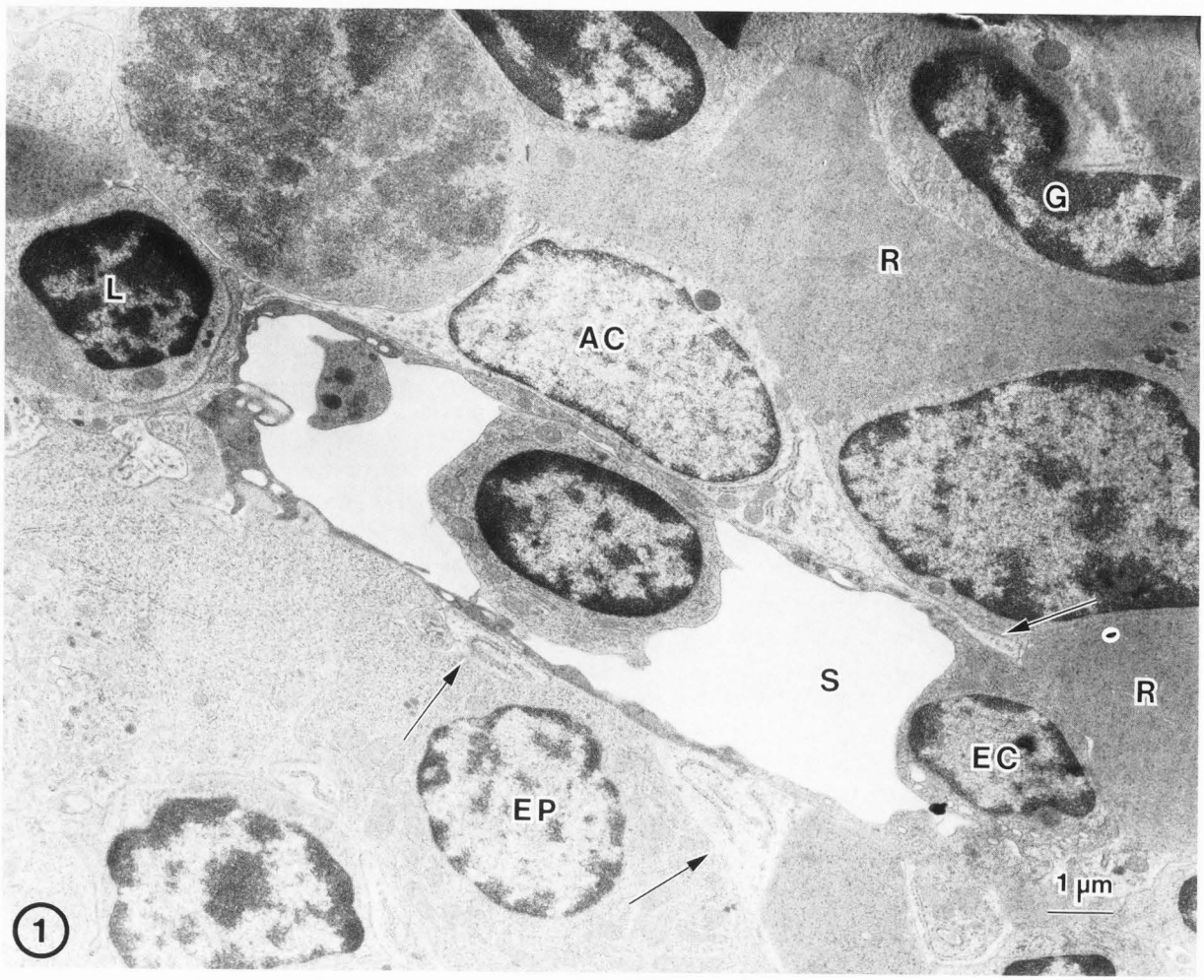


Figure 1. Normal bone marrow. Cross section of sinus. Endothelial cells (EC) lining this sinus (S) are devoid of cells in transit. Adventitial cells (AC) and their processes (⌈) cover 55% of the abluminal surface. Hemopoietic cords contain reticulocytes (R), granulocytes (G) and erythroid precursors (EP).

