To the Graduate Council:

I am submitting herewith a thesis written by Daniel Gregorio Deana entitled "Lipopolysaccharide Enhancement of Colony-Stimulating Factor-1 Induced Monocytopoiesis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Robert N Moore

Robert N. Moore, Major Professor

We have read this thesis
and recommend its acceptance:

J. B. Jones

Gary Thorne

Accepted for the Council:

C W Minkel

The Graduate School

LIPOPOLYSACCHARIDE ENHANCEMENT OF COLONY-STIMULATING FACTOR-1 INDUCED MONOCYTOPOIESIS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Daniel Gregorio Deana

December 1984

DEDICATION

This thesis is dedicated to my parents, Americo Francis and Cecilia Pauline Deana, who taught me to pursue my goals with passion, yet enjoy the rewards in moderation. Their support, encouragement, and guidance have been, and will continue to be, paramount to my success.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my committee members: Dr. Robert N. Moore, Dr. J. B. Jones, and Dr. Barry T. Rouse. Each was quick to recognize strengths and weaknesses as the data developed, and indispensible in pointing the way towards future work.

I would especially like to thank Dr. Moore who carefully guided the path of my work, and kept me on that path as I strayed. He taught me that science is a dynamic process of thought and labor, and the key to that process lies not only in finding the answers to questions, but in discovering the questions to answer.

Finally, in addition to Dr. Moore, I wish to thank my laboratory partners who I have worked with for the last three years, Dave Horohov and Vic Candler. Special thanks also to Sandi Woolf. Memories of these years will not quickly fade. As I leave the university, I take a part of each of them with me.

ABSTRACT

This investigation examines the effects of bacterial lipopolysaccharide (LPS) on the proliferative response of murine femoral bone marrow myeloid precursors induced by colony-stimulating factor-1. This proliferative response was measured both as [^{3}H]-thymidine incorporation in liquid culture and colony formation in semisolid agar. Lipopolysaccharide exposure produced dose-dependent effects on CSF-induced myeloid proliferation, ranging from significant suppression ($p \leq 0.01$) at LPS concentrations greater than 10^{-1} mcg/ml to significant enhancement ($p \leq 0.01$) at 10^{-3} to 10^{-5} mcg LPS/ml. Similar LPS-induced enhancement occurred both in the presence and absence of anti-IFN (α + β) antiserum and indomethacin, thus the LPS effect was independent of CSF-induced interferon and prostaglandin E. Enhancement was not due to LPS-stimulated CSF production, as proliferation was not observed in cultures receiving LPS alone. The lack of LPS-induced enhanced proliferation by LPS-hyporesponsive C3H/HeJ cells indicated enhancement is due to lipid A. Removal of more mature adherent cells via plating did not prevent the LPS stimulation effect, nor did co-culture of C3H/HeJ and LPS-responsive C3H/AnF nonadherent cells result in proliferation more than additive with respect to proliferation seen in cultures incubated separately. These results indicated that LPS acts directly on myeloid precursors and not via a secondary soluble mediator produced by LPS-stimulated cells. Finally, the ability of contaminating serum endotoxin to mask the stimulatory effects of experimentally administered nanogram levels of LPS is presented.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	SURVEY OF THE LITERATURE	2
III.	MATERIALS AND METHODS	16
	Mice	16
	Lipopolysaccharide (LPS)	16
	Anti-Interferon (IFN)	16
	Indomethacin	17
	Colony-Stimulating Factor (CSF)	17
	Assays for CSF.	20
	Statistical Analysis.	22
IV.	RESULTS.	23
	Comparison of Thymidine Incorporation and Soft Agar Colony Formation by Bone Marrow Cells Stimulated with CSF	23
	LPS Effect on CSF-Induced ^{3}H-TdR Incorporation and Colony Formation by Marrow Cells in the Presence or Absence of Anti-IFN (α + β) Antiserum and Indomethacin.	25
	LPS-Enhanced Responsiveness of Marrow Cells to CSF is Associated with Lipid A	33
	LPS Acts Directly on CSF-Stimulated Bone Marrow Cells.	36
	Serum Endotoxin Contamination Influences Bone Marrow Response to CSF.	40

CHAPTER PAGE

V. DISCUSSION. 44

LIST OF REFERENCES 52

VITA 62

LIST OF TABLES

TABLE PAGE

1. Colony formation by CSF stimulated nonadherent marrow from C3H/AnF and C3H/HeJ mice in the presence of LPS 41

2. Influence of contaminating serum LPS on CSF-induced colony formation 42

LIST OF FIGURES

FIGURE PAGE

1. Comparison of ^{3}H-TdR incorporation and colony formation by bone marrow cells stimulated with CSF 24
2. Dose dependency of LPS effect on CSF-induced ^{3}H-TdR incorporation and colony formation by C3H/AnF cells . . . 26
3. Comparison of ^{3}H-TdR incorporation and colony formation by bone marrow cells stimulated with CSF and LPS 27
4. Effect of anti-IFN (α + β) antiserum and indomethacin on CSF-induced proliferation of 1 x 10^5 marrow cells in the presence or absence of LPS. 29
5. Effect of anti-IFN (α + β) antiserum and indomethacin on CSF-induced proliferation of 2 x 10^5 marrow cells in the presence or absence of LPS. 30
6. Effect of LPS on CSF responsiveness of C3H/AnF marrow cells determined by colony formation in the presence or absence of anti-IFN (α + β) antiserum and indomethacin 32
7. Effect of LPS on CSF-induced proliferation by C3H/AnF and C3H/HeJ marrow cells in the presence or absence of anti-IFN (α + β) antiserum and indomethacin 34
8. Dose dependency of the LPS effect on CSF-induced colony formation by C3H/AnF and C3H/HeJ marrow cells in the presence or absence of anti-IFN (α + β) antiserum and indomethacin 35

FIGURE PAGE

9. Effect of LPS on the CSF responsiveness of total and nonadherent C3H/AnF marrow cells determined by colony formation in the presence or absence of anti-IFN (α + β) antiserum and indomethacin 37

10. Effect of LPS on the CSF responsiveness of total and nonadherent C3H/HeJ marrow cells determined by colony formation in the presence or absence of anti-IFN (α + β) antiserum and indomethacin 39

LIST OF ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
CFUc	colony forming unit-committed
cGMP	cyclic guanosine monophosphate
cpm	counts per minute
CSA	colony-stimulating activity
CSF	colony-stimulating factor
FBS	fetal bovine serum
^{3}H-TdR	tritiated thymidine-radioactive
IFN	interferon
LCM	L-cell conditioned medium
LPS	lipopolysaccharide
LPS^{d}	lipopolysaccharide defective responder
LPS^{n}	lipopolysaccharide normal responder
LTBMC	long-term bone marrow culture
mcg	microgram
mcM	micromolar
PEG	polyethylene glycol
PGE	prostaglandin E
U	units
SEM	standard error measurement

CHAPTER I

INTRODUCTION

The mechanism of mononuclear phagocyte expansion from the bone marrow in response to extramedullary events remains elusive. Despite the isolation and purification (86, 94) of colony-stimulating factor-1 (CSF-1), a soluble mediator that supports bone marrow macrophage progenitor proliferation and differentiation in in vitro bioassay, no data demonstrate enhanced myelopoiesis upon injection of exogenous CSF into an animal.

The purpose of this thesis is to examine the role bacterial endotoxin (lipopolysaccharide-LPS) plays in proliferation of bone marrow myeloid progenitors. In an historical review, important data defining the status of CSF as a myeloproliferative agent is discussed. Furthermore, the relationship of LPS to CSF as a probable myelopoietic mediator is reviewed.

CHAPTER II

SURVEY OF THE LITERATURE

Colony stimulating factors (CSF) are soluble glycoproteins that promote the clonal proliferation and differentiation of myeloid stem cells (7, 63, 85). CSF can be detected in a variety of mammalian tissues and fluids. These molecules may be cytokine (68), lymphokine (67, 70, 76), or monokine (23, 70) in origin and are found in media conditioned by pancreatic (100), uterine (6), placental (62), and lung tissue (8). CSF is excreted in urine at low levels; interestingly, in patients over 65, urine CSF levels are increased 10-fold (52, 85). In abnormal physiologic conditions, CSF may be found in moderate levels in the serum; for example, in human patients exhibiting lung cancer associated with excessive leukocytosis (38) and in test animals following partial hepatectomy (61), serum CSF levels are significantly increased.

The granulopoietic effect of CSF on bone marrow cells is an *in vitro* phenomenon observable either by culturing bone marrow cells with an appropriate CSF source in soft agar and observing colony formation, or by measuring tritiated thymidine uptake and estimating DNA synthesis (52, 85). A common source of CSF for experimentation is from the neoplastic murine fibroblast L-cell line designated clone 929 (87). These cells constitutively produce CSF in culture. Media conditioned by these cells (L cell conditioned medium, LCM) have been used as a standard CSF source (1). Recently, researchers have successfully devised a purification scheme for LCM; using ultrafiltration, alcohol

precipitation, and various forms of column chromatography Stanley et al. (86) and Waheed and Shadduck (94) obtained a 65,000 to 70,000 dalton glycoprotein of reasonable purity with the functional properties of CSF--the molecule, in fact, comprised only 0.1% of the original LCM protein (the L-cells were grown serum free) (94).

