Effect of Endotoxin on Granulopoiesis and the In Vitro Colony-forming Cell

By Peter J. Quesenberry, Alec Morley, Marilyn Miller, Kevin Rickard, Donald Howard, and Frederick Stohlman, Jr.

The effect of endotoxin on the tibial in vitro colony-forming cell (CFC) and the differentiated granulocyte compartment was evaluated. The injection of 5 μg of Salmonella typhosa endotoxin into CF₁ mice leads to elevated serum colony-stimulating factor (CSF) levels along with a transient granulocytopenia followed by the release of granulocytes from the marrow. The present study demonstrates that the number of tibial CFC, as assayed on soft agar, decreases within 20 min after endotoxin, reaches a nadir at 6 hr (65.8 ± 4.9% of control), and then returns to control values by 48 hr. Concomitantly, over the 48 hr after endotoxin, there are sequential increases in the marrow myeloblast-promyelocyte compartment (MPC), myelocyte compartment (MC), and the PMN compartment. Radioautographic studies utilizing ³HThrd showed increased labeling indices and grain counts in the MPC and MC at 24 or 48 hr after endotoxin, indicating a shortening of generation time and perhaps of the DNA synthetic period in these compartments. The present data suggest that elevated CSF levels seen after endotoxin administration may lead to differentiation of the marrow CFC into the granulocyte pathway. There are also changes in the differentiated granulocyte compartment, suggesting a decreased generation time that may be due to CSF or a separate regulator such as antichalone.

The humoral regulation of granulopoiesis has received increasing attention since the introduction of methods for growing granulocyte and macrophage colonies in soft agar.¹² Evidence has accumulated that the in vitro colony-forming cell (CFC) monitored by this system is a committed granulocytic stem cell,³⁻⁶ and it has been proposed that colony-stimulating factor (CSF), which in the mouse is a prerequisite for growth of colonies, may be a physiologic regulator of granulopoiesis.⁷ We have recently observed a striking rise in serum colony-stimulating factor in CF₁ mice after intraperitoneal injection of Salmonella typhosa endotoxin, which correlated with an acute drop in blood neutrophil levels. This was followed by release of granulocytes from the marrow and differentiation along the granulocytic
pathway. If the CFC is a granulocytic stem cell and if CSF is a regulator of granulopoiesis, one would expect marked changes in serum CSF levels to have an effect on the CFC in addition to the differentiated granulocytic compartment of the bone marrow. We report here our observations on the marrow CFC and the proliferative status of the marrow granulocytic precursors after endotoxin injection.

MATERIALS AND METHODS

Twelve to 16-wk-old virgin female CF, mice (Carworth Farms) were injected with 5 μg of S. typhosa endotoxin (Difco, No. 3946) intraperitoneally, and studies were done at varying times after injection. The endotoxin was suspended in normal saline immediately prior to use. Five to six mice were included in each assay group. Bone marrow cells were flushed from five to six tibias with Eagle's solution and pooled. The nucleated cell concentration of the pooled suspensions was measured in hemacytometers by two observers using four diluting WBC pipettes; the results were expressed as the number of nucleated cells per tibia. Marrow differentials were based on 250 cells. Myeloblasts were defined as cells with fine nuclear chromatin but without a nuclear opening or cytoplasmic granules; promyelocytes were classified as cells with azurophil granules with or without a nuclear opening, and myelocytes were cells with neutrophilic granulation and with a nuclear-cytoplasmic ratio of greater than one-third. Metamyelocytes were defined as cells with a nuclear-cytoplasmic ratio of less than one-third but without well-developed chromatin condensation of their nuclei; band neutrophils were defined as cells showing well-clumped nuclear chromatin but not nuclear segmentation, and segmented neutrophils were defined as cells showing nuclear segmentation. Peripheral WBC counts and differentials were measured using standard techniques. Marrow and peripheral blood films were stained with Wright-Giemsa.

To measure turnover of bone marrow cells, control and endotoxin-treated mice were injected intravenously with 1 μCi/g body weight of tritiated thymidine (Schwarz Bio-research, 1.9 Ci/mole) and were sacrificed 1 hr later. Smears were made from the femoral marrow, fixed in methanol, dipped in Kodak NTB3 emulsion, and exposed for 25 days. After developing, these smears were stained with Wright-Giemsa. The labeling data on the myeloblast-promyelocyte compartment (MPC), myelocyte compartment (MC), and erythroid compartment were based on the evaluation of 100-150 labeled cells/slide and four to five such slides for each time point in each experiment. A labeled cell was regarded as one with four or more grains over the nucleus.

