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A Dichotomy between the Release of Bone Marrow Granulocytes and Stem Cells (39602)

P. QUESENBERRY,[2] K. ZUCKERMAN, M. RYAN, AND F. STOHLMAN, JR.[3]

Departments of Medicine and Research, St. Elizabeth's Hospital and Tufts University School of Medicine, and Division of Hematology, Department of Medicine, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Massachusetts 02135

The administration of endotoxin to a number of experimental animals including mice results in a release of marrow granulocytes (1-3) possibly mediated by a humoral factor termed leukocytosis-inducing factor (4, 5). Changes in peripheral blood granulocyte-macrophage *in vitro* colony-forming cells (CFC) after endotoxin injection parallel the changes in blood mature granulocyte levels, with marked elevations seen 6 hr after endotoxin (6, 7). That these peripheral blood elevations of CFC are also due to a release from the bone marrow is suggested by the decrease in marrow CFC with either increased or no change in splenic CFC seen over the 6-h interval after endotoxin injection (7, 8). When endotoxin is administered repeatedly to CF1 or C57 black mice, a "tolerance" or resistance develops to the serum colony-stimulating factor (CSF), elevating effect of endotoxin although granulocytes continue to be released from the bone marrow (9). In the present study, we have evaluated the effect of endotoxin preinjection on the peripheral blood granulocyte and CFC response to endotoxin.

Methods. The *in vitro* soft agar culture technique is a modification of a double-layer agar technique (10) which has previously been reported (8). The present studies were carried out over a 2-year time interval during which the final concentration of fetal calf serum was increased gradually from 13 to 19%. During this time, there was a progressive increase in plating efficiency of peripheral blood; however, qualitatively the results of experiments were similar. Whole heparinized peripheral blood (Panheparin without preservative, Abbot Laboratories; final concentration approximately 25 units/ml) was added directly to the agar-culture medium mixture and plated; colonies of over 50 cells were counted on Day 10. This time interval of growth was selected because at this time there was enough lysis of red cells to allow for accurate colony counts. Peripheral blood granulocyte values were determined on individual blood samples anticoagulated initially with versenate (K_2EDTA), but in later experiments granulocyte counts were determined on pooled heparinized blood: the results were the same with either method. Bone marrow cells were flushed from four to five tibias with single-strength Eagle's medium and pooled. The nucleated cell concentrations of the pooled suspensions were determined and 25,000 marrow cells were plated in the soft agar culture technique. The methods employed were the same as those for peripheral blood CFC except that the colonies were counted on Day 8. In the present experiments, control CF1 mice (Carworth Farms) were injected for 7 days with 10 μg of *Salmonella typhosa* endotoxin (Difco 3946-10) intraperitoneally (ip): 24 hr after the seventh injection, mice from control and tolerant groups were injected with either 0.1 ml of saline or 5 μg of *S. typhosa* endotoxin ip, and 6 hr later the peripheral blood CFC and granulocyte values were determined. In some experiments, peripheral blood CFC were also determined at earlier time points after the injection of either saline or endotoxin. Each experimental group at each point consisted of from four to nine individual mice.

Marrow and peripheral blood differentials were measured as previously reported (8). At least 100 cells per animal were

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[2] Scholar, Leukemia Society of America.

[3] Deceased.

counted for peripheral blood. Paint-brush bone marrow smears were made from individual femurs and from 410 to 515 cells were counted for each femoral smear.

Statistical significance was determined utilizing the Wilcoxon rank order test (two tails).

Results. Figure 1 shows the peripheral blood granulocyte values in control and tolerant mice 6 hr after the ip injection of 0.1 ml of saline or 5 μg of *S. typhosa* endotoxin ip. Both endotoxin-injected groups show significant increases in blood granulocytes. Table I presents the values of differentiated marrow cells expressed as the absolute number per tibia. Absolute increases in total granulopoiesis inthe tolerant groups are apparent and a significant decrease in marrow mature neutrophils in both of the endotoxin-injected groups is clearly shown, indicating that the increase in peripheral blood granulocyte values in these animals was indeed due to a release of granulocytes from the bone marrow storage pool.