CSF may be divided into four subclasses depending on target cell specificity (85). CSF-1 stimulates proliferation of committed macrophage precursors, whereas CSF-2 acts on both neutrophilic granulocyte and macrophage precursors. CSF-3 stimulates only neutrophilic granulocytes, while CSF-4 stimulates eosinophilic granulocyte production. Biochemically, the different CSFs are similar; of the four, CSF-1 has been best characterized (L cell CSF is CSF-1). CSF-1 has an M_r of 45,000 to 86,000 with the range dependent on the glycosylation of the polypeptide chain. It is a sialic acid containing molecule that upon reduction with 2-mercaptoethanol loses biological activity and is reduced to half the original M_r (85).

CSF is believed to have two sites of action in the body: 1) the progenitor cell, as mentioned above, where the molecule induces clonal proliferation and differentiation, and 2) the mature cell, where CSF has the ability to stimulate functional capacity. CSF has been shown to induce or increase production of interleukin 1 (IL 1), E series prostaglandin (PGE), interferon, and plasminogen activator (52) by macrophages, as well as induce antitumor activity in normal macrophages (99) and candidacidal activity in infected macrophages (65). Also, CSF affects expression of surface enzymes such as aminopeptidase (49). In addition, CSF-2 enhances antibody-dependent cell-mediated cytotoxicity

(ADCC) by purified eosinophils and neutrophils (92). Thus, CSF both stimulates proliferation of, and regulates many functions of macrophages and granulocytes.

In spite of the proliferative activity induced by CSF as seen in in vitro assays, evidence for in vivo stimulation of myelopoiesis remains somewhat elusive. In vivo myelopoietic studies may be accomplished using the diffusion chamber technique (78). In this method, chambers composed of nucleopore filter walls are inoculated with non-adherent bone marrow cells syngeneic with respect to the test animals. The chambers are sealed with nylon plugs and inserted into the peritoneal caviety of recipient test animals. An initial decline in recoverable granulocytic cells is followed by a "wave" of granulocyte production; neutropenia induced by irradiation or cyclophosphamide treatment enhances this post-implantation granulocyte maturation. Thus, presumably some humoral factor in the neutropenic animal causes the diffusion chamber granulopoietic effect.

Injection of anti-CSF antisera into mice previously exposed to whole body X-irradiation (700 Rads at 120 Rad/min) was found to significantly reduce the granulopoietic activity of the diffusion chambers (78). If, however, repeated injections of anti-CSF was administered to non-irradiated mice, no suppressive effect were observed in numbers of peripheral blood neutrophils, marrow granulocytic precursors, or marrow committed granulocyte/macrophage colony forming cells (CFUc). Thus, CSF produced exogenously to the bone marrow may affect extramedullary myelopoiesis, but perhaps not myelopoiesis within the bone marrow.

Other researchers have established in vitro long term hematopoietic cultures (13) wherein pluripotent stem cells and CFUc are produced for up to six months. The cellular contents of a single femur from a young adult mouse are plated with an enriched medium into a culture flask. Within a month, an adherent layer of endothelial-like cells, fat cells, fibroblasts, and macrophages is formed. Syngeneic bone marrow cells are inoculated onto this layer, and the cultures are fed by the removal and subsequent replacement of half the medium biweekly; cells removed in the media may then be assayed. Using this system it was found first that no biodetectable levels of CSF were present in the cultures. Since the same marrow cells exhibit a marked response to CSF in soft agar cultures some "subtle mechanism" was assumed responsible for their sustained growth in the long term cultures. Secondly, it was found that the addition of either purified CSF or anti-CSF had no effect on hematopoiesis in the long term cultures. Thus, if CSF is involved, low levels of endogenously produced CSF may be responsible for the proliferation and subsequent differentiation of the CFUc. This endogenous CSF must be either antigenically different from the CSF used in the preparation of the antisera, or the CSF exchange is an intimate interaction between producer and responder and is thus inaccessible to antisera.

Specific membrane receptors for CSF-1 have been identified and are restricted to cells of the mononuclear phagocyte system, their precursors, and established macrophage cell lines (26). Radiolabeled CSF is not bound by freshly isolated marrow cells unless those cells have been previously incubated for a short period of time (26). Since

the CSF receptors are most likely not simply induced or made functional on in vitro incubation, the lack of binding with the labeled CSF to freshly isolate marrow cells is most likely a consequence of those receptors being saturated with endogenously produced CSF. Seemingly, this proposition could experimentally be observed with fluorescently-labelled anti-CSF; however, there is no reference to such an assay in the literature.

Recent work has substantiated the theoretical production and consumption of CSF by cells in long-term bone marrow cultures (29). A bilayer agar culture was devised in which a dense layer of agar is plated over the adherent cells of long-term bone marrow cultures (LTBMC --as before); a second agar layer containing fresh myeloid cells as a source of CFUc is added on top. These researchers found a significant level of granulocyte/macrophage colony production in the upper layer when the lower layer of LTBMC was hematopoietically inactive (production of only a few non-adherent cells). The CSF activity was not found, however, when the LTBMC was hematopoietically active (proliferation of adherent cells in lower layer). Adherent cells in the CFUc cell source did not affect colony formation of the CFUc. Confirmation of CSF consumption in situ by the adherent cells was found by incubating exogenous CSF over an adherent layer of hematopoietically active LTBMC's; such incubation was found to deplete the added CSF activity as shown by a marked reduction in CFUc proliferation as compared to control incubation of exogenous CSF without adherent cells. No CSF inhibitor was found in this system. Thus, granulocyte/macrophage clonal proliferation in LTBMC is a result of CSF production

and consumption. Previous reports of a failure to detect CSF activity in LTBMC are a consequence of low (but sufficient) CSF production and consumption by the regenerating adherent layer. The results are consistent with the cell-cell transmission theory of CSF activity, but suggest long-range CSF activity is possible when the adherent feeder layer is hematopoietically inactive, and consumption, as a result, low (29).

The dilemma facing CSF research presently is the inability to demonstrate CSF activity on myelopoiesis upon injection of exogenous CSF into an animal--no *in vivo* evidence is as yet described. Infusion of human urinary colony-stimulating factor, into leukocytopenic patients has been shown to increase the serum colony stimulating activity (CSA) levels of those individuals. This probably occurs through the stimulation of CSA-producing cells *in vivo*; however, no true enhancement of myelopoiesis occurs (60). Thus, as has been previously mentioned, evidence supports the hypothesis that CSF exogenous to the bone marrow functions to stimulate only extramedullary proliferation as well as a plethora of "activating" functions unrelated to proliferation. Also, evidence exists that bone marrow myeloid precursors may exist in an environment actively supplied with endogenous CSF. Obviously, these observations leave the question of what mechanisms function to enhance myelo-granulopoiesis within the bone marrow in response to events external to the bone marrow.

Evidence tends to support a complex bioregulation of myeloproliferation. Either: (1) myeloproliferation is regulated by a second inhibitory signal, with proliferation proceeding in the inhibitor's

absence, or (2) myeloproliferation is regulated by a second stimulatory signal, with proliferation proceeding in the stimulator's presence, or (3) both inhibitory and stimulatory second signals are intertwined in a complex web of interaction leading ultimately to control of the hematopoietic response.

A number of factors have been described which influence the activity of CSF on pluripotent stem cells leading to CFUc differentiation. A number of authors have described what is probably a single factor, but is known by a variety of names. Endogenous pyrogen, interleukin 1 (IL 1), leukocytic endogenous mediator (LEM), and synergistic activity (SA) are all very similar molecules with similar activities (35, 39). This factor appears to act as a stimulator of endogenous marrow CSF production and a neutrophil releasing factor (36, 37). It is found in spleen and placental conditioned media and is produced by macrophages. This factor's ability to act as a neutrophil releasing factor is a prime example of the relation of inhibitors and stimulators. Neutrophils have been shown to produce lactoferrin (also called CIF, colony inhibitory factor) which acts as a negative feedback control to shut down colony proliferation by decreasing CSF production in the marrow (5). Thus, LEM acts to delete the marrow of neutrophils, which in turn may reduce lactoferrin levels leading to increased myeloproliferation. Others (2) have described a non-dialyzable, heat labile factor from monocyte-conditioned medium that enhances CSA production by endothelial cells 4-15 fold. Lactoferrin does not inhibit CSA production by the endothelial cells, but does inhibit the CSA-stimulating monokine.

Prostaglandin E (PGE) biosynthesis appears to be intrinsically linked to both the synthesis of, and response to, CSF by normal macrophages (43). Dose-dependent stimulation of PGE synthesis by macrophages in response to increasing concentrations of CSF is concommitantly associated with progressively inhibitory activity for CFUc proliferation (40). It is important to note that the concentration of CSF capable of stimulating macrophage PGE synthesis is greater than that required to stimulate CFUc proliferation. Reports have shown that murine bone marrow progenitor cells that form macrophage colonies are more sensitive to the effects of PGE than those giving rise to granulocytes or megakaryocytes (98).

Another inhibitory molecule, LIA (leukemia-associated inhibitory activity), formed in bone marrow, spleen, and blood cells of patients with both acute and chronic myeloid and lymphoid leukemia has been found to suppress CSF activity on bone marrow progenitors. LIA has been identified as an acidic isoferritin (3).

