

## Granulocyte Modulation of Endotoxin-stimulated Colony-stimulating Activity (CSA) Production

By Tariq Mahmood and William A. Robinson

Incubation of human macrophages with endotoxin resulted in significantly enhanced colony-stimulating activity (CSA) production by these cells. Preincubation of endotoxin with mature granulocytes abolished this stimulatory effect. The stimulatory effect of endotoxin on macrophage CSA production was not abolished, however, by preincubation with NaF-treated granulocytes, granulocyte membranes, or nonphagocytic cells (lymphocytes or erythrocytes). These data suggest that mature granulocytes may play a role in the modulation of CSA production and granulopoiesis by inactivation of stimulatory materials such as endotoxin.

**H**UMORAL REGULATION of red blood cell (RBC) production through erythropoietin (Ep) is now well established. The control mechanism for RBC production lies in its main physiologic function, i.e., oxygen carrying capacity.<sup>1</sup> The mechanism for the regulation of granulocyte production has remained considerably more elusive. The best candidate for a physiologic, humoral regulator of granulopoiesis is the colony-stimulating factor (CSF).<sup>2</sup> Substances containing CSF have the ability to promote the growth of granulocytic and macrophage colonies in vitro in semisolid tissue culture systems and are referred to as having colony-stimulating activity (CSA).<sup>3,4</sup>

The major cellular source of CSA in humans is the monocyte-macrophage system.<sup>5-9</sup> It has been noted that when these cells are exposed to endotoxin derived from gram-negative bacteria in vitro they respond with enhanced CSA production.<sup>10-12</sup> It has also been noted that injection of endotoxin in humans and rodents results in elevated serum CSA levels and stimulation of granulopoiesis.<sup>13-15</sup> These facts have led to the suggestion that bacteria, or their products, particularly endotoxin, may play an important role in modulating normal granulopoiesis by enhancing CSA production and hence stimulating granulocyte production.<sup>13</sup> It has also been suggested that the major regulatory arm inhibiting granulocyte production (negative feedback) is modulated through mature granulocytes, but the exact mechanisms by which this is accomplished have not been fully elucidated.<sup>16-18</sup>

In the present studies we explored the relationship between these two previously described granulopoietic stimulatory and inhibitory phenomena by determining the effect of mature granulocytes on endotoxin-stimulated CSA production by human macrophages. The present experiments showed that CSA production by macrophages is significantly enhanced by incubation with endo-

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*Submitted June 27, 1977; accepted November 29, 1977.*

*Supported by grants from the National Institutes of Health, National Cancer Institute (1ROCA11305-08 and CA05058-10), American Cancer Society (CH-8G), Monfort Hematology Fund, and the General Clinical Research Centers Program of the Division of Research Resources, NIH (RR-51).*

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toxin and that this effect can be abolished by preincubation of the endotoxin with mature granulocytes.

## MATERIALS AND METHODS

### *Separation of Leukocyte Subpopulations From the Peripheral Blood*

Peripheral blood was obtained from normal human subjects after informed written consent was obtained. Blood was collected in heparinized tubes, and erythrocytes were allowed to settle at room temperature for 1–2 hr. The leukocyte-rich plasma was subjected to centrifugation at 350 g for 10 min and the pellet was resuspended in modified McCoy's 5A medium. The leukocytes were separated into various subpopulations by modifications of the procedures described by Böyum.<sup>19</sup>

Erythrocytes, granulocytes, and mononuclear cell fractions were separated by the use of Ficoll-Hypaque solutions of two different densities. A Ficoll-Hypaque solution of density 1.12 (mixture of 18 parts Ficoll and 12 parts 50% Hypaque) was layered at the bottom of a pyrex glass tube (12 × 120 mm). On top of this, a Ficoll-Hypaque solution of density 1.077 (Lymphoprep, Nyegaard, Oslo, Norway) was layered. The leukocyte-rich suspension was placed on top of the gradient and the tube subjected to centrifugation at 400 g for 35 min. The erythrocytes sedimented to the bottom. Granulocytes were recovered from the interface of the two Ficoll-Hypaque solutions. The mononuclear cell fraction was obtained from the top of the gradient.