The tibial CFC at 6, 24, and 48 hr after endotoxin was assayed on soft agar by the technique of Metcalf and Foster as modified by Rickard et al. In more recent experiments, which include the studies from 20 min to 3½ hr after endotoxin, a modification of the double-layer soft agar technique described by Bradley and Sumner was used. The source of CSF was mixed with Eagle's media and agar, so that the final concentration of agar was 0.5%. One cubic centimeter of this mixture was used for the underlayer. The overlayer consisted of 1.0 cc of Eagle's media and agar, with a final concentration of agar of 0.3%. The marrow cells to be assayed were mixed with the latter to give the appropriate concentration of cells (usually 0.5 × 10⁶/cc). This modification was adopted because of better growth of murine CFC and less variability within and between experiments. Growth of marrow CFC was found to be linear when the concentration of cells was between 1.25 × 10⁴ and 1.5 × 10⁵ cells/cc and when either mouse embryo-conditioned media or sera from endotoxin-injected mice were used as the source of CSF. This method also permitted the detection of smaller amounts of CSF in mouse sera.

The proliferative status of CFC was evaluated after hydroxyurea (OHU), 900 mg/kg body weight, as previously described by Rickard et al. Hydroxyurea is a rapidly metabolized agent that is selectively lethal for cells in DNA synthesis. The decrease in cell number 2 hr after OHU provides an estimate of the number of cells synthesizing DNA at the time of OHU administration.
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Fig. 1. Mean values of various morphologic compartments of tibial marrow ± 1 SEM at varying time points after endotoxin. Each point represents data from four experiments with four to six mice per time point in each experiment. Hatched areas in A indicate mean ± 1 SE for control animals. RBC refers to nucleated red blood cells.

Data were expressed as the weighted mean ± one standard error of the mean. Probability figures were calculated using Student's t test.

RESULTS

Data on the changes within various morphologic compartments of the bone marrow in animals treated with endotoxin are given in Fig. 1. There was a decrease in the neutrophils within 6 hr, followed by increases in the myeloblast and promyelocyte compartments at 24 hr (p <0.001) and in the MC at 45 hr (p <0.001). The neutrophil count had nearly returned to the control value by 48 hr. The erythroid and lymphoid compartments decreased in size over the 48 hr after endotoxin.

The proliferative status of the differentiated granulocytic compartment after endotoxin was evaluated by estimating the labeling indices after tritiated thymidine, and these are given for the MPC and the MC in Fig. 2. There was a significant increase in the labeling indices of the MPC and MC at 24 hr (p <0.001) and also in the MC at 6 and 48 hr (p <0.001).

The grain count distributions of the MPC and MC from a representative experiment are presented in Fig. 3. There was a significant increase in the percentage of heavily labeled cells (more than 20 grains) in both compartments at 24 hr and in the MPC at 48 hr. The increase noted in the MC at 48 hr was significant at the 0.01 level. Erythroid precursors were evaluated for labeling index and grain count distribution at the time of and 24 hr after endotoxin in the above experiment (Table 1). This was done to assess the specificity of changes in the granulocytic precursors. The changes in erythroid labeling did not parallel the changes seen in granulocytic labeling.
There was a significant decrease in the number of CFC per tibia 20 min after the administration of endotoxin ($p < 0.001$). This reached its nadir at 6 hr ($p < 0.001$) and was also seen after 24 hr (Figs. 4 and 5). Forty-eight hours after endotoxin the number of tibial CFC had returned to the control level (Fig. 4). The number of CFC in DNA synthesis, as determined by the OHU technique, appeared to have increased 24 and 48 hr after endotoxin, although this was not statistically significant (Fig. 5).

**DISCUSSION**

The injection of *S. typhosa* endotoxin into female C57 mice leads to a
Fig. 4. Tibial CFC assayed by single-layer agar technique at varying times after 5 μg of endotoxin, expressed as % of control ± 1 SEM. Lower solid line represents % decrease caused by OHU at various time points. Numbers in parentheses are the number of experiments. At least five mice were used per time point in each experiment.