Cyclic nucleotides have been shown to influence CSF activity. Proliferation of bone marrow precursors stimulated by CSF is antagonized by factors that elevate cyclic AMP, including PGE (43, 64), and potentiated by factors that elevate cyclic GMP, including PGF (41). Recent work in this laboratory has also shown that interferons affect CSF activity. Partially purified murine beta interferon preparations and Con-A stimulated splenocyte supernatants, both free of myeloid CSF, were found to enhance the <u>in vitro</u> response of CFUc to CSF-1 (51, 55). Highly purified alpha and beta interferon preparations mimicked this response. Enhancement of CSF-1 responsiveness required pre-exposure to

the interferon preparations with subsequent removal or neutralization of the interferon prior to addition of sub-optimal levels of CSF to the cells. The interferon preparations appeared to enhance the responsiveness of CFUc to CSF-1-induced cloning rather than increasing the total number of committed precursors, as judged by analysis of soft-agar cultures. However, this laboratory has also shown that simultaneously incubation of murine femoral cells with CSF-1 and antiserum directed against interferons α and β result in a significant enhancement of mononuclear phagocyte expansion as compared to cells incubated with CSF-1 only. Stimulation of femoral marrow cells with CSF-1 was shown to result in production of α/β interferons which appears to act as a feedback regulators of CSF-1 induced proliferation (53).

Infection with Gram-negative bacteria (90) or exposure to preparations of bacterial endotoxin (LPS-lipopolysaccharide) isolated from the outer cell membranes of a variety of Gram-negative bacteria (9, 12, 46, 69, 84) both enhance serum CSF levels and stimulate myelopoiesis in mammals in vivo, as well as a variety of other immunologic or immunopathologic responses (58). LPS is a polyclonal stimulant of B cell proliferation (21). Endotoxin also activates macrophages to become tumoricidal (15, 73, 74); in fact the gene for control of macrophage-tumoricidal capacity is either closely linked, or identical, to the LPS gene (75). Macrophages release a variety of immunoregulatory factors in addition to CSF, such as interleukin-1 (91), interferon (30, 57, 81), tumor necrosis factor (79), E prostaglandins (42), and plasminogen activator (24) in response to LPS.

Lipopolysaccharide can be lethal at high doses, inducing hypotension, leukopenia, thrombocytopenia, and disseminated intravascular coagulation (59). At sublethal doses, however, the LPS effect is stimulatory, as evidenced by an increase in peripheral mononuclear phagocytes and granulocytes (56). Endotoxin also increases the numbers of myeloid stem cells in cell cycle in treated animals (14), as well as increasing the mass of organs rich in reticuloendothelial cells (90). All of these responses are characteristic of a variety of Gram-negative bacterial infections and suggest that LPS can trigger protective responses in infectious disease.

Structurally, LPS consists of three distinct domains. The O-specific antigen region which comprises the hydrophilic outermost portion is comprised of repeating oligosaccharide units. These are attached to the core region which is composed of approximately ten oligosaccharide residues including L-glycero-D-mannoheptose and the unusual sugar 2-keto-3-deoxy-octulosonic acid. The core region is attached to lipid A, the hydrophobic portion of LPS that is embedded in the outer membrane of the bacterium. Lipid A of the enteric bacteria is made up of a $\beta(1-6)$ diglucosamine backbone that carries long chain fatty acid residues at the hydroxyl and amino groups, and one or more phosphate residues (45, 93).

It is the Lipid A portion of LPS that is responsible for endotoxic activity as measured by pyrogenicity and mouse lethality (44), B-cell proliferation (25), and macrophage activation (16). The carbohydrate moieties of LPS have been implicated as mitogenic in a few reports (22, 77); however, their major role appears in receptor interaction.

Plasma membrane receptors for LPS have been identified on erythrocytes (83), platelets and granulocytes (82), lymphocytes (34), mast cells (31), and hepatocytes (71). LPS binds to a lectin-like receptor on macropahges (28) which is distinct from the mannose receptor (20). Once ingested by the macrophage LPS is not degraded, although the parent bacterium is extensively. Although not degraded, LPS may be modified by the host phagocyte; however, the molecule retains biologic activity (17).

The binding of CSF-1 is altered by lipopolysaccharide presence. ^{125}I-CSF-1 at 0°C binds directly and specifically to membrane receptors. At 37°C, the radio-labeled CSF is rapidly internalized and degraded by macrophages which then release radioactive iodo-tyrosine (10). Bacterial endotoxin at nanogram quantities induces a temperature dependent and time dependent inhibition of ^{125}I-CSF-1 binding to peritoneal exudate macrophages. This inhibition is non-competitive, and is neutralized via polymyxin B addition, which binds to and effectively neutralizes the Lipid-A portion of endotoxin. This inhibition is not permanent, and CSF-1 binding recovery can be blocked by addition of cycloheximide, suggesting *de novo* receptor production and insertion into the membrane (10).

C3H/HeJ mice are refractory to all known *in vivo* biologic effects of LPS. This lack of responsiveness is controlled by a single autosomal codominant gene (72) located on chromosome 4 (97). This gene with the mutant allele has been designated LPS^{d} (defective response) (96) and is only 0.06 $\pm$ 0.02 recombination units from the major urinary protein locus Mup-1 (96, 97). Immature B-cells from C3H/HeJ mice will

not express cell surface antigens in response to LPS as normal LPS responders (designated LPS^n, normal response) would, nor will more mature B cells from C3H/HeJ mice respond mitogenically to LPS (95). LPS will not act as an adjuvant in C3H/HeJ mice (80), and it will not elevate serum levels of the serum precursor SAA of the secondary amyloid protein AA (47). Endotoxic shock is not induced in these LPS^d mice (89).

Macrophages from C3H/HeJ mice are not as tumoricidal as those from LPS^n mice (19). LPS levels that are lethal to macrophages from responder strains of mice are not cytotoxic for C3H/HeJ macrophages (25). The LPS^d macrophages will not exhibit increased adherence and phagocytosis in response to endotoxin (10), nor will they show enhanced glucose utilization after LPS treatment (10)--characteristics seen in LPS^n macrophages. Activation of C3H/HeJ macrophages, as measured by tumoricidal capacity, does, however, occur in response to BCG (Bacille Calmette-Guerin, an attenuated strain of Mycobacterium tuberculosis). Such activation by LPS is not overcome via co-culture of LPS^n responder spleen cells with LPS^d non-responder C3H/HeJ macrophages, indicating that LPS acts directly with macrophages to induce tumor-cell lysis (16). The same autosomal co-dominant gene controls both B-lymphocyte and macrophage LPS sensitivity (72).

Numerous explanations for the expansion of the reticuloendothelial pool following LPS exposure concentrate on LPS-induced production of CSF. LPS exposure at sublethal doses leads to enhanced serum myelopoietic activity (Colony Stimulating Activity, CSA); this elevated serum CSA response is seen with both Gram-negative bacterial infection

(90) and with injections of purified LPS (9, 11, 46, 69, 84). The cellular origin of this CSA (and the molecule responsible, CSF) has been identified tentatively as macrophages (18). It is thus implied that LPS stimulates peripheral CSF production which then enhances proliferation of the myeloid progenitor cells. Available evidence, including the long-term bone marrow cultures previously described (29), fails to support this contention. CSF produced external to the bone marrow appear to have effects on macrophage/monocytic cells in the periphery, whereas those cells of the marrow produce and consume their own CSF. In fact, it was work with adoptive transfer of bone marrow and chimera formation between LPS^d and LPS^n strains of mice that provided direct evidence for CSF production by cells of bone marrow origin (32, 50). Lethal irradiation of C3H/HeJ mice, with subsequent reconstitution of bone marrow cells from C3H/HeN (LPS^n), will render the chimeric C3H/HeJ mice responsive to LPS with concommitant CSF production after exposure.

Other researchers have examined the kinetics of CSF production in response to LPS (88). They found that human monocytes, after exposure to 1 mcg endotoxin/ml produce colony stimulating activity (CSA) after a lag period of 1-2 hours with most activity produced within the first 6-12 hours of incubation with the endotoxin. Puromycin and cyclohexamide significantly inhibited CSA, suggesting production requires *de novo* synthesis. CSA production ceased by 72 hours incubation.

Recently, Moore *et al.* (56) have found that addition of LPS to CSF-stimulated macrophage populations had very different effects, depending on the concentration of CSF employed. Macrophage colony

formation by murine peritoneal exudate cells is actually inhibited by LPS at doses $\geq$ 0.01 mcg/ml in the presence of optimal to supra-optimal CSF doses. This inhibitory activity was not due to LPS cytotoxicity and was not mediated through prostaglandin synthesis. In the presence of sub-optimal CSF doses however, minute LPS concentrations (10^{-7} mcg/ml) significantly enhanced macrophage colony formation. Polymyxin B, which binds to the lipid A portion of LPS neutralized both the stimulatory and inhibitory effects of LPS.

The purpose of this investigation is to compare the effect of LPS on murine bone marrow myeloid precursors with that seen with peritoneal exudate cells described above, and determine if marrow cells behave in a similar manner. Data presented suggest that the responses are similar in that LPS does enhance the CSF-responsiveness of the marrow myeloid precursors. Additional evidence is presented that suggest a significant proportion of the progenitor population actually requires LPS to exhibit CSF responsiveness.

CHAPTER III

MATERIALS AND METHODS

Mice

C3H/AnF and Ha/ICR female mice were purchased from Cumberland View Farms, Clinton, TN. C3H/HeJ female mice were obtained from the breeding facilities at the Medical Research Center for the Health Sciences, Knoxville, TN. The animals were maintained on standard lab food and acidified water *ad libitum*. These animals were used between the ages of 5 and 7 weeks as the source of femoral bone marrow.