The mononuclear cell fraction was then separated into monocyte and lymphocyte populations by glass adherence. The ratio of monocytes to lymphocytes was 1:4. To obtain  $1 \times 10^6$  monocytes, four times the desired number, i.e.,  $4 \times 10^6$  mononuclear cells, were added to 35-mm plastic Petri dishes and allowed to adhere for 2 hr at 37°C. The plates were then vigorously washed with McCoy's 5A medium to remove the nonadherent lymphocytes. The nonadherent fraction constituted the lymphocyte population, and the adherent fractions constituted the monocytes. Each of the resultant populations was at least 95% pure as judged by morphology on Wright-stained smears and by positive peroxidase staining for monocytes and the absence of peroxidase-positive granules for lymphocytes.

### *Preparation of Macrophages*

Macrophages were obtained by incubating  $1 \times 10^6$  peripheral blood monocytes prepared as described above in McCoy's 5A medium with 20% autologous serum for 48 hr. The resultant cells had the morphology of macrophages on Wright-stained smears as described by Bennett and Cohn.<sup>20</sup>

### *Liquid Culture Procedure*

Liquid cultures (2 ml) were used to prepare conditioned medium for CSA assay. McCoy's 5A medium with 20% autologous human serum was used throughout these experiments.

Cultures were performed in 35-mm plastic Petri dishes at 37°C in 7.5% CO<sub>2</sub> in air with 100% humidity. *Salmonella typhosa* endotoxin (Difco, Detroit, Mich.) at a concentration of 10 ng in 0.1 ml McCoy's 5A medium was used throughout these experiments. Erythrocytes and subpopulations of leukocytes at various concentrations were incubated with endotoxin for 2 hr. Cells were then removed by centrifugation, and the residual endotoxin-containing material was incubated with human macrophages for 2 days under the conditions described above. At the end of this incubation period, the conditioned medium was centrifuged at 400 g for 20 min to remove cellular debris and stored at 4°C prior to assay.

### *Preparation of Bone Marrow Cells*

Bone marrow was obtained from normal volunteers after informed written consent was obtained (as approved by the Human Subject Research Committee, University of Colorado Medical Center). Bone marrow samples were aspirated into a heparinized plastic syringe from the posterior iliac crest using a Jamshidi needle. The marrow samples were layered over a cushion of Ficoll-Hypaque (density 1.077) and centrifuged at 400 g for 35 min. The mononuclear cells were obtained from the surface of the gradient. Adherent CSA-producing cells were removed from the bone marrow by glass adherence as described by Messner et al.<sup>21</sup>

### CSA Assay

The assay for CSA was done using a modification of the technique of Robinson and Pike, described in detail elsewhere.<sup>22</sup> In brief, 0.1 ml of the test material was mixed with 1 ml of McCoy's 5A medium with 15% fetal calf serum and 0.3% agar containing 50,000 nucleated human bone marrow cells in 35-mm plastic Petri dishes. Plates were incubated at 37°C with 100% humidity in an atmosphere of 7.5% CO<sub>2</sub> in air.

Colony counts were performed on day 14 with the aid of a dissecting microscope. All experiments were done in triplicate. Data were expressed as the mean  $\pm$  SEM. Bone marrow when cultured without CSA did not produce colonies.

### Preparation of Granulocyte Membranes

Granulocytes ( $8 \times 10^6$ ) prepared from a Ficoll-Hypaque gradient as described earlier were suspended in 8 ml hypotonic saline. The suspension was subjected to centrifugation at 1500 g for 10 min and the cell pellet resuspended in 8 ml of hypotonic saline. The granulocyte suspension was sonicated twice for 15 sec. After these procedures no intact granulocytes were found on Wright-stained preparations of the suspension. Membranes were obtained by centrifugation of the suspension at 12,000 g for 30 min. The membrane pellet was washed thrice with McCoy's 5A medium prior to use.

### Sodium Fluoride (NaF) Treatment of Granulocytes

A 5% solution of NaF in McCoy's 5A medium was used in these experiments.<sup>23</sup> Granulocytes ( $8 \times 10^6$ ) were incubated in a 0.1% solution of NaF. The incubation was carried out at 37°C in 7.5% CO<sub>2</sub> in air with 100% humidity for 2 hr. Granulocytes were washed with McCoy's 5A medium thrice to remove the NaF and the viability of granulocytes determined by trypan blue dye exclusion.