Table. 1 Absolute Peripheral Neutrophil Counts[§] in Endotoxin and Saline Injected Mice (mean ± SE)

Time After Injection	Endotoxin	Saline
2 h	0.18 ± 0.11*	0.65 ± 0.10
4	0.19 ± 0.06*	0.82 ± 0.31
6	1.74 ± 0.77	1.05 ± 0.09
8	4.16 ± 3.00	1.64 ± 0.87
12	5.92 ± 1.13*	2.11 ± 1.50
24	1.34 ± 0.55	1.04 ± 0.22
48	1.01 ± 0.87	0.69 ± 0.26
72	0.48 ± 0.33	0.83 ± 0.66
96	0.90 ± 0.50	0.72 ± 0.18
120	1.14 ± 0.92	0.51 ± 0.22
144	1.17 ± 0.39*	0.62 ± 0.25
168	1.01 ± 0.43	0.62 ± 0.14
192	0.96 ± 0.02	0.84 ± 0.08

§ Neutrophils x 10^3 per cubic mm.
* Endotoxin vs saline: 2 h $p<0.001$, 4 h $p<0.001$, 12 h $p<0.05$, 144 h $p<0.05$.

The ultrastructural differences between control and endotoxin treated groups that we measured are shown in Table 2 and represent the mean and standard errors of measurements made in 60 or more sinuses for each study group.

The number of leukocytes in transit in the endotoxin group at 2 h (5.7 ± 2.2 per mm sinus wall) was increased compared to 2.3 ± per mm sinus wall in the control ($p,0.05$) and had returned to base line values by 8 h. A second rise in leukocytes in transit at 144 h in endotoxin treated mice nearly reached statistical significance.

Marrow sinus circumference significantly increased within 2 h of endotoxin administration