Lipopolysaccharide (LPS)

Escherichia coli K235 LPS, prepared by a water-phenol extraction procedure described by McIntire *et al.* (48), was used in all experiments. This LPS was free of protein and was available as a standard laboratory preparation.

Anti-Interferon (IFN)

Rabbit anti-mouse IFN (α + β) antiserum was obtained from Enzo Biochem Inc., New York, NY. This antiserum was heat inactivated and reported to have neutralizing titres of 1:28,000 against mouse IFNα and 1:10,000 against mouse IFNβ. The antiserum had no neutralizing activity for CSF-1, nor did it possess colony-stimulating activity (55). The antisera was produced via repeated immunization of rabbits with Erlich Ascites tumor cell interferon (specific activity, 5×10^7

U/mg of protein), and neutralized at least 300 U/ml of IFN (α + β) at 1:1,000, the final dilution employed in all assays.

Indomethacin

Indomethacin (Sigma, St. Louis, MO) was prepared by rapidly dissolving 5 to 10 mg in 1.0 ml of 0.1 M sodium carbonate solution. Within thirty seconds, tissue culture medium was added preventing alkaline hydrolysis and diluting the indomethacin to a stock concentration of 1 mcg/ml. This preparation was filtered through a 0.2 micrometer filter (Gelman, Ann Arbor, MI). Indomethacin was used at a final concentration of 0.1 mcg/ml (0.3 mcM) in all experiments.

Colony-Stimulating Factor (CSF)

CSF-1 (hereafter referred to as CSF) was purified from serum-free culture medium conditioned by murine L929 cells as described by Waheed and Shadduck (94). L929 cells were suspended at 1 x 10^5 cells/ml in Dulbecco's Modified Eagle Medium (M.A. Bioproducts, Walkersville, MD) containing 5.6 mM sodium bicarbonate (Fisher, Fairlawn, NJ), 200 mM L-glutamine (GIBCO, Grand Island, NY) and antibiotics (100 U/ml penicillin and 100 mcg/ml streptomycin, GIBCO, Grand Island, NY). All media used in these experiments were filtered through 0.2 micrometer filters (Nalgene, Sybron Corp., Rochester, NY). The cell suspension was added to 150 cm^2 tissue culture flasks (Corning, Corning, NY) at 50 ml/flask and incubated in a humidified CO_2 incubator for 5 days. Supernatants were drawn from these cultures, centrifuged in a Sorvall RC2-B centrifuge (Sorvall, Norwalk, CT) at 10,000 x g relative

centrifugal force to remove cells and debris and the resulting supernatant medium stored frozen at -40°C as L-cell conditioned medium (LCM).

Ten liter starting pools of LCM were concentrated sequentially through a 10,000 MW exclusion laminar flow apparatus (Millipore Pellicon Cassette System, Millipore, Bedford, MA) and with Spectra/Por Stirred Cells (Spectrum Medical, New York, NY) equipped with 10,000 MW exclusion filters (Amicon, Lexington, MA) to approximately 50 ml. The concentrated supernatant was mixed with an equal volume of 0.01 M $NaPO_4$ buffer, pH 7.5. Ethanol was added at 5°C to 40%, and the precipitate forming after 1 hr was pelleted by centrifugation at 10,000 x g and discarded. A 50% ethanol concentration was then achieved, and the precipitate again discarded. The resulting supernatant was washed by continuous ultrafiltration with eight equal volumes of the phosphate buffer and concentrated with the 30,000 MW exclusion filter (Amicon) in a Spectra-Por Stirred Cell to approximately 10 ml.

Ion-exchange chromatography was performed with a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column (1.0 cm x 50 cm). The buffer used was 0.01 M $NaPO_4$, pH 7.5, containing 0.05% polyethylene glycol (PEG) 3350 mw (Sigma, St. Louis, MO). Eluting buffers were 0.1 M, 0.18 M, and 0.3 M NaCl in the basic phosphate buffer at pH 7.5. The flow rate was 10 ml per hour and 4 ml fractions were collected on an ISCO fraction collector, model 272 (Instrumentation Specialties, Lincoln, NE). This, and all other columns were run at 4°C. CSF eluted from this column at the 0.18 M NaCl buffer step, and those fractions were pooled and concentrated. CSF activity in column fractions was

monitored via bioassay and protein determination (described below). The concentrate was dialyzed in standard cellulose dialysis tubing of 12,000 to 14,000 MW exclusion (Spectropor, Fisher, Pittsburgh, PA) against 0.02 M $NaPO_4$ with 0.15 M NaCl and 0.05% PEG, the starting buffer for the next step involving chromatography on a Concanavalin A-Sepharose 4B column (0.7 cm x 20 cm) (Sigma, St. Louis, MO). After the start buffer, buffer containing 0.1 M 1-O-methyl-alpha-D-gluco-pyranoside (Sigma, St. Louis, MO) was added which elutes CSF from the column. The column flow rate was 12 ml per hour, and 4 ml fractions were collected. CSF containing fractions were pooled, concentrated and dialyzed against PBS (phosphate buffered saline - 0.02 M $NaPO_4$ pH 7.2 with 0.15 M NaCl) containing 0.05% PEG. Gel filtration was then performed using a Bio-Gel P-300 column (2 cm x 100 cm) (BIO-RAD, Richmond, CA) having a 60,000 to 400,000 MW fractionation range. The elution buffer (PBS with 0.05% PEG) flow rate was 10 ml per hour and 4 ml fractions were collected. CSF-containing fractions eluted between the markers aldolase (158,000 mw, Sigma, St. Louis, MO) and bovine serum albumin (67,500 mw, Sigma, St. Louis, MO).

Protein determinations were performed spectrophotometrically on a Beckman DU-2 spectrophotometer (Beckman Instruments, Fullerton, CA) using the formula: mg protein/ml = 1.55 (OD_{280}) - 0.76 (OD_{260}). Final units of CSF activity were determined via serial dilution in soft agar assay (described below), where one unit is that amount calculated to yield one colony. From these determinations, the specific activity of the final CSF preparation was calculated to be

more than 1 x 10^7 U/mg protein. The CSF preparation contained less than 100 pg of LPS per 50,000 U CSF as determined by enhancement of superoxide anion production by macrophages stimulated with phorbol myristate acetate (66).

Assays for CSF

Two different assays were used to determine proliferative responses to CSF. One was the classical assay where colony formation in semisolid agar is determined (55). The second was an assay based on incorporation of [^{3}H] thymidine (^{3}H-TdR) by CSF-stimulated marrow preparations as described by Moore and Rouse (55).

Bone marrow cells were prepared from the femurs of 5 to 7 week old female mice. The animals were killed by cervical dislocation, and the femurs were removed under aseptic conditions and placed in cold RPMI 1640 medium (GIBCO, Grand Island, NY) with 5.6 mM sodium bicarbonate (Fisher, Fairlawn, NJ), 200 mM L-glutamine (GIBCO, Grand Island, NY) and antibiotics (100 U/ml penicillin and 100 mcg/ml streptomycin, GIBCO, Grand Island, NY). The bone marrow was released following gentle grinding using a mortar and pestle. After dispersal, the supernatant cell suspension was vortexed for 20 seconds, and large particles were allowed to settle. The supernatant cell suspension was removed, and the cells were centrifuged at 250 x g (IEG HN-5II Centrifuge, Damon, Needham Heights, MA) and resuspended in RPMI 1640 medium. The viability of bone marrow cells prepared by this method exceeded 90% as determined by exclusion of trypan blue dye. In some experiments, this total nucleated cell population was fractionated over

a mouse density centrifugation medium (Lympholyte M, Cedarlane Laboratories, Ontario), selecting for low density nucleated cells. In other experiments, total nucleated cell population suspensions in RPMI 1640 at 1 x 10^7 cells/ml were allowed to adhere to tissue culture flasks (Corning, Corning, NY) for three hours with subsequent removal of nonadherent cells for assay.

In the semi-solid agar assay, either 1 or 2 x 10^5 marrow cells were suspended in warm RPMI 1640 containing sodium bicarbonate, glutamine, antibiotics, and 15% fetal bovine serum (FBS) (Hyclone, Sterile Systems, Logan, UT) and 0.3% agar (Bacto-Agar, Difco, Detroit, MI). In some experiments, a 1:1000 final dilution of the rabbit anti-mouse IFN (α + β) was incorporated into the suspending medium. In one experiment eight separate lots of FBS (4A040, 3G079, 4B083, 4B028, 4C049, 4B069, 3H032, and 4C056, MA Bioproducts, Walkersville, MD) varying in endotoxin content were employed rather than Hyclone FBS. One milliliter of the above mixture was added to a 35 mm petri dish (Corning, Corning, NY) containing up to 0.25 ml of CSF, LPS, and/or indomethacin at 0.1 mcg/ml (0.3 mcmol). After cooling, cultures were incubated at 37°C in 5° CO_2 for 6 or 7 days. The dishes were inspected with an inverted microscope at 35x magnification, and proliferative centers ($\geq$25 cells) were scored.