## RESULTS

The effect of endotoxin on CSA production by macrophages in liquid culture is shown in Fig. 1. In these studies endotoxin at various concentrations was incubated with macrophages for 48 hr. At the end of the culture period the conditioned medium was removed and centrifuged at 400 g for 20 min and assayed for CSA using normal human bone marrow target cells from which all CSA

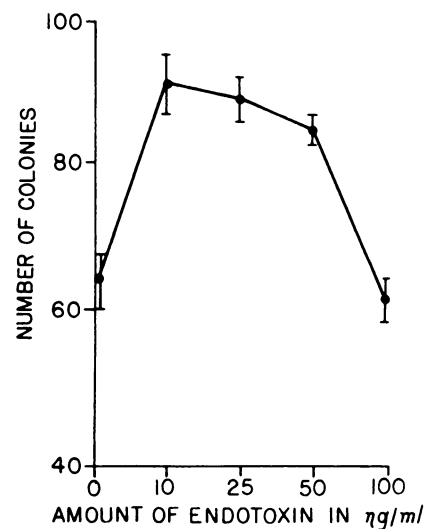


Fig. 1. Effect of increasing concentrations of endotoxin on CSA production by human macrophages. Each point, mean colony count of three plates. Bars, SEM.

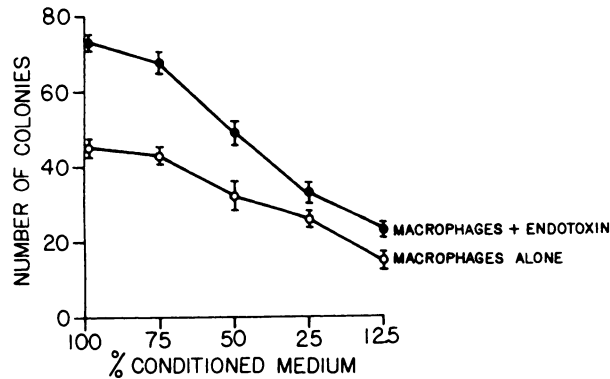
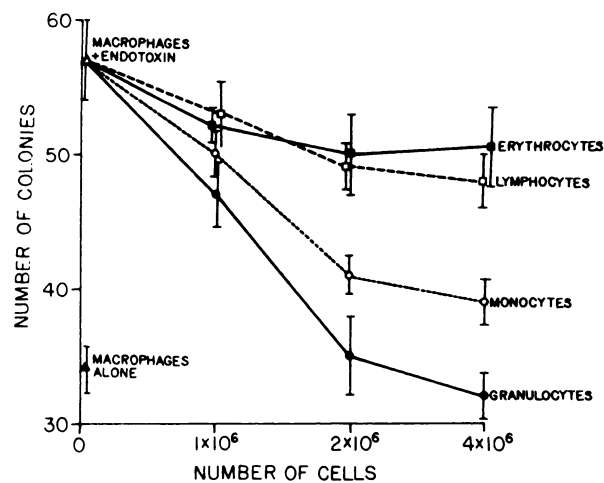


Fig. 2. Dose-response curves using different dilutions of conditioned medium from macrophages incubated with and without endotoxin;  $1 \times 10^6$  macrophages incubated with (10 ng/ml) and without endotoxin for 48 hr. Conditioned medium diluted with McCoy's 5A medium to obtain concentrations of 75%, 50%, 25%, and 12.5% prior to assay. Each point, mean colony count of three plates. Bars, SEM.

producing cells had been removed. Incubation of macrophages with 10, 25, and 50 ng/ml of endotoxin significantly enhanced CSA production ( $p < 0.01$ ). Concentrations higher than 50 ng/ml did not show this effect. Dose-response curves using different dilutions of conditioned medium obtained from macrophages incubated with 10 ng/ml of endotoxin (the concentration that stimulated macrophages maximally) and those incubated alone were also determined. Figure 2 shows that macrophages incubated with endotoxin produced more CSA. The two dose-response curves were not parallel. The CSA induced in the presence of endotoxin appeared to dilute out faster than the CSA from macrophages alone. Addition of endotoxin directly to bone marrow cells from which adherent cells had been removed did not result in enhanced colony formation (data not shown).

