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Effects of endotoxin on proliferation of human hematopoietic cell precursors

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Abstract

In examining the effects of corticosteroids on hematopoiesis *in vitro*, we observed that results were highly dependent on the lot of commercial fetal calf serum (FCS) utilized. We hypothesized that this variability correlated with the picogram (pg) level of endotoxin contaminating the FCS. Randomly obtained commercial lots of FCS contained 0.39 to 187 pg/ml of lipopolysaccharide (LPS). Standard FCS concentrations in hematopoietic precursor proliferation assays (granulocyte-marcrophage colony forming units [CFU-GM]) resulted in final LPS levels as high as 40 pg/ml. LPS (2–5 pg/ml) added to essentially endotoxin-free cultures, induced human mononuclear cell release of interleukin (IL)-1, IL-6 and granulocyte colony stimulating factor (G-CSF). Lots of FCS induced the release of IL-1, IL-6, and G-CSF from human mononuclear cells and the release of these factors correlated with the level of contaminating LPS. Human bone marrow CFU-GM proliferation, in response to granulocyte-macrophage colony stimulating factor (GM-CSF), positively correlated with the level of LPS contaminating the FCS and the FCS-induced release of IL-6 from mononuclear cells. CFU-GM proliferation of human bone marrow cluster of differentiation (CD) 34+CD14-cells were not affected by the presence of endotoxin. These data suggest that LPS at 2–5 pg/ml may induce bone marrow accessory cell release of hematopoietic growth factors, thus altering proliferative response of hematopoietic precursors and confounding the study of exogenously added cytokines to culture systems.

Introduction

Bacteremia and endotoxemia induce marked hematopoietic changes including leukocytosis and increased bone marrow production of hematopoietic precursors (Kuhns *et al*., 1995; Mackensen *et al*., 1991). One possible mechanism of increased leukocyte production is hematopoietic growth factor, or cytokine release from accessory cells that have been stimulated by bacterial products such as endotoxin or lipopolysaccharide (LPS) (Kuhns *et al*., 1995; Mackensen *et al*., 1991). LPS has been demonstrated to induce the release of cytokines or cytokine mRNA through the interaction of LPS with LPS binding protein. Interaction of this complex and CD14 on accessory cell surfaces results in the intracellular release of nuclear factor (NF)-κB (Kopp and Ghosh, 1995; Raetz *et al*., 1991; Tobias and Ulevitch, 1988).

In a series of experiments designed to evaluate the effects of corticosteroids on hematopoiesis, we observed that the results were highly dependent upon the lot of fetal calf serum (FCS) utilized. We hypothesized that these variations may have been due to different contaminating levels of LPS in each FCS lot. The effect of LPS, which is usually detectable in FCS from commercial sources, on *in vitro* hematopoiesis has not been systematically evaluated. Published studies of *in vitro* hematopoiesis usually do not describe the level of endotoxin in cultures or ensure levels of endotoxin in culture conditions to be < 5 pg. Our data suggest that some of the results previously attributed to hematopoietic growth factors and their effect on human bone marrow hematopoiesis precursor proliferation *in vitro*,

may have been partly induced by cytokines released from LPS-stimulated accessory cells.

Methods

Endotoxin levels. In the described experiments, we eliminated as completely as possible, cell exposure to endotoxin; endotoxin-free plastic, water, media, methylcellulose, cytokines, and heated glassware (410 °C for 4 hours) were used. All media were tested for endotoxin and discarded if endotoxin was detected. Endotoxin was assayed utilizing a timed gel formation system (LAL vials, Sigma, St. Louis, MO). The lots of FCS were obtained from Hyclone (Logan, UT) and Gibco BRL (Grand Island, NY). Endotoxin (LPS from *E. coli* K-235) was obtained from Sigma (St. Louis, MO).

Cell populations. Bone marrow (BM) aspirates and peripheral blood were collected from normal donors after obtaining informed consent.

Blood mononuclear cells (BMNC) were obtained by Ficoll-Hypaque density gradient separation (Pharmacia, Piscataway, NJ), washed by centrifugation, counted, and suspended to 2×10^6 cells/ml in RPMI-1640 (Gibco) with 20% FCS and 2 mM-glutamine.