In the ^{3}H-TdR assay, 0.5 to 2 x 10^5 marrow cells were incubated in a total volume of 0.2 ml in individual wells of 96-well flat-bottomed plates (Corning, Corning, NY) containing CSF, LPS, 1:1000 anti-IFN, and 0.1 mcg/ml (0.3 mcM) indomethacin. The medium used and incubation periods were the same as those employed in the

cloning assay, with the deletion of the semisolid agar. The cultures were incubated for a total of 72 hours with the addition of 1 mcCi of ^{3}H-TdR (specific activity of 2 Ci/mmol, ICN, Irvine, CA) during the last 6 hours. The cultures were harvested onto glass fiber filters (Skatron, Sterling, VA) with a cell harvester (Titertek, Flow Laboratories, Rockville, MD) and ^{3}H-TdR incorporation was determined by liquid scintillation spectrometry (Scinti Verse E, Fisher, Fairlawn, NJ) on a Beckman LS7000 Liquid Scintillation Counter (Beckman, Fullerton, CA).

Statistical Analysis

All experiments were performed in triplicate. All results are expressed as the mean value $\pm$ standard error measurement (SEM = Standard deviation/$\sqrt{N}$). The significance of differences between means was determined by Student's *t*-test. Linear regression analysis was performed using a Texas Instrument TI-55 Calculator.

CHAPTER IV

RESULTS

Comparison of Thymidine Incorporation and Soft Agar Colony Formation by Bone Marrow Cells Stimulated with CSF

Correlation of the two assays used in this investigation for CSF-induced proliferation of bone marrow cells is presented in Figure 1. ^{3}H-TdR incorporation and soft agar colony-stimulating assays were performed with marrow cells from the same preparation. Eight doses of CSF doubling from 30 to 3840 U/ml were used. ^{3}H-TdR incorporation stimulated by highly purified CSF was as sensitive for measuring CSF responsiveness as was the classical colony-forming assay. Using linear regression analysis, a correlation co-efficient of 0.98 was generated by comparing the two assays indicating significance $p \leq 0.01$. The ^{3}H-TdR incorporation assay was developed for use in these assays by Moore and Rouse (55) for several reasons. Most importantly, cultures could be established and treated with a variety of agents, including LPS, anti-IFN (α + β), and indomethacin, without manipulating the cells within the established culture. Also, the data generated via this assay are not subject to possible bias as are data compiled via the subjective visual determination of colony formation in soft agar. Finally, the ^{3}H-TdR assay can be performed in 72 hours, whereas colony formation requires 6 to 7 days. The soft agar cloning assay, however, was deemed preferable in later assays as this assay may detect events that occur later than 3 days in culture. Also, the colony-formation assay detects a proliferation aspect undetectable in

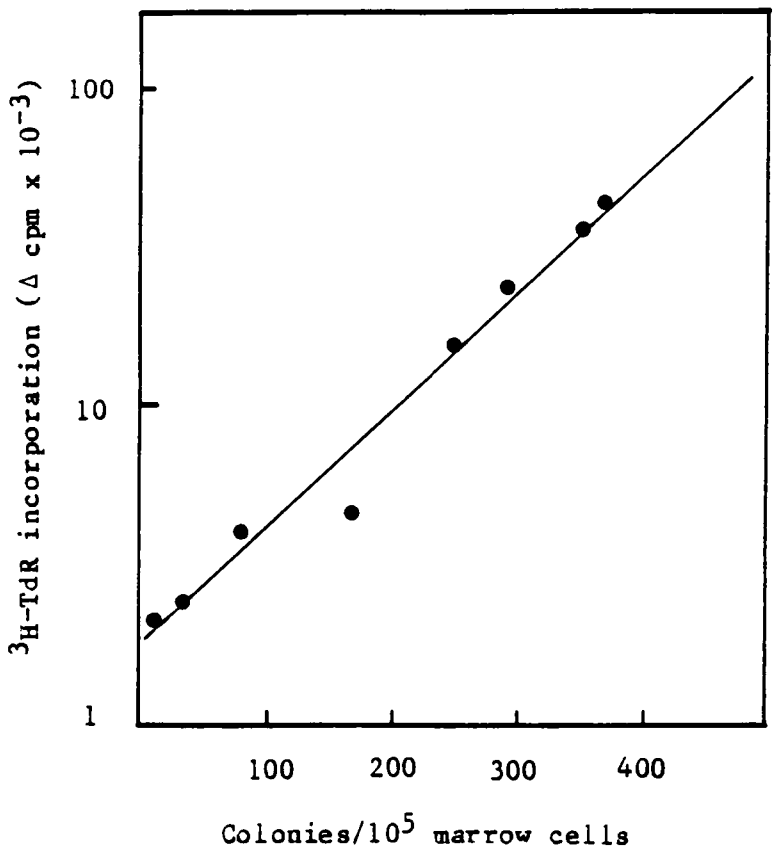

Figure 1. Comparison of ^{3}H-TdR incorporation and colony formation by bone marrow cells stimulated with CSF.

Both assays were performed with cells from the same marrow preparation. Low density nucleated Ha/ICR marrow cells (1 x 10^5) were incubated with 8 doses of CSF doubling from 30 to 3840 U/ml. ^{3}H-TdR incorporation was measured by thymidine pulse during the last four hours of 72 hr incubation of 1 x 10^5 cells/well in 96 well plates. Corresponding colony formation was measured in one-milliliter semisolid agar cultures containing 1 x 10^5 cells/ml after 7 days incubation. Linear regression analysis indicated significance at $p \leq 0.01$.

^{3}H-TdR incorporation assays, the enumeration of individual CFUc (committed colony forming units, CSF responsive marrow precursor cells). The enumeration of clonal proliferative centers [>25 cells (55)] is actually a measurement of CSF-responsive CFUc in the marrow preparations after experimental treatment.

LPS Effect on CSF-Induced ^{3}H-TdR Incorporation and Colony Formation by Marrow Cells in the Presence or Absence of Anti-IFN ($\alpha + \beta$) Antiserum and Indomethacin

Moore, et al. (56) has shown that LPS at low concentrations enhances colony formation by peritoneal exudate macrophages stimulated with sub-optimal doses of CSF. Therefore, a similar LPS dose response was performed with femoral bone marrow cells stimulated with LPS in the presence of a suboptimal dose of 960 U CSF/ml (2000 U/ml had been predetermined to be optimal). The results are presented in Figure 2. Both ^{3}H-TdR and colony-stimulating assays were performed with marrow cells from the same preparation. Both CSF-induced ^{3}H-TdR incorporation and soft agar colony formation were significantly enhanced ($p \leq 0.01$) by the presence of 10^{-3} to 10^{-5} mcg of LPS/ml, with peak enhancement seen at 10^{-4} mcg LPS/ml. LPS at 1 mcg/ml actually suppressed ($p \leq 0.01$) colony formation by the marrow cells. LPS by itself did not stimulate either ^{3}H-TdR incorporation or colony formation and these values are not shown in the figure.

Comparison of the two assays in measuring LPS effects on CSF-induced marrow cell proliferation is presented in Figure 3. A suboptimal dose of 960 U of CSF/ml was used (2000 U/ml had been predetermined to be optimal) with eight doses of LPS ranging from

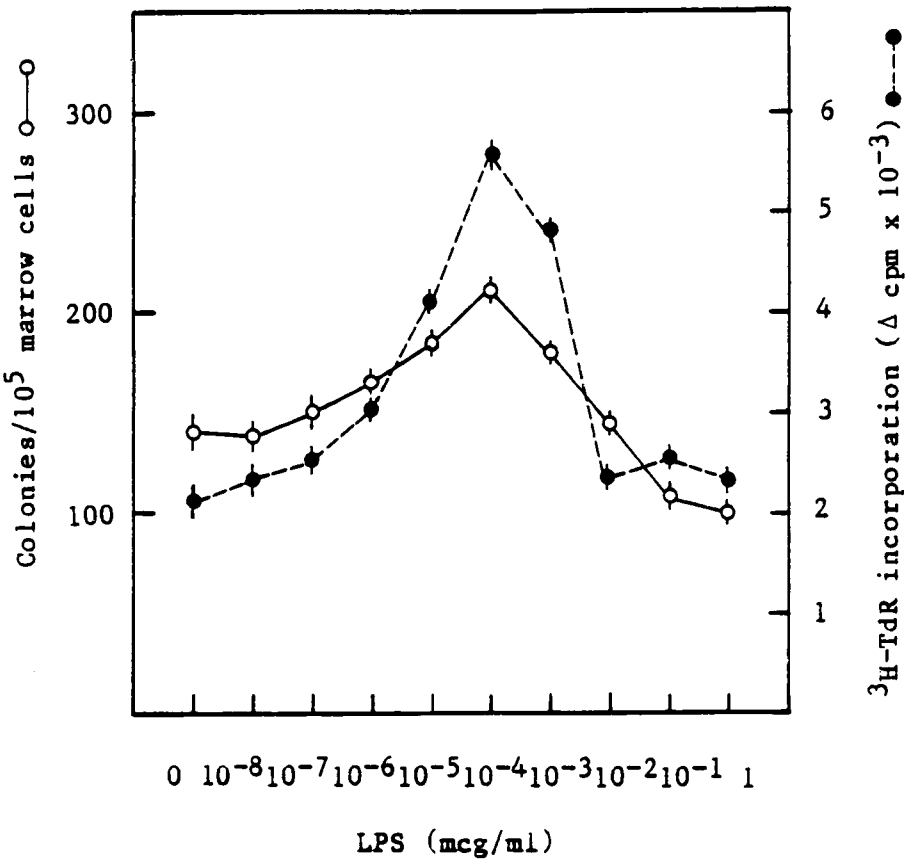

Figure 2. Dose dependency of LPS effect on CSF-induced ^{3}H-TdR incorporation and colony formation by C3H/AnF cells.