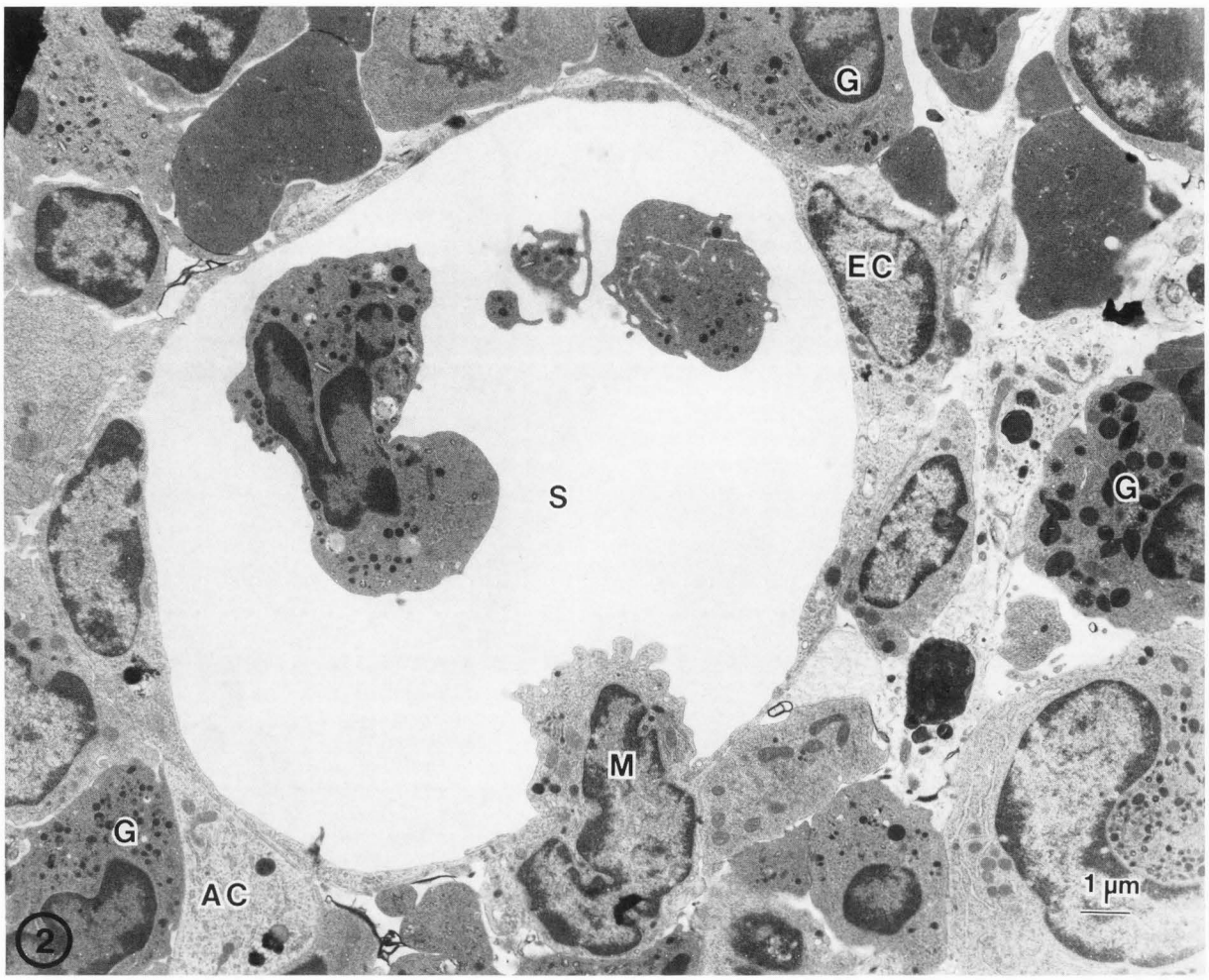


Figure 2. Bone marrow 2 h after endotoxin. A monocyte (M) is migrating through an endothelial cell (EC) into the sinus lumen (S). Granulocytes (G) can be seen clustering around the sinus. Adventitial cells (AC) cover 40% of the abluminal surface.

Figure 3. Bone marrow 120 h after endotoxin. Adventitial cells (AC) cover only 20% of the endothelial cell (EC) abluminal surface. Hemopoietic cords are filled with mature granulocytes (G). Reticulocytes are not apparent. A monocyte (M) is migrating through the endothelium into the sinus (S). →

(43.5 ± 1.5 µm vs 61.1 ± 2.0 µm, $p<0.05$) and was associated with decreased endothelial cell overlap (0.57 ± 0.04 µm vs 0.45 ± 0.04 µm, $p<0.05$). By 48 h sinus circumference returned to base line. Values statistically different from the control were demonstrated only at 2 h. Endothelial cell overlap was most at 24 (0.93 ± 0.07 µm) and 72 h (0.95 ± 0.07µm) both significantly different from the control ($p<0.01$) and least at 2 hours (0.45 ± 0.4 µm, $p<0.05$).

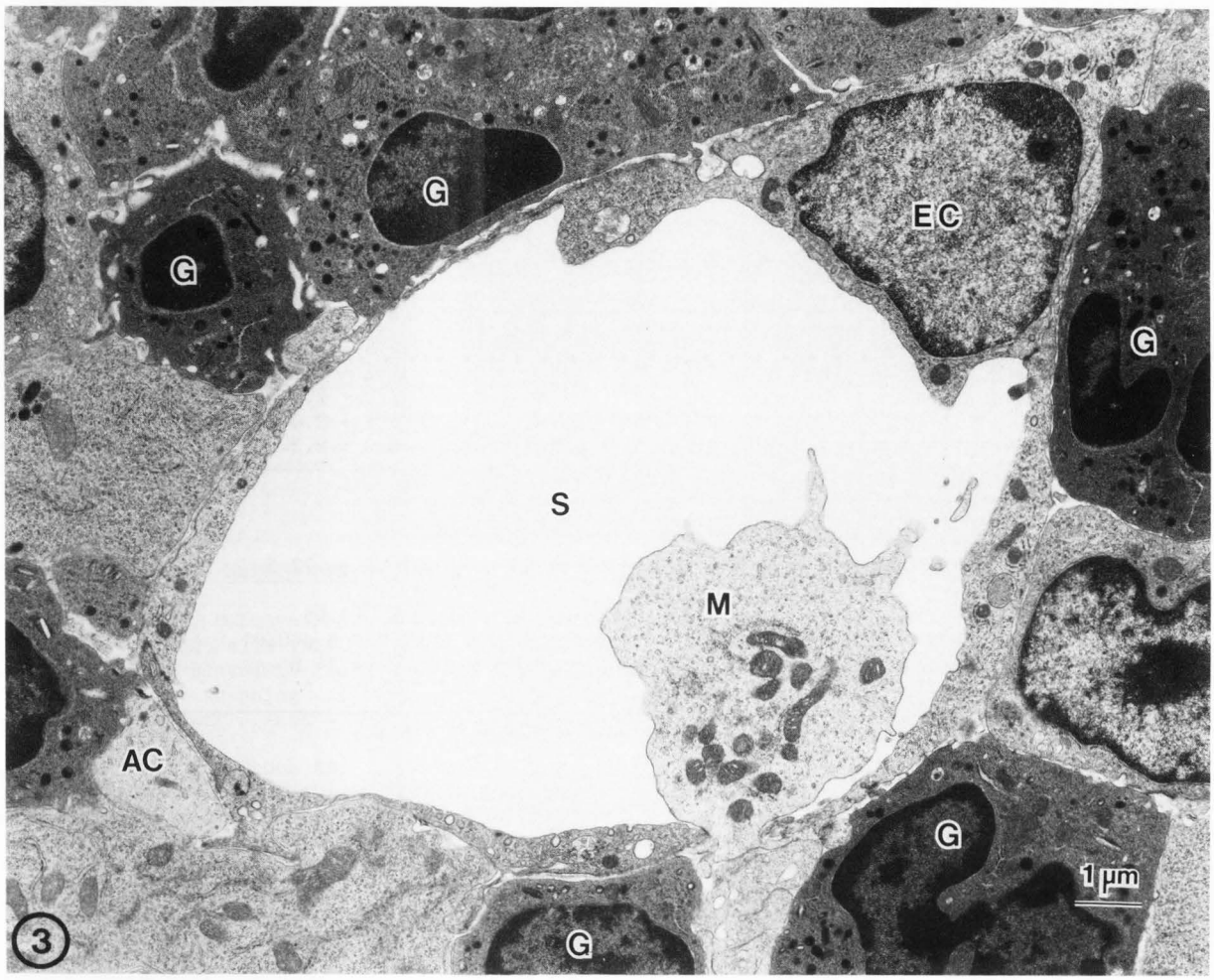
No significant differences in endothelial cell thickness compared to control could be demonstrated at any time but endothelial cell thickness was least at 2 h (0.74 ± 0.07 µm), the time of maximum cell migration, and greatest at 72 h (1.24 ± 0.10 µm), a time of less migration. The thickness change at 2 h compared to 72 h, however, was significant ($p<0.05$).