Figure 2 shows the peripheral blood CFC values in tolerant and control mice at varying times out to 6 hr after 5 μg of endotoxin ip. The values at 6 hr are from the same experiments presented in Fig. 1. There are marked increases in the numbers of peripheral blood CFC in control mice 6 hr after the injection of 5 μg of endotoxin as compared to the saline-injected control mice ($P < 0.05$). The peripheral blood CFC levels in tolerant mice 6 hr after 5 μg of endotoxin were not significantly different from those seen in the saline-injected tolerant mice. There were also no increases in peripheral blood CFC in tolerant mice 1¾, 2½, and 4 hr after endotoxin, indicating that an earlier release of marrow CFC had not occurred in the tolerant mice. The absolute numbers of CFC per milliliter of peripheral blood ± 1 standard error of the mean (SE) in control and tolerant mice 6 hr after ip injection with saline were 1686 ± 733 and 1460 ± 473, respectively. The absolute numbers of CFC per milliliter of blood ± 1 SE 6 hr after 5 μg of endotoxin in control and tolerant mice were 5692 ± 1970 and 1175 ± 381, respectively.

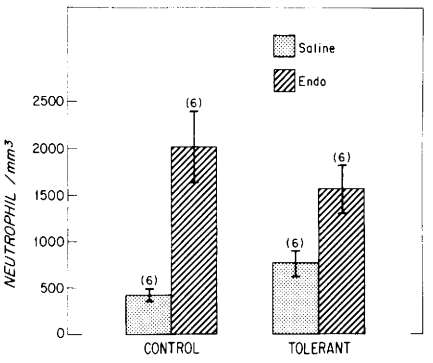

FIG. 1. The peripheral blood neutrophils (band and polymorphonuclear) per cubic millimeter ± 1 SE from control and tolerant mice 6 hr after either 5 μg of endotoxin or 0.1 ml of saline ip. Numbers in parenthesis represent the number of separate experiments from which these data were derived.

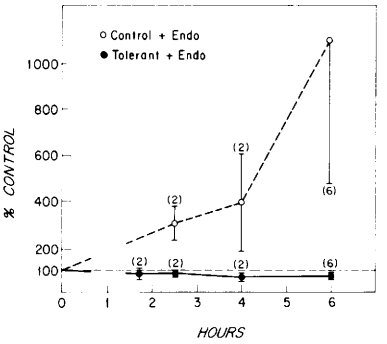

FIG. 2. Peripheral blood CFC from tolerant and control mice at varying time intervals after 5 μg of endotoxin expressed as a percentage of the saline-injected controls ± 1 SE. The numbers in parenthesis represent the number of separate experiments from which these data were derived.

TABLE I. TIBIAL CELL VALUES ($\times 10^{-6}$).[a]

Group	Proliferative granulocytes	Metamyelocytes	Bands	PMN	Lymph	RBC
Control Saline	0.707 ± 0.230	0.826 ± 0.028	1.910 ± 0.513	2.18 ± 0.356	3.574 ± 1.02	3.491 ± 0.846
Control Endo	0.539 ± 0.189	0.884 ± 0.014	1.263 ± 0.454	0.485 ± 0.235	3.404 ± 0.275	3.641 ± 0.802
Tolerant Saline	1.117 ± 0.142	1.664 ± 0.298	2.867 ± 0.543	5.910 ± 1.24	0.761 ± 0.266	0.983 ± 0.095
Tolerant Endo	1.231 ± 0.249	1.609 ± 0.075	2.885 ± 0.217	3.13 ± 0.189	1.006 ± 0.547	1.580 ± 0.114

[a] The number of various cell types per tibia in control and tolerant mice 6 hr after either 5 μg of endotoxin or 0.1 ml of saline. These data are derived from two experiments included in the data in Fig. 2, and each point is based on data derived from 13–18 individual femoral smears.