Both assays were performed with cells from the same marrow preparation. Low density nucleated C3H/AnF marrow cells (1 x 10^5) were incubated with 960 U CSF/ml and eight doses of LPS ranging from 1 to 10^{-8} mcg E. coli K235 LPS/ml. ^{3}H-TdR incorporation, ●, was measured by thymidine pulse during the last four hours of 72 hr incubation of 1 x 10^5 cells/well in 96 well plates. Corresponding colony formation, O, was measured in one-milliliter semisolid agar cultures containing 1 x 10^5 cells/ml after 7 days incubation. Values represent the mean ± 1 standard error of triplicate determinations.

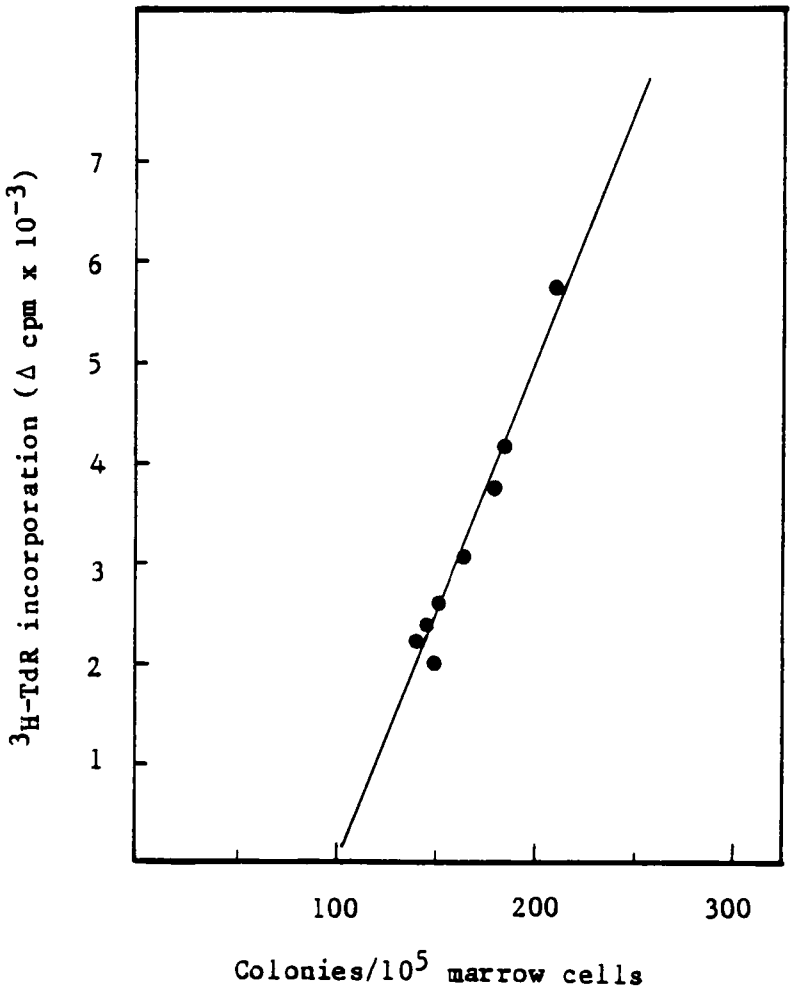

Figure 3. Comparison of ^{3}H-TdR incorporation and colony formation by bone marrow cells stimulated with CSF and LPS.

Both assays were performed with cells from the same marrow preparation. Low density nucleated C3H/AnF marrow cells (1×10^5) were incubated with 960 U CSF/ml and eight doses of LPS ranging from 10^{-2} to 10^{-10} mcg E. coli K235 LPS/ml. ^{3}H-TdR incorporation was measured by thymidine pulse during the last four hours of 72 hr incubation of 1×10^5 cells/well in 96 well plates. Corresponding colony formation was measured in one-milliliter semisolid agar cultures containing 1×10^5 cells/ml after 7 days incubation. Linear regression analysis indicated significance at $p \leq 0.01$.

10^{-2} to 10^{-10} mcg E. coli K235 U/ml. Once again, ^{3}H-TdR incorporation and colony-stimulating assays were performed with marrow cells from the same preparation. LPS by itself did not cause either ^{3}H-TdR incorporation or clone formation. ^{3}H-TdR incorporation due to B cell mitogenesis requires up to 100 mcg LPS/ml (21). Using linear regression analysis, a correlation co-efficient of 0.98 was generated by comparing the two assays indicating significance at $p \leq 0.01$.

Kurland, et al. (40) proposed that CSF-induced prostaglandin-E (PGE) synthesis acted as a negative-feedback mechanism to inhibit CSF-induced proliferation. Interferons, like PGE, are also potent inhibitors of CSF-induced stem cell proliferation (4). Using anti-IFN (α + β) antiserum to inhibit the action of IFN (α + β) and indomethacin, an inhibitor of the cyclo-oxygenase pathway responsible for PGE synthesis, this laboratory (54) recently has shown that IFN (α + β) and PGE act independently as feedback regulators of CSF-induced proliferation. In addition, LPS stimulates production of both PGE and IFN α/β by macrophages (4, 40). Therefore, assays were performed to determine if these endogenously produced feedback regulators might act to suppress further LPS enhancement of CSF-induced proliferation. ^{3}H-TdR incorporation assays were performed using 0.25, 0.5, 1, and 2 x 10^{5} marrow cells/ml culture. At lower cell doses, 0.25 and 0.5 x 10^{5} cells/ml, the data was generally unreliable with large standard error measurements (data not shown). Data from cultures containing 1 x 10^{5} and 2 x 10^{5} cells/ml are presented in Figures 4 and 5 respectively. The results from both cultures were virtually identical with an overall enhancement of ^{3}H-TdR incorporation resulting from

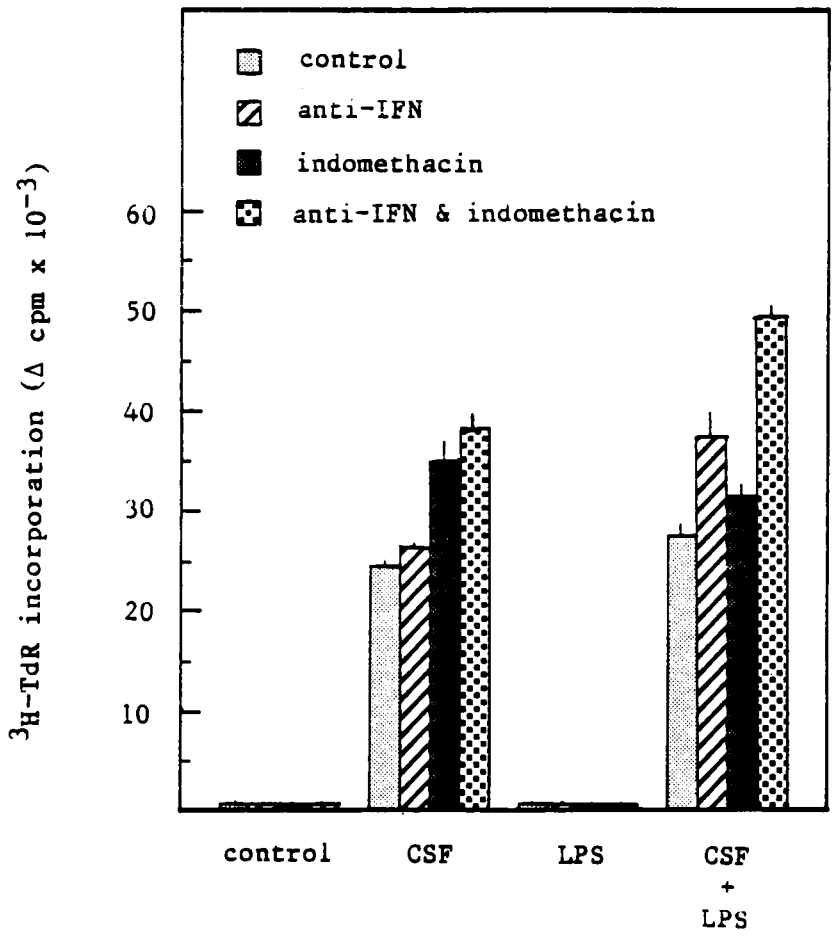

Figure 4. Effect of anti-IFN (α + β) antiserum and indomethacin on CSF-induced proliferation of 1 x 10^5 marrow cells in the presence or absence of LPS.

Low density nucleated Ha/ICR bone marrow cells (1 x 10^5/well) were incubated with or without 960 U CSF/ml in the presence or absence of 10^{-4} mcg/ml E. coli K235 LPS. Control cultures represented by light shaded bars (▨) did not receive either anti-IFN (α +β) antiserum or indomethacin. Cultures represented by diagonally striped bars (▨) received a 1:1000 dilution of anti-IFN (α +β) whereas cultures receiving 0.3 mcM indomethacin are shown as dark shaded bars (■). Results obtained with cultures that received both anti-IFN (α + β) and indomethacin are represented as stippled bars (▩). Incorporation of ^{3}H-TdR was measured during the last 4 hr of the 72 hours incubation period. Values represent the mean ± 1 standard error of triplicate determinations.