Adventitial cell cover was significantly reduced within 2 h of endotoxin administration (46.8 ± 2.1% to 35.6 ± 1.8%, $p,0.05$). At 24 h adventitial cover had again increased and by 48 h had returned to base line. A second significant decrease in adventitial cell cover occurred at 96 and 120 h (33.6 ± 2.6% and 32.3 ± 1.8% from 46.8 ± 2.1%, $p<0.01$). Recovery to base values was apparent by 168 h.

The ME ratio was used to quantify the numbers of granulocytes in the marrow. It reached its nadir from 4 to 24 h (4 h 0.8 ± 0.1, 8 h 1.0 ± 0.2 and 24 h 0.7 ± 0.2). These values were significantly different from the control ($p<.05$). After starting to rise at 48 h the ME ratio peaked at 120 h (24.6 ± 8.2 vs 2.4 ± 0.4, $p<0.01$).

Discussion

The purpose of this investigation was to examine whether endotoxin administration *in vivo* might act indirectly on bone marrow by stimulating the production or release of a hemopoietic



activity capable of producing a wave of marrow granulocyte proliferation and differentiation, a peripheral blood neutrophilia, and a decrease in the marrow-peripheral blood barrier analogous to the action of erythropoietin on erythropoiesis.

Endotoxin administration has been demonstrated to produce an initial neutropenia and then a striking increase in serum CSF. [28,29] The increase in CSF is followed by a release of marrow granulocytes and a wave of differentiation along the granulocytic pathway. [29,32] In addition, endotoxin given to mice shortly before radiation hastens hemopoietic recovery and improves survival. [35,36] These effects are thought to be due at least in part to an action on stem cells. [37,38] Serum levels of CSF and absolute granulocyte counts in germ free mice are lower than normal mice and no rise in CSF occurs after radiation in germ free mice. [27] Several stromal cell lines in culture have been demonstrated to increase CSF production 70 to 200 fold following exposure to endotoxin. [31] Thus, evolution of CSF after endotoxin administration with subsequent granulocytic hyperplasia suggests that endotoxin has a role in modulating hemopoietic factor production and that production of neutrophils can be upregulated in response to bacterial infection via endotoxin production.