BM cells (anticoagulated with heparin) were separated by Ficoll-Hypaque gradients. To obtain CD34+CD14-cells, BM mononuclear cells were washed with buffered media (phosphate buffered saline; 0.6% anticoagulant citrate dextrose solution, formula A [ACDA]; 1% human serum albumin). BM mononuclear cells in buffered media (10^7 cells/80 μl), and anti-CD14 magnetic microbeads (20 μl/10^7 cells) (Miltenyi Biotec, Sunnyvale, CA) were added and reacted at 6–8 °C for 20 minutes. The Supermacs magnetic cell sorter (Miltenyi Biotec) was used to separate the positive and negative fractions. The CD14-fraction was then labeled using a CD-34 isolation kit (Miltenyi Biotec) and was passed through two successive magnetic columns. Resultant positively selected cells were 98% CD34+ by flow cytometry and contained no detectable CD14+ cells.

CFU-GM cultures and cytokines. BM mononuclear cells (MC) were cultured in methylcellulose/IDMEM at 4×10^5/ml as previously described (Rinehart *et al.*, 1994). CD34+ cells were then cultured at 1×10^5/ml. Interleukin (IL)-1 (IL-1β, Syntex, Palo Alto, CA), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Sandoz, East Hanover, NJ) (both cytokines were donated) were added at the final concentrations indicated in individual experiments. IL-6, stem cell factor (SCF), IL-3 and granulocyte colony stimulating factor (G-CSF) were obtained from R&D Systems (Minneapolis, MN). Granulocyte-macrophage colony forming unit (CFU-GM) colonies were counted in triplicate 1.0 ml/16 mm wells at 7 days.

Table 1. Endotoxin levels in random lots of fetal calf serum

Laboratory number	Supplier/number	Endotoxin[a] (pg/ml)
1	Gibco/48N3440	0.39
2	Gibco/35K3541	1.3
3	Hyclone/15022394	1.3
4	Gibco/34N2048	31.5
5	Gibco/40K0242	62.5
6	Gibco/37K2043	125.0
7	Gibco/42K2442	187.0

[a] See Methods for assay system.

Supernatants. BMNC were cultured at 2×10^6/ml in RPMI-1640 with 20% of FCS lots as indicated. Resultant supernatants were centrifuged at 200G × 10 minutes and frozen at –20 °C.

Assay of G-CSF and other cytokines. G-CSF, IL-1, IL-3, IL-6, GM-CSF, and SCF levels in human BMNC supernatants were determined using an enzyme-linked immunoabsorbent assay (ELISA, Quantikine, R&D Systems). Known standards were assayed simultaneously, and the standard curve was plotted using the optical density versus the known cytokine concentrations.

Results

The level of endotoxin contamination in seven lots of commercially available FCS was highly variable and ranged from 0.39 to 187 pg/ml (Table 1). To determine if contaminating levels of endotoxin alone induced CFU-GM proliferation, we added endotoxin (LPS) to bone marrow CFU-GM assayed with our standard lot of serum (contains 1.3 pg/ml endotoxin) (Table 2). CFU-GM proliferation was induced by endotoxin at 200 pg/ml but was not observed at endotoxin levels that would be representative of the highest concentration in FCS. Thus, a significant number of colonies was not seen at 50 pg/ml (Table 2), which is comparable to