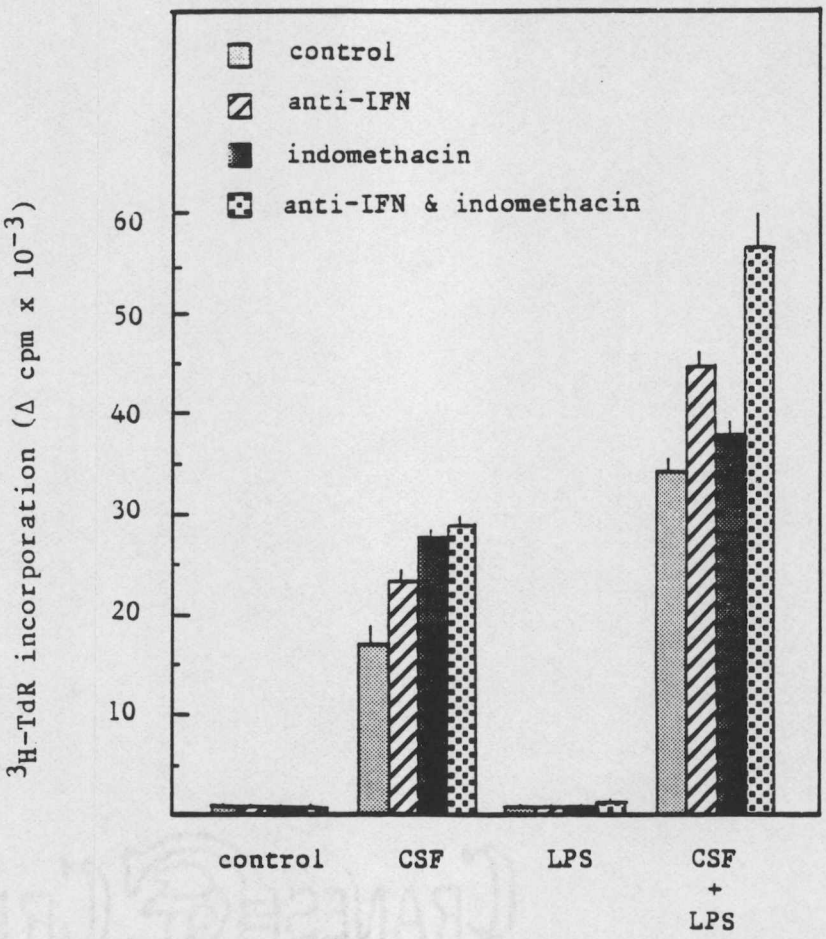

Figure 5. Effect of anti-IFN (α + β) antiserum and indomethacin on CSF-induced proliferation of 2 x 10^5 marrow cells in the presence or absence of LPS.

Low density nucleated Ha/ICR bone marrow cells (2 x 10^5/well) were incubated with or without 960 U CSF/ml in the presence or absence of 10^{-4} mcg/ml E. coli K235 LPS. Control cultures represented by light shaded bars (▩) did not receive either anti-IFN (α +β) antiserum or indomethacin. Cultures represented by diagonally striped bars (▨) received a 1:1000 dilution of anti-IFN (α + β) whereas cultures receiving 0.3 mcM indomethacin are shown as dark shaded bars (■). Results obtained with cultures that received both anti-IFN (α + β) and indomethacin are represented as stippled bars (⊡). Incorporation of ^{3}H-TdR was measured after 72 hours incubation of cells in 96 well plates. Values represent the mean ± 1 standard error of triplicate determinations.

the addition of both antagonists with or without additional LPS. In Figure 5, cell cultures which received both anti-IFN (α + β) antiserum and indomethacin in addition to 960 CSF/ml showed a 44% increase in ^{3}H-TdR incorporation over cultures receiving CSF alone ($p \leq 0.01$). Cultures receiving CSF and 10^{-4} mcg LPS/ml showed a 50% increase in ^{3}H-TdR incorporation over those receiving CSF alone ($p \leq 0.01$), and, importantly, in cultures containing both anti-IFN (α + β) and indomethacin, in addition to CSF the addition of 10^{-4} mcg LPS/ml enhanced thymidine incorporation 50% over those containing no LPS ($p \leq 0.01$). Cultures containing anti-IFN (α + β), indomethacin, and/or LPS showed no ^{3}H-TdR incorporation, indicating enhancement was not due to elevated endogenous CSF levels. Thus, while significantly enhancing the CSF response under all conditions, the two antagonists did not dramatically enhance the LPS effect.

More dramatic effects of LPS on CSF-induced proliferation were observed in soft-agar cloning assays. Moore, et al. (56) had shown that LPS enhances the clonal response of peritoneal exudate cells to suboptimal doses of CSF causing a shift in the dose response curve; however, LPS did not enhance colony formation beyond the maximum number of CSF-responsive cells in the peritoneal exudate population. Thus, a CSF dose response for marrow progenitors exposed to LPS was performed, both in the presence and absence of anti-IFN (α + β) and indomethacin. As seen in Figure 6, at each CSF dose ranging from 100 to 2560 U CSF/ml, the addition of 10^{-4} mcg LPS/ml or the addition of both anti-IFN (α + β) and indomethacin caused a significant ($p \leq 0.01$) increase in colony formation. The addition of LPS to cultures

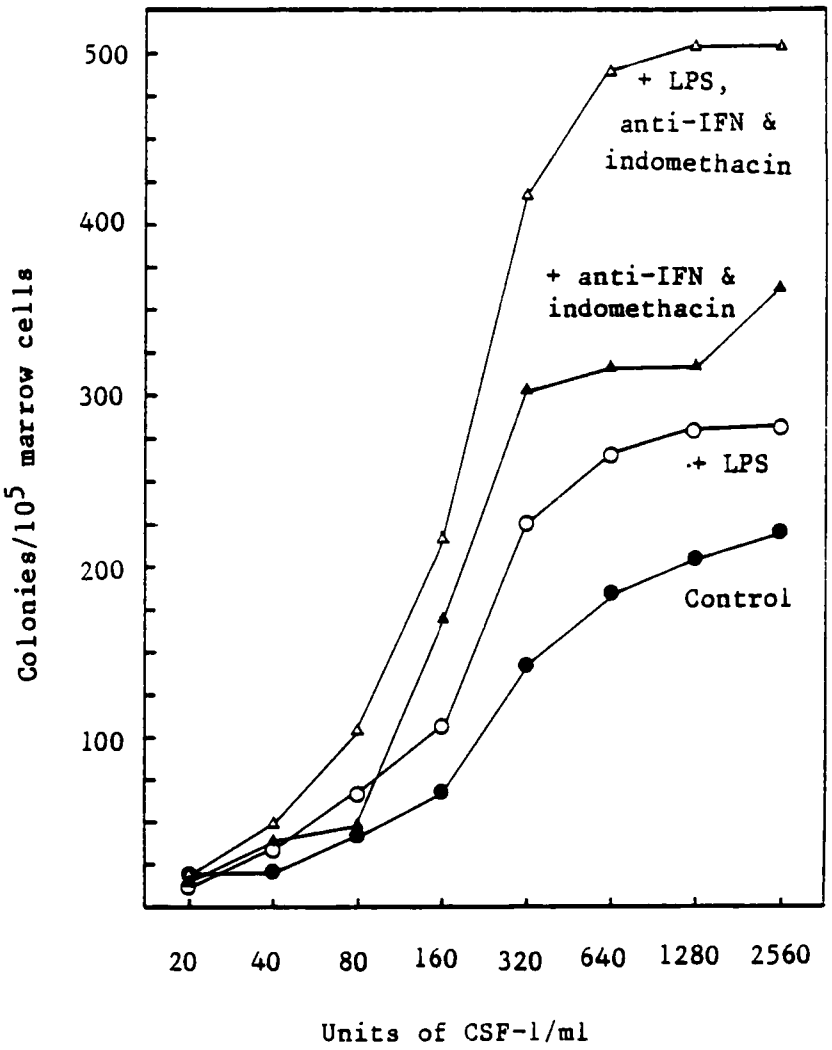

Figure 6. Effect of LPS on CSF responsiveness of C3H/AnF marrow cells determined by colony formation in the presence or absence of anti-IFN ($\alpha + \beta$) antiserum and indomethacin.

One-milliliter semisolid agar cultures containing 1 x 10^5 low density nucleated C3H/AnF marrow cells were incubated for 7 days with 960 U of CSF/ml with or without 10^{-4} mcg E. coli K235 LPS/ml in the presence or absence of both a 1:1000 dilution of anti-IFN ($\alpha + \beta$) and 0.3 mcM indomethacin. ●, CSF only; ○, CSF + LPS. ▲, CSF + anti-IFN ($\alpha + \beta$) and indomethacin; Δ, CSF + LPS + anti-IFN ($\alpha + \beta$) and indomethacin. Values are the mean ± standard error of triplicate determinations. No colonies were observed in any group without addition of CSF.

containing anti-IFN (α + β) and indomethacin enhanced colony formation 30% or more using 320 to 2560 U CSF/ml ($p \leq 0.01$). Thus, it appears that only two-thirds of the cells in the marrow capable of responding to CSF will do so while one-third requires stimulation by LPS before responding to CSF. In contrast to earlier work with peritoneal exudate cells (56), colony formation was enhanced by LPS at optimal CSF doses.