The sequence of the peripheral blood and bone marrow ultrastructural changes that we observed following endotoxin administration is summarized in Tables 1 and 2 and is illustrated by Figures 1, 2, and 3. Endotoxin injected into mice produces an initial transient neutropenia that is associated with a number of rapid onset, short duration changes that quickly reduce the marrow-peripheral blood barrier: (1) the adventitial cell cover of the marrow sinus wall decreases rapidly converting large areas of sinus wall from a trilaminar structure to a single endothelial cell layer that can be penetrated more easily by cells ready to migrate, (2) a transient decrease in mean endothelial cell thickness occurs. By becoming thinner, the endothelial cell may offer less resistance to penetration by migrating cells since fusion of abluminal and luminal endothelial cell plasma membrane precedes the formation of a migration pore, [1, 13, 25], (3) a rapid decrease in endothelial cell junction overlap is observed. (0.57 ± 0.03 µm to 0.44 ± 0.2 µm, $p<0.05$). This event is associated with (4) significant sinus dilatation that could enhance delivery of marrow cells.

The absence of tight junctions between endothelial cells of the marrow sinus may permit endothelial cells to slide over one another,

Table 2. Endotoxin Induced Changes in Bone Marrow Ultrastructure mean ± SE)

	Leukocytes in Transit	Sinus Circumference #	Endothelial Cell Overlap #	Endothelial Cell Thickness #	% Adventitial Cell Cover	Myeloid Erythroid Ratio
C	2.3 ± 1.2	43.5 ± 1.5	0.57 ± .03	0.86 ±.06	46.8 ± 2.1	2.4 ± 0.4
2 h	5.7 ± 2.2*	61.1 ± 2.0*	0.45 ± .02*	0.74 ±.07*	35.6 ± 1.8*	1.3 ± 0.3
4	4.2 ± 1.1	51.1 ± 1.6	0.56 ± .06	0.90 ±.06	37.1 ± 2.2	0.8 ± 0.1*
8	2.7 ± 1.1	52.9 ± 1.6	0.80 ± .06	1.09 ±.10	37.6 ± 2.9	1.0 ± 0.2*
24	2.9 ± 1.2	51.0 ± 1.9	0.93 ± .07*	1.20 ±.10	57.2 ± 2.4	0.7 ± 0.2*
48	1.1 ± 0.7	43.6 ± 1.4	0.68 ± .04	1.05 ±.06	46.0 ± 2.2	9.4 ± 3.5
72	3.8 ± 1.3	44.3 ± 1.4	0.95 ± .07*	1.24 ±.10*	47.2 ± 2.6	12.1 ± 6.2
96	2.1 ± 0.7	43.9 ± 1.7	0.81 ± .06	0.99 ±.10	33.6 ± 2.6*	13.3 ± 5.9
120	2.2 ± 0.7	42.0 ± 1.2	0.77 ± .06	1.08 ±.07	32.2 ± 1.8*	24.6 ± 8.2*
144	5.0 ± 2.0	40.9 ± 1.8	0.75 ± .07	1.04 ±.09	43.0 ± 2.3	15.8 ± 7.7
168	4.0 ± 1.5	42.2 ± 1.0	0.78 ± .06	0.86 ±.06	46.0 ± 2.0	3.1 ± 1.1
192	2.7 ± 1.0	43.3 ± 1.2	0.89 ± .26	0.86 ±.05	43.9 ± 2.3	1.9 ± 0.6

Units are μ m

* Leukocytes in Transit: C vs 2 h $p<0.05$
Sinus Circumference: C vs 2 h $p<0.05$
Endothelial Cell Overlap: C vs 2 h $p<0.05$, C vs 24 h $p<0.01$, C vs 72 h $p<0.01$
Endothelial Cell Thickness: C vs all others NS, 2 h vs 72 h $p<0.05$
Adventitial Cover: C vs 2 h $p<0.05$, C vs 96 h $p<0.01$, C vs 120 h $p<0.01$
Myeloid Erythroid Ratio: C vs 4 h $p<0.05$, C vs 8 h $p<0.05$, C vs 24 h $p<0.05$, C vs 120 h $p<0.01$

thereby changing the size of the lumen [40]. Indeed, during periods of high marrow activity, rhythmic sinus dilatation has been demonstrated by in vivo microscopic studies. [5] Expansion of the vascular lumen within a bony encasement of fixed volume may increase the internal pressure of the hemopoietic cords and provide an additional propulsive force to displace cells already in transit into the sinus lumen. [40] The net result of these rapid, short duration changes is a rapid flux of leukocyte migration as demonstrated by an increase of leukocytes in transit. This rapid flux of leukocytes from the marrow produces an abrupt neutrophilia of large magnitude but short duration and is associated with a temporary depletion of the storage pool of mature granulocytes as evidenced by a decrease in the ME ratio.