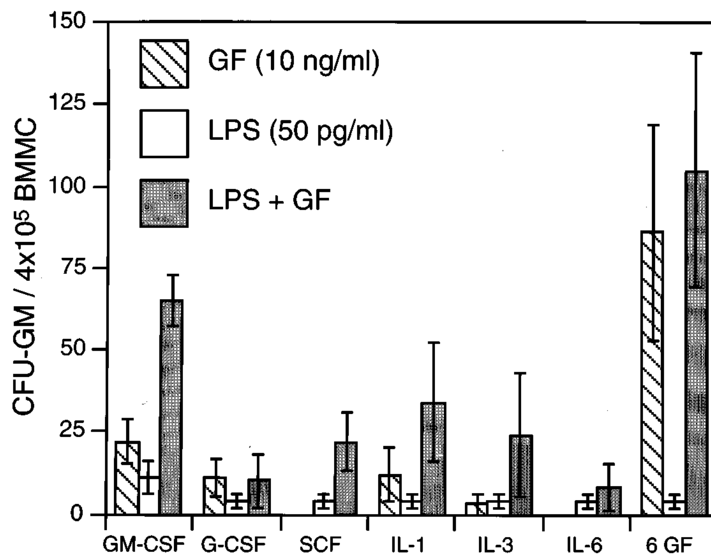

Figure 1. Bone marrow mononuclear cells were cultured in the methylcellulose system described in the Methods section in the presence of one or all six hemapotopoietic growth factors (GF, concentrations of all were 10 ng/ml) indicated in the Figure and in the presence or absence of LPS (50 pg/ml). The data are presented as the mean $\pm$ standard error of 5 separate experiments with normal human bone marrow mononuclear cells. LPS significantly enhances proliferative response to GM-CSF ($p < 0.01$). Apparent LPS enhancement of proliferative response to SCF, IL-3, and IL-1 did not reach statistical significance ($0.05 < p < 0.1$). The presence of LPS did not significantly alter response to all six cytokines.

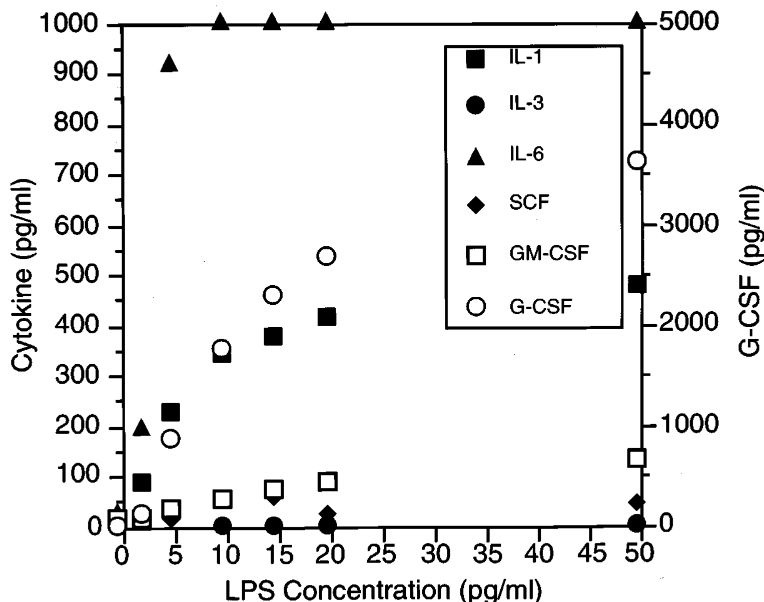

Figure 2. LPS (*E. coli*), at concentrations indicated in the Figure, was added to human blood mononuclear cells cultured in RPMI 20% FCS (Hyclone, see Table 1) at 2×10^6/ml for five days at 37 °C. Supernatants were cleared by centrifugation, and cytokine levels were determined by ELISA, as described in the Methods section.

20% of 187 or 37 pg/ml (FCS lot number 7, Table 1). However, we hypothesized that sub-stimulatory levels of endotoxin may synergize with hematopoietic growth factors to induce CFU-GM proliferation (Figure 1). The CFU-GM number induced by six individual cytokines was small when we used our standard serum