In the next set of experiments, the effect of preincubation of endotoxin with various white blood cell subpopulations and erythrocytes was studied. Endotoxin at a concentration of 10 ng/ml was incubated with each cell population in liquid culture for 2 hr. The cells were then removed and the medium incubated with macrophages for a further 48 hr. As shown in Fig. 3, preincubation of endotoxin with mature granulocytes in concentrations of  $\geq 2 \times 10^6$  cells/ml

Fig. 3. Effect of preincubation of endotoxin (10 ng/ml) for 2 hr with various cellular elements of the blood. After incubation with the cells shown in the right-hand column, and at concentrations shown, cell-free conditioned medium was incubated with macrophages for a further 48 hr. Also shown, controls of macrophages incubated alone and with endotoxin not exposed previously to cells. Points shown, mean colony counts of three plates with SEM.



abolished the enhancement effect of endotoxin on CSA production by macrophages. Medium derived from granulocytes incubated alone (without endotoxin) and in the same concentration for a 2-hr period and subsequently added to macrophage cultures stimulated by endotoxin (10 ng/ml concentration) did not inhibit endotoxin-stimulated CSA production (data not shown). This finding indicated that the effect of granulocytes noted above was not the result of an inhibitory factor produced by these cells.

Preincubation of endotoxin with nonphagocytic cells (erythrocytes or lymphocytes) did not abolish the enhancement effect of endotoxin on CSA production by macrophages. These data are also shown in Fig. 3. Incubation of peripheral blood monocytes in various concentrations with endotoxin (10 ng/ml) in liquid culture for 2 hr (with subsequent removal of monocytes) indicated that monocyte concentrations  $\geq 2 \times 10^6$ /ml also neutralized the endotoxin-mediated enhancement effect, although not to the same extent as granulocytes (Fig. 3).

In order to investigate the mechanism of endotoxin inactivation by granulocytes, the following studies were performed: Viable granulocytes, or the membranes derived from an equivalent number of granulocytes, were incubated with endotoxin at a concentration of 10 ng/ml. Incubation of endotoxin with granulocyte membranes, with subsequent removal of the membranes by centrifugation, did not inactivate endotoxin or abolish its stimulatory effect on macrophages (Fig. 4), suggesting that the removal of endotoxin was not simply the result of adherence to cellular membranes. Granulocytes incubated for 2 hr with NaF, a compound known to inhibit glycolysis and phagocytosis by these cells, likewise did not abolish endotoxin-stimulated CSA production by macrophages (Fig. 4). This failure was not related to granulocyte cell death, as evidenced by the fact that 99% of the granulocytes were viable by trypan blue dye exclusion test after incubation with NaF and prior to incubation with endotoxin. These studies indicated that the effect of granulocytes in endotoxin inactivation was the result of a specific phenomenon requiring active cellular metabolism and probably phagocytosis.<sup>24</sup>

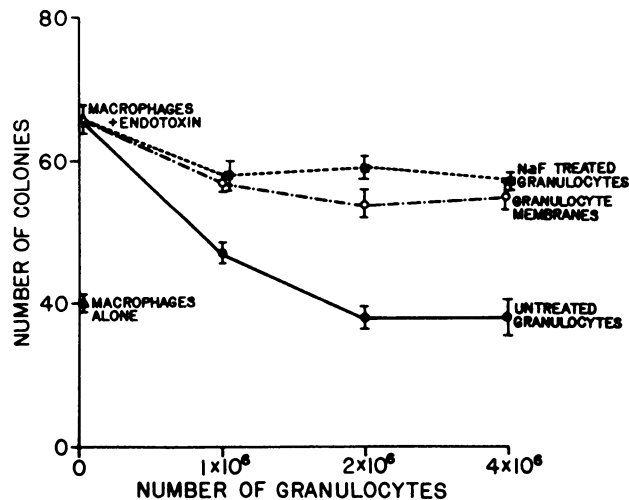


Fig. 4. Effect of preincubation of endotoxin (10 ng/ml) with untreated granulocytes, granulocyte membranes, and NaF-treated granulocytes. Points shown, mean colony counts of three plates with SEM.