The later occurring more sustained leukocytosis that we observed was preceded (1) by a wave of granulocyte proliferation, evidenced by a rising ME ratio (2.4 ± 0.4 to 24.6 ± 8.2, $p<0.01$) at 120 h (5 days), (2) a gradual but more sustained reduction in adventitial cell cover, and (3) a gradual sustained increase in leukocytes in transit that was near statistical significance. This lack of statistical significance is understandable since electron microscopy is an insensitive method for detecting cells in migration. It demonstrates only cells caught in the act of passage, not cells that have completed migration and would be found in the perfusate. That an increase in migrating leukocytes really does exist is supported by the presence of a significant peripheral blood leukocytosis and an increased ME ratio at the same time. We found little or no change in endothelial cell thickness, endothelial cell overlap or sinus circumference at this later time. The reason for a lack of change perhaps is that the initial early effect was caused by a direct vasoactive action by endotoxin on the sinus wall or that sinus dilatation was hindered mechanically by the marked granulocytic hyperplasia which produced a packed marrow at the later times.

The studies we did from 2 to 24 h confirm previous observations that endotoxin causes an initial leukopenia followed by a neutrophilic leukocytosis related to the mobilization of granulocytes from the bone marrow storage pool. [3, 4, 11, 15, 17, 29, 32, 43] The ultrastructure adaptations of the marrow include reduction in adventitial cell cover with an increase in numbers of leukocytes in transit. These changes have been observed in a variety of situations associated with increased delivery of marrow cells to the peripheral blood. [6, 7, 8, 9, 12, 38, 41, 42] In addition, previously undescribed with endotoxin, we found a transient thinning of endothelial cells and a decrease in endothelial cell overlap associated with dilatation of the marrow venous sinuses. These changes not only reduce the marrow-blood barrier but may provide an additional propulsive force to facilitate cell migration.

Our studies from 48 to 192 h (2 to 8 days) indicate endotoxin administration in mice is followed by the gradual onset of a sustained leukocytosis that is preceded by granulocyte proliferation and is associated with marrow ultrastructural changes found in situations associated with increased delivery of marrow cells to peripheral blood.

Our data indicate that endotoxin has two effects on bone marrow ultrastructure: (1) an early direct effect on the endothelial cell and adventitial cell, unrelated to cell proliferation, that reduces the marrow-peripheral blood barrier and may provide an additional propulsive force enhancing cell delivery; and (2) a later effect that appears to be indirect which also diminishes the marrow-blood barrier but which is associated with granulocyte proliferation. We suggest that the second effect is consistent with the action of a hemopoietic factor induced by endotoxin. Since the administration of endotoxin has been shown to rapidly stimulate the production of CSF from macrophages, granulocytes and several stromal cell lines [31] and the kinetic timing of the response we observed is consistent with the generation time of murine granulocytes, [29, 32] we suggest that endotoxin in the mouse is capable of modulating granulocyte production probably via stimulation of CSF release or production.

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Discussion with Reviewers

R.P. Becker: Oroszlan et al. ((1966) Ann NY Acad Sci 133, 622-628), has shown that alpha 2 and beta globulins inactivate endotoxin in rat serum. How then do you know that the changes you report are due even indirectly to administration of endotoxin?

Authors: In the paper cited above, tumor damage in the sarcoma 37 tumor bearing mouse is used to assay the effect of endotoxin. Although tumor damage induced by endotoxin could be prevented by incubation with rat plasma and its Beta or alpha-2 fractions, 15 μg of endotoxin (alone) injected intraperitoneally invariably produced tumor damage.

When the tumor bearing mouse assay is used to measure endotoxin inactivation, the effect of endotoxin injection is the positive control. Hence, the question of endotoxin inactivation by incubation with rat serum is not relevant.

Reviewer III: The morphometric measurements in this paper are improperly done and there was no effort to make sure that an unbiased sample was taken. The authors measured ten sinuses from 6 animals to arrive at the figures presented. The proper method for making these measurements would have been to make cuts on random blocks, take micrographs at a single set magnification with a calibrated microscope (using a calibration grid each time pictures of the samples were taken), and determine the proper number of micrographs by the progressive mean technique (Williams, Quantitative Methods in Biology, Volume VI, Practical Methods in Electron Microscopy, A.M. Glauert, (ed.), North Holland). Perhaps more significant differences would have been found if the proper number and method of analysis of micrographs had been done.