In five experiments the number of CFC per tibia were determined. The mean number of CFC per tibia ± 1 SE in tolerant mice 6 hr after 5 μg of endotoxin was 142 ± 17% of the saline-injected tolerant mice. The absolute values for tibial CFC ± 1 SE in tolerant mice 6 hr after either endotoxin or saline were 54,165 ± 18,120 and 40,200 ± 11,547, respectively. These differences were not statistically significant.

Discussion. In the present studies tolerant CF1 mice continued to release marrow granulocytes in response to endotoxin, but no longer showed increases of peripheral blood CFC after endotoxin. The increases of peripheral blood CFC seen after endotoxin injection of CF1 mice were presumably secondary to marrow release, since in previous studies utilizing the same strain of mouse and dose of endotoxin, we found a drop in marrow CFC without any decrease in splenic CFC over the 6 hr after endotoxin (7, 8). Accordingly, the absence of an increase in peripheral blood CFC in endotoxin-injected tolerant mice was presumably due to a failure of release of CFC from the marrow. The absence of decrease in tibial CFC in the endotoxin-injected tolerant mice is also consistent with this conclusion. It must be acknowledged that changes in marrow CFC numbers after endotoxin may be secondary to a number of mechanisms other than migration from the marrow and thus should be interpreted with caution. Changes in marrow CFC may be secondary to alterations in fluxes between the marrow pluripotent stem cell (CFU) compartment, the CFC compartment, and the differentiated granulocyte compartment. The decrease in marrow CFC seen after endotoxin injection of control mice could, in part, be due to differentiation in the marrow granulocytic pathway while the suggestion of an increase in tibial CFC (not statistically significant) in the tolerant endotoxin-injected mice could be due to an increased differentiation of CFU into CFC or perhaps a decreased outflow from the CFC compartment into the differentiated marrow compartment or peripheral blood pool. In addition, as we have no specific label for the CFC, we cannot directly assess peripheral blood turnover or distribution and thus changes in the latter cannot at present be ruled out as contributing to our results. It seems likely, from the studies sited above that we were, in fact, dealing with a marrow release of stem cells, a phenomenon well documented in irradiated leg-shielded animals (11, 12).

These data imply that there may be basic differences in the manner in which granulocytes and stem cells are released from the bone marrow. There is evidence that a humoral entity termed leukocytosis-inducing factor may mediate endotoxin-induced granulocyte release (4, 5); perhaps stem cell release is mediated by a separate factor or a direct effect of endotoxin. Alternatively, differences intrinsic to the cell could underlie differences in marrow release. Lichtman and Weed (13, 14) have presented evidence that polymorphonuclear neutrophils (PMN) may be more deformable and adhesive than their less mature, morphologically recognizable progenitors, and it is possible that similar differences exist between PMN and CFC. In this case, the CFC would be less deformable and adhesive than PMN and thus less likely to exit from the bone marrow. If there were a simple quantitative decrease in the release stimulus, as might occur with the development of endotoxin tolerance, a decrease in the release of marrow CFC might be expected prior to any demonstrable decrease in granulocyte release.

The above studies do not deal directly with the mechanism of the stem cell release seen after endotoxin injection. In normal CF1 mice injected with 5 μg of endotoxin, there is a temporal correlation between increases in serum CSF levels and stem cell release (7, 9). Tolerant CF1 mice injected with 5 μg of endotoxin fail to release stem cells and do not show increases of serum CSF levels over the 6 hr after injection. This suggests that CSF may play a role in stem cell release.

Summary. Control CF1 mice injected ip with 5 μg of *S. typhosa* endotoxin release marrow granulocytes and granulocyte-macrophage *in vitro* colony-forming cells (CFC). Mice repetitively injected with 10 μg of endotoxin daily for 7 days and then challenged on Day 8 with 5 μg of endotoxin release granulocytes but not CFC from the marrow, indicating that the mechanisms

governing release are dissimilar for the two cell types.

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