Table 1. Grain Count Distributions of 100 Marrow Erythroid Precursors After Endotoxin

<table>
<thead>
<tr>
<th>Grain Count</th>
<th>0-3</th>
<th>4-10</th>
<th>11-20</th>
<th>21-30</th>
<th>31-40</th>
<th>40-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.6 ± 1.3</td>
<td>36.2 ± 1.1</td>
<td>10.4 ± 0.8</td>
<td>1.8 ± 0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 Hr</td>
<td>66.8 ± 2.7</td>
<td>24.4 ± 2.4</td>
<td>7.6 ± 2.1</td>
<td>1.4 ± 0.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are based on one experiment with five animals per time point. One hundred cells were read per slide, and results are expressed as the mean ± 1 SE.

Fig. 5. Tibial CFC assayed by double-layer agar technique at varying time points up to 3½ hr after 5 μg of endotoxin, expressed as % of control ± 1 SEM. Lower solid line represents % decrease caused by OHU. Numbers in parentheses are the number of experiments per time point. At least five mice were used per time point in each experiment.
transient granulocytopenia within 45 min, followed by a granulocytosis that is apparently secondary to the release of granulocytes from the bone marrow. There is also a sequential wave of differentiation along the granulocytic pathway during the 48 hr after endotoxin. We have previously reported that CSF levels are elevated within 45 min after endotoxin and more recently, utilizing the double-layer agar technique, we have been able to demonstrate increased CSF activity as early as 10 min after intraperitoneal endotoxin. CSF has been proposed as a hormonal regulator of granulopoiesis. If this is the case, one would expect elevated CSF levels to affect the candidate granulocytic stem cell, i.e., the CFC. In the present studies, the tibial CFC decreased within 20 min and returned to control values from 12 to 48 hr after endotoxin. There was also a suggestion, although the data were not conclusive in this regard, that the return to control values was mediated in part by increased proliferation in the CFC compartment. It is possible, however, that increased influx from the more primitive pluripotent stem cell compartment may have accounted in significant measure for the repletion of the CFC. The observed sequential changes of a decrease in tibial CFC followed by increases in the MPC and MC seem most compatible with the thesis that the decrease in tibial CFC accompanying increased CSF levels is due to differentiation into the granulocytic pathway.

It might also be suggested that either a direct toxic effect of endotoxin on CFC or a decrease in plating efficiency, as has been reported for colony-forming units (CFU) in transplantation studies, accounted for the decrease in CFC. There is evidence for migration to the spleen of both marrow CFU and CFC in response to various perturbations including endotoxin and it is possible that the decrease in marrow CFC described herein may reflect, in part, migration from the marrow.

The studies on the labeling of myeloid precursors with 3HTdR after endotoxin indicated changes in the generative cycle that, as previously noted, contributed in small part to the granulocytic response. Thus, after 24 hr there was an increased labeling index in the MPC and MC compartments that, together with an increased grain count, indicated a shortening of the generation time and perhaps of the DNA synthetic period. A recent study of labeled mitosis in animals receiving endotoxin indicates that the shortening of generation time is accomplished primarily by a shortening of G1 rather than by any important change in DNA synthetic time. The change in the generative cycle of the differentiated granulocytic elements might be attributed to CSF, but the possibility should be considered that it is the result of a separate stimulator such as antichalone. The relationship between CSF and antichalone has yet to be established, but in our view, they are most likely distinct.

The sequence of events observed in these studies suggests that CSF is a physiologic regulator of granulopoiesis. There was an early rise in CSF followed by an effect on the committed myeloid stem cell and by a wave of granulocytic differentiation. Although providing circumstantial evidence, we feel that these data are not definitive enough to permit the conclusion that CSF occupies the central role in the regulation of granulopoiesis that has
been established for erythropoietin in the case of red cell production. Thus, we would favor withholding the designation of CSF as granulopoietin until such time as in vivo administration of this protein has been shown to produce a sustained increase in granulopoiesis.

The effects of endotoxin on marrow erythroid elements described here are similar to those that have been previously reported. No completely satisfactory explanation of these changes has been advanced. Stem cell competition probably plays a role in the depletion of marrow erythroid cells, and migration of pluripotent and perhaps of committed erythroid stem cells may also be involved. In the case of the depletion of marrow lymphoid elements seen after endotoxin, migration or a lethal effect produced by either endotoxin directly or through cortisol release might be invoked as an explanation. In addition, if a subpopulation of cells usually classified by morphologic criteria as lymphocytes are, in fact, myeloid precursor cells, then differentiation might also contribute to the depletion of marrow lymphocytes.

REFERENCES


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