The measurements presented must, therefore, be taken as gross approximations of the real state of the sinus wall. The numbers presented are to this reviewer almost unbelievable in the smallness of their variance. For example, anyone familiar with bone marrow sinus structure is aware that there is a hierarchy of successively larger vessels which empty into the central sinus. Likewise, there is considerable variability in junctional overlap in endothelial cells from simple abutment (close to 0 μm) to long stretches (measuring several μm, as in the author's figure 3, next to the nucleus). These are dependent on the plane of section through the junction to some extent. Why aren't these large differences reflected in the numbers on table 2?

Authors: Precautions were taken to insure an unbiased sample. Random blocks from each animal and random sections were obtained. Measurements of at least 10 sinuses from randomly selected micrographs were utilized for each animal.

We agree that there is a hierarchy of vessel size, but felt that the random selection of the specimens and micrographs would balance variation due to anatomy and plane of section in our experimental groups. The expression of variation which we used was the standard error and this may give a false impression of smallness of variance. When viewed as a percentage, variations of 10-60% are reflected in the endothelial cell overlap measurements. The numbers in table 2 are the findings we observed.

Reviewer III: There is implicit in both the design and discussion of this paper that a thinner, single endothelial cell layer would offer less "resistance to penetration" than the endothelium plus adventitial cells (See "Discussion"). There is no solid morphometric evidence in the literature that a thinner endothelium or the presence or absence of an adventitial layer has anything to do with the ease of migration. In fact, it makes very little sense that it would have an effect. The majority of the sinus wall is devoid of adventitial undercoating. If the adventitial cells presented a barrier, it would not be a difficult thing for the highly motile white cells to move to a more likely spot within a few seconds. Further, DeBruyn has shown that blood cells are capable of interacting with adventitial cell to make a migration pore through them and then through endothelial cells.

Authors: DeBruyn has suggested in his publications the reason why a thinner cell would offer less "resistance to penetration." Migration pores are formed by apposition and subsequent fusion of abluminal and luminal endothelial cell membranes. Intuitively, it seems that if the surfaces were closer together, fusion could be accompanied more readily. Adventitial cell cover does reproducibly vary with amount of cell migration and has been reported by Weiss and Tavassoli as well as the authors. Whether the cells retract or are pushed aside is a moot point. We have also observed migration pores in adventitial cells but this has always been associated with an endothelial cell pore and is a rare occurrence. Since migration occurs preferentially adjacent to an endothelial cell junction, movement or displacement of adventitial cells from this site may be a prerequisite for migration.

Reviewer III: There is the notion stated that endothelial cells in the bone marrow may slide past one another to increase luminal diameter (See "Discussion"). In a cell biological sense, it is probably not possible for cells with adhesive junctions of the type found in bone marrow endothelium to slide past each other within a short length of time. This would require the dissolution of these symmetrical plaques which are attached to the endothelial cell's cytoskeleton in a short period of time. It is doubtful that this occurs, but it might be possible. Do the authors have any evidence for change in junctional morphology, such as a decrease in junctional adhesive densities, which might make the notion of sliding endothelial cells more consistent with current cell biological thinking?

Authors: Fluctuations in sinus size have been seen by in vivo microscopy by Branemark as well as McCuskey and are associated with variations in marrow blood flow. Since the endothelial cell junctions in the marrow are not tight junctions, (Tavassoli (1979) Br J Haematol 41:303-307, we felt that the endothelial cells might be capable of sliding at their junctions. The densities usually observed at the cell junctions were less prominent but this is difficult to quantify.

Reviewer III: The authors state that a transient decrease occurs in endothelial cell thickness (See "Discussion") despite the fact that their statistics indicate that there is no significant change. No significant change is no change at all.

Authors: Endothelial cell thickness was least at the time of maximum migration and most at a time of less migration. There is a significant difference between these times but not in respect to the control.

Reviewer IV: The authors state that they cut the portal vein in order to allow blood to escape from the vascular system. I suspect this would route all the perfusate through the gastrointestinal system and result in little fixative reaching the femurs. Do the authors mean inferior vena cava?

Authors: The portal vein was severed because we wished to fix the spleen and liver as well as bone marrow. Perfusate reached the hind limbs in quantity sufficient to empty the blood and produce hardening of the marrow.