## DISCUSSION

It is now well established that the major cellular source of CSA in humans is the monocyte-macrophage system.<sup>5-9</sup> The factors modulating and controlling the production of CSA by these cells have not, however, been clearly demonstrated. It has been shown that CSA production by mononuclear cells can be enhanced by microorganisms and materials derived from them. These include whole live Staphylococci, bacterial conditioned medium, lipopolysaccharides, *Trichinella spiralis*, and bacillus Calmette Guérin.<sup>10, 15, 25, 27</sup> The counterpart in vivo of this phenomenon, i.e., increased granulopoiesis and elevation of serum and urine CSA levels in response to endotoxin and infection, has also been well documented in the literature.<sup>13, 15, 28, 29</sup> On the other hand, in the germ-free state decreases in absolute granulocyte counts and serum CSA levels have been reported.<sup>30, 32</sup> In view of these findings, it has been suggested that bacterial products, particularly endotoxin, may play an important role in the regulation of granulopoiesis by stimulating production of granulopoietic factors by the monocyte-macrophage complex.<sup>13, 26</sup> Our data further corroborate this contention by showing enhanced CSA production by human macrophages after exposure to endotoxin.

It is generally believed that mature peripheral blood granulocytes must in some way exert a negative feedback control over the regulation of granulopoiesis.<sup>33, 34</sup> Several investigators have demonstrated that mature granulocytes and/or their products inhibit granulocyte colony formation in vitro, suggesting a negative feedback mechanism for maintenance of homeostasis.<sup>16, 17, 35, 36</sup> On the other hand, investigations in experimental animals have indicated that CSA production and granulopoiesis are dependent on the antigenic load rather than the peripheral granulocyte count.<sup>37</sup> In this context it is also noteworthy that a consistent relationship between the peripheral blood granulocyte count and urine or serum CSA levels has not been demonstrated.<sup>38, 39</sup>

Our study confirms the previous observations that endotoxin stimulates CSA production by macrophages.<sup>11, 12</sup> It should be noted that the dose-response curves of conditioned medium obtained by incubation of macrophages alone and with endotoxin were not parallel. The CSA produced in the presence of endotoxin diluted out faster than the CSA produced by macrophages alone. This observation suggests that endotoxin induces elaboration of a different type of CSA, present in lower amounts, than the CSA produced by macrophages alone. In this regard it is interesting that Sheridan and Metcalf<sup>40</sup> noted a low molecular weight CSA in lung tissue of endotoxin-injected mice. This CSA was shown to be different from the CSA produced by lungs from saline-injected animals. Price et al. also showed that CSA derived from human leukocytes is heterogeneous in nature.<sup>41</sup>

We believe that these findings (the stimulation by microorganisms and their products and inhibition by mature granulocytes) may be closely interrelated in the control of granulopoiesis. The present and previous data from our laboratory<sup>25, 26</sup> suggest that the inhibitory role of mature granulocytes in granulocyte regulation may be through removal, and inactivation, of microorganisms and their products. In such a system the functional mass of mature circulating and tissue granulocytes is the main determinant of antigenic load from micro-

organisms reaching the reticuloendothelial system (RES) and hence of CSA production and granulopoiesis. A system where the functional mass of a particular cell type regulates its own production by inhibition of the stimulus responsible for elaboration of the regulating hormone is well illustrated by the erythrocyte system. The model we propose for granulocyte regulation is clearly analogous. The function of red blood cells is to carry oxygen. Their production is not simply controlled by numbers of circulating RBC but also by the amount of oxygen delivered to tissues. The role of granulocytes is to control microorganism invasion. It is likely that the production of granulocytes is controlled by their main physiologic function and hence by the load of microorganisms and their products reaching the RES and not simply by the number of circulating granulocytes.

It should be pointed out that endotoxin is known to have effects on multiple cellular systems.<sup>42</sup> Endotoxin activates the complement system, enhances properdin titers, and mobilizes interferon.<sup>43 45</sup> It also causes release of neutrophils from the bone marrow and induces blastogenesis of bone marrow derived lymphocytes (B cells).<sup>46,47</sup> In view of the complex and multiple effects of endotoxin, further work is necessary to establish firmly the interrelationship of microorganisms and their products, CSA-producing cells, and mature granulocytes in the regulation of granulopoiesis. The data presented here suggest one possible scheme by which this may be accomplished.

#### ACKNOWLEDGMENT

The authors gratefully acknowledge the capable technical assistance of M. Entringer, S. Redecker, and C. Drebing and the expert secretarial assistance of E. Fitzgerald.

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