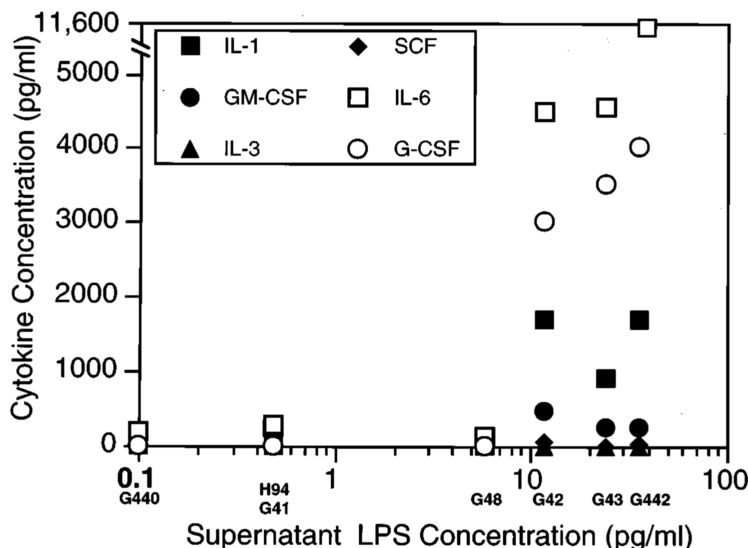

Figure 3. Human blood mononuclear cells were cultured in RPMI and 20% of the FCS in the Figure (see Table 1) at 2×10^6/ml for five days at 37 °C. The resultant calculated concentration of LPS (i.e, 20% of the level determined, Table 1) is presented on the horizontal axis. Supernatants were cleared by centrifugation and cytokine levels were determined by ELISA as described in Methods. Induced release of G-CSF, IL-1, and IL-6 correlated with the level of contaminating LPS ($r = 0.96$, $p < 0.01$; $r = 0.85$, $p < 0.02$; $r = 0.78$, $p < 0.05$).

Table 2. Induction of CFU-GM by *E. coli* endotoxin (LPS)

Concentration of added LPS (pg/ml)	CFU-GM/4×10^5 bone marrow cells
control[a]	0 ± 0[b]
200	22 ± 12
50	4 ± 2
10	0 ± 0

[a] Fetal calf serum lot #3 (Table 1) was used in these and all experiments, except as indicated, so that the baseline concentration of endotoxin in the control cultures was approximately 0.26 pg/ml. Added endotoxin was *E. coli* derived (see Methods).
[b] Standard methylcellulose assay of normal human bone marrow was used (see Methods), and the data are expressed as mean ± standard error (m ± SE).
LPS = lipopolysaccharide
FCS = fetal calf sera

containing minimal endotoxin contamination (Figure 1, hatched bars), but large numbers of colonies were observed when all six cytokines were used together to induce CFU-GM proliferation. The presence of 50 pg/ml of LPS increased CFU-GM proliferation in response to single cytokines or growth factors (GM-CSF, SCF, IL-1, and IL-3), but not in response to all six cytokines (Figure 1).

LPS at nanogram (ng) and microgram (μg)/ml concentrations, have been reported to release several cytokines and induce cytokine mRNA from mononuclear cells. We hypothesized that LPS enhancement of CFU-GM proliferation in response to cytokines was due to an LPS-induced release of additional cytokines by blood and bone marrow mononuclear cells. To examine this enhancement, human mononuclear cells were incubated with 2.5 to 50 pg/ml LPS (Figure 2). LPS at 5 pg/ml induced significant release of G-CSF, IL-1, and IL-6 and a maximum release was induced between 20 and 50 pg/ml of LPS.

To determine if the LPS contaminating the FCS induced similar release of cytokines as exogenously added LPS, human mononuclear cells were incubated at 37 °C for 5 days in RPMI containing 20% of the FCS lots under study. Resultant supernatants were assayed for cytokines by ELISA (Figure 3). Release of G-CSF, IL-1 and IL-6 was induced by LPS contaminating the FCS (Figure 3) at levels comparable to those with added LPS (Figure 2) i.e., between 5–10 pg/ml. Further, there was significant correlation between the level of LPS contaminating the lots of FCS and the level of cytokine released into the supernatants: G-CSF, $r = 0.92$, $p < 0.01$; IL-1, $r = 0.85$, $p < 0.02$; IL-6, $r = 0.78$, $p < 0.05$. Significant correlation was not observed between the level of contaminating endotoxin and supernatant IL-3, GM-CSF or SCF (Figure 3).

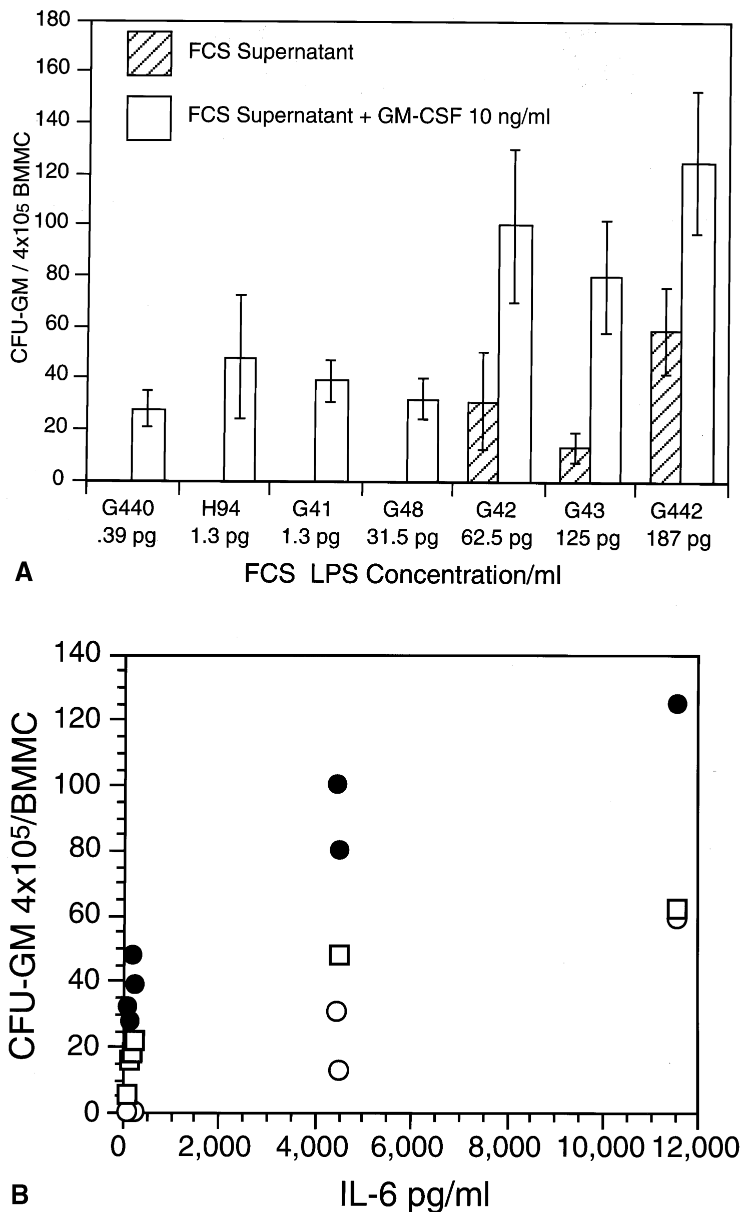

Figure 4A–B. (A) Bone marrow mononuclear cells were cultured in the methylcellulose system as described in the Methods section with 10% of the FCS supernatants (see Figure 3) ± GM-CSF. The FCS used in the culture system at 10% concentration was Hyclone 15022394, which contained minimal LPS (Table 1). In the absence of FCS supernatants < 5 CFU-GM/4 × 10^5 bone marrow mononuclear cells were observed. Data are presented as mean ± standard error in the five experiments. (B) The level of IL-6 in the FCS supernatants (see Figure 4A) is plotted against the number of CFU-GM induced by FCS supernatants alone (□), FCS supernatant + GM-CSF (●), or GM-CSF in the presence of various FCS at 10% concentration (○).

To determine if the cytokines released in response to contaminating endotoxin altered CFU-GM proliferation, we further studied the supernatants described in the previous paragraph (Figure 4). CFU-GM assays of normal bone marrow were established containing 10% supernatant with or without 10 ng/ml GM-CSF. The three supernatants with the highest levels of contaminating endotoxin and also containing G-CSF, IL-1 and IL-6, induced the highest number of CFU-GM colonies with or without added GM-CSF (Figure 4A). We also

Table 3. Comparative effect of LPS on CFU-GM proliferation in unfractionated bone marrow mononuclear cells and isolated CD34+ cells

Culture additions[a]	CFU-GM[b]	
	mononuclear cells 4×10^5/BM cells	CD34+ cells 1×10^5/BM cells
none (control)	$12 \pm 4/0$	0/0
GM-CSF 10 μg/ml	$16 \pm 3/14 \pm 3$	$11 \pm 4/2 \pm 1$
LPS 50 pg/ml	2 + 1/0	0/0
GM-CSF+LPS	$72 \pm 4/44 \pm 8$	$15 \pm 3/4 \pm 1$
Six cytokines[c]	50 + 3/27 $\pm$ 5	$24 \pm 7/40 \pm 13$

[a] Bone marrow mononuclear cells or bone marrow CD34+CD14- cells were cultured at the indicated concentration for 7 days in the methylcellulose system described in the Methods Section and the number of CFU-GM colonies were enumerated in triplicate wells.
[b] Data are expressed as mean $\pm$ standard error of three triplicate cultures in two identical experiments. The number of CFU-GM is expressed per 4×10^5 bone marrow mononuclear cells and per 1×10^5 CD34+CD14- cells.
[c] Cytokines were all added at 10 ng/ml (IL-1, IL-3, IL-6, SCF, G-CSF, GM-CSF).
CFU-GM = granulocyte macrophage colony forming units; GM-CSF = granulocyte macrophage colony stimulating factor; BM = bone marrow.

examined the relationship between the capacity of the various sera to induce cytokines and to support CFU-GM proliferation in response to GM-CSF (Figure 4B). The two FCS with the highest LPS concentrations supported the best CFU-GM proliferation in response to GM-CSF. Significant correlation was noted between the capacity of sera to induce IL-6 in mononuclear cell supernatants and to support bone marrow CFU-GM proliferative response to GM-CSF (Figure 4B).

We hypothesized that LPS enhanced CFU-GM proliferation indirectly by the induction of monocyte or other accessory cell release of cytokines. Therefore, we fractionated human bone marrow by sequential Ficoll-Hypaque gradient centrifugation, depletion of CD14+ cells by a monoclonal antibody magnetic microbead system, and then positive selection of CD34+ cells by the same technique. CD34+ cells were cultured with LPS 50 pg/ml, GM-CSF 10 mg/ml, both, or with six cytokines. Bone marrow mononuclear cells were cultured with the same additions (Table 3). As predicted by our hypothesis, LPS enhanced CFU-GM proliferation only in the bone marrow mononuclear cell population.

Discussion

Our data demonstrate that tissue culture media containing > 5.0 pg/ml of LPS, induce human accessory cell production of cytokines important in hematopoiesis. As a result, these released cytokines and hematopoietic growth factors (IL-1, IL-6, G-CSF) induce human bone marrow CFU-GM proliferation and modulate proliferative response to GM-CSF. The data strongly suggest that conclusions drawn regarding the effects of added hematopoietic growth factors to hematopoietic precursor proliferation assays must be assessed critically unless LPS levels of < 5 pg/ml are assured.

Studies evaluating the effects of LPS on cytokine and hematopoietic growth factor secretion or gene expression have utilized concentrations of LPS in the nanogram or microgram range (e.g., references 1 and 9). Clearly, CD14+ cells, in the presence of LPS binding protein (LBP), respond to LPS in the pg/ml concentrations (Kopp and Ghosh, 1995; Raetz *et al.*, 1991; Tobias and Ulevitch, 1994).

In our present study we evaluated only CFU-GM. However, the hematopoietic growth factors induced by 2–5 pg/ml LPS (IL-1, IL-6 and G-CSF) have the potential to alter the growth of other hematopoietic precursors and our presented finding may be important in the study of other hematopoietic precursors. These data present the potential impact of LPS on hematopoiesis *in vivo*, since in the course of gram negative infections, LPS blood concentrations of > 2 pg/ml are frequently achieved (Endo *et al.*, 1992). Further, our data suggest that the effect of LPS on hematopoiesis is indirect and due to cytokine or hematopoietic growth factor release from CD14+ accessory cells.

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