LPS-Enhanced Responsiveness of Marrow Cells to CSF is Associated with Lipid A

Cells from C3H/HeJ mice are unresponsive or poorly responsive to a variety of effects elicited by LPS and the lipid A portion of LPS (10, 19, 25). Assays using LPS-hyporesponsive C3H/HeJ mice along with normal LPS-responsive C3H/AnF mice were performed to determine if lipid A, the biologically active portion of LPS (16, 25, 44), was responsible for the enhancement of CSF-responsiveness of marrow cells seen in earlier experiments with LPS. In this study, the bone marrow precursor cells of C3H/HeJ failed to respond to the stimulatory effect of LPS at concentrations which are stimulatory for the LPS-responsive C3H/AnF. Lipopolysaccharide at 10^{-4} mcg/ml significantly enhanced ($p \leq 0.01$) CSF-induced ^{3}H-TdR incorporation by C3H/AnF marrow cells both in the presence and absence of anti-IFN (α + β) and indomethacin (Figure 7). The same LPS dose failed to elicit any response in the C3H/HeJ marrow cells. Similar results were observed in soft-agar cloning assays (Figure 8). While anti-IFN (α + β) and indomethacin significantly enhanced colony formation by C3H/AnF cells and, to a lesser degree, C3H/HeJ cells at each LPS dose, the LPS effect was only seen in the

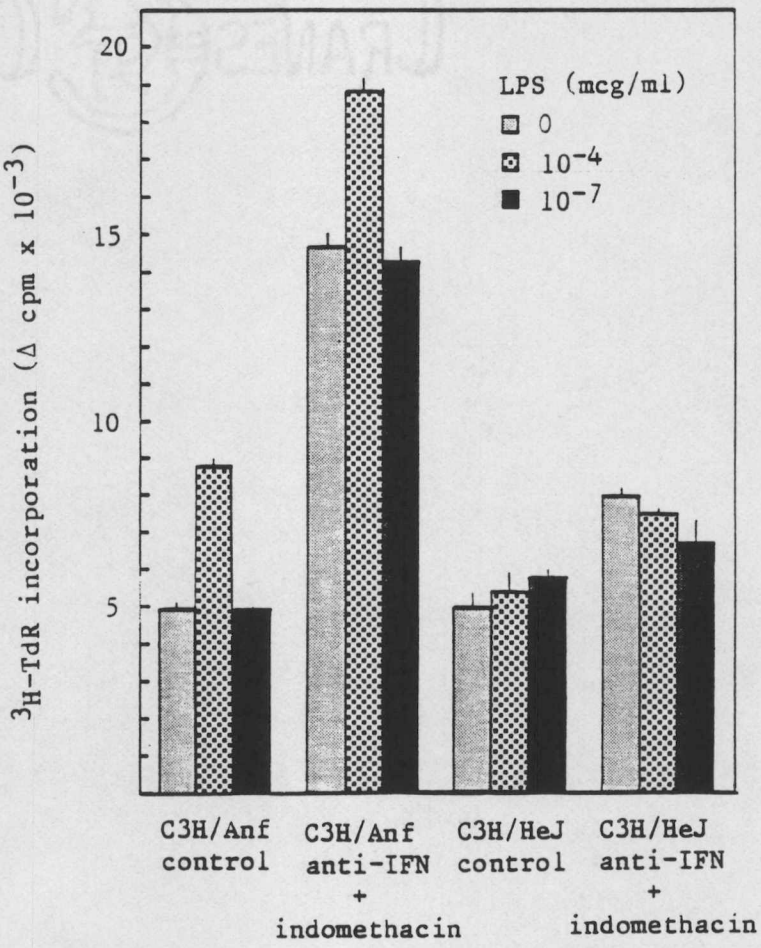

Figure 7. Effect of LPS on CSF-induced proliferation by C3H/AnF and C3H/HeJ marrow cells in the presence or absence of anti-IFN ($\alpha + \beta$) antiserum and indomethacin.

Low density nucleated C3H/AnF or C3H/HeJ marrow cells (1 x 10^5/well) were incubated with 960 U of CSF/ml in the presence or absence of both a 1:1000 dilution of anti-IFN ($\alpha + \beta$) and 0.3 mcM indomethacin. Control cultures represented by light shaded bars (▨) received no LPS. Cultures receiving 10^{-4} mcg E. coli K235 LPS/ml are shown as stippled bars (⊡), whereas those receiving 10^{-7} mcg LPS/ml are represented by dark shaded bars (■). ^{3}H-TdR incorporation was measured after 72 hours incubation of cells in 96 well plates. Values represent the mean ± 1 standard error of triplicate determinations.

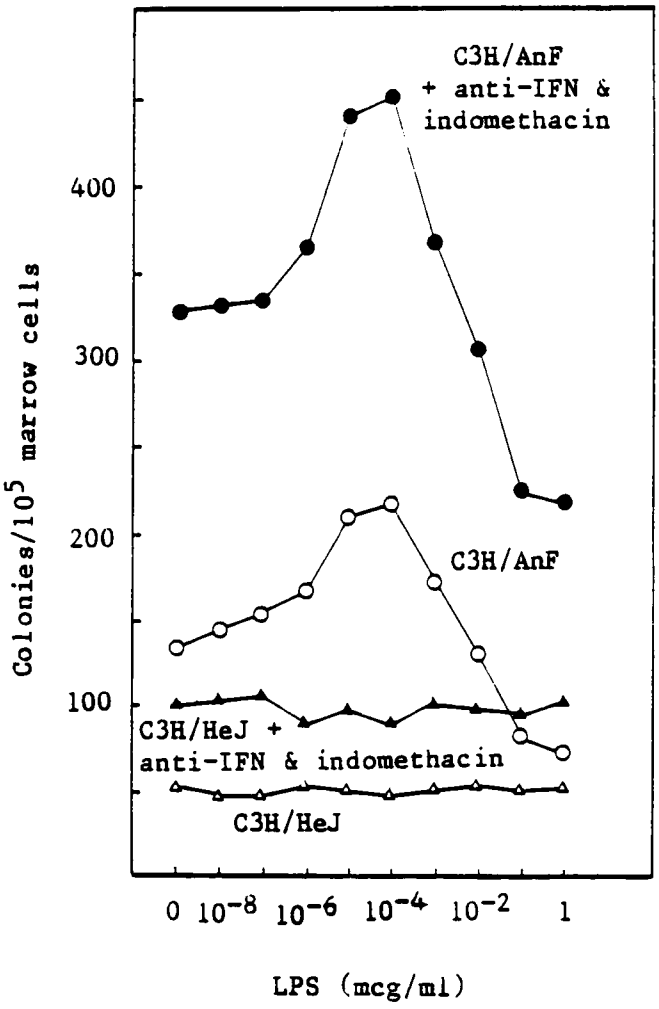

Figure 8. Dose dependency of the LPS effect on CSF-induced colony formation by C3H/AnF and C3H/HeJ marrow cells in the presence or absence of anti-IFN ($\alpha + \beta$) antiserum and indomethacin.

One-milliliter semisolid agar cultures containing 1 x 10^5 low density nucleated C3H/AnF or C3H/HeJ marrow cells were incubated for 7 days with dilutions of E. coli K235 LPS and 960 U of CSF/ml in the presence or absence of both a 1:1000 dilution of anti-IFN ($\alpha + \beta$) and 0.3 mcM indomethacin. ○, C3H/AnF cultures; ●, C3H/AnF cultures containing anti-IFN ($\alpha + \beta$) and indomethacin. △, C3H/HeJ cultures; ▲, C3H/HeJ cultures containing anti-IFN ($\alpha + \beta$) and indomethacin. Values are the mean ± 1 standard error of triplicate determinations. No colonies were observed in any group without addition of CSF.

C3H/AnF, cells and not the LPS-nonresponsive C3H/HeJ cells. In the C3H/AnF cultures, 10^{-6} to 10^{-3} mcg LPS/ml significantly enhanced ($p \leq 0.01$) colony formation, while 10^{-1} to 1 mcg LPS/ml significantly suppressed ($p \leq 0.01$) colony formation both in the presence and absence of anti-IFN (α + β) and indomethacin. It is significant to note that the LPS dose response curve for the CSF-responsive C3H/AnF cultures receiving anti-IFN (α + β) and indomethacin, although augmented, parallels that of cultures that did not receive the two antagonists.

LPS Acts Directly on CSF-Stimulated Bone Marrow Cells

Experiments were performed to determine if mature mononuclear phagocytes in the marrow population were indirectly affecting the progenitor response. Unlike stem cells the more mature cells are adherent to plastic surfaces (29). Experiments to test this hypothesis were performed by exposing both CSF-stimulated total and nonadherent marrow cells to LPS. In Figure 9, CSF-stimulated total C3H/AnF marrow cells and 3 hour plastic-nonadherent C3H/AnF cells were exposed to 10^{-4} mcg LPS/ml in the presence and absence of anti-IFN (α + β) and indomethacin. The removal of adherent cells from the marrow preparations resulted in colony formation equal to that seen in cultures containing anti-IFN (α + β) and indomethacin, confirming prior observation (54) that the adherent cells are the source of IFN (α + β) and PGE. Adherent cell removal, however, did not prevent a significant ($p \leq 0.01$) LPS-mediated enhancement of colony formation, suggesting that LPS indeed acts directly on the marrow cells, and not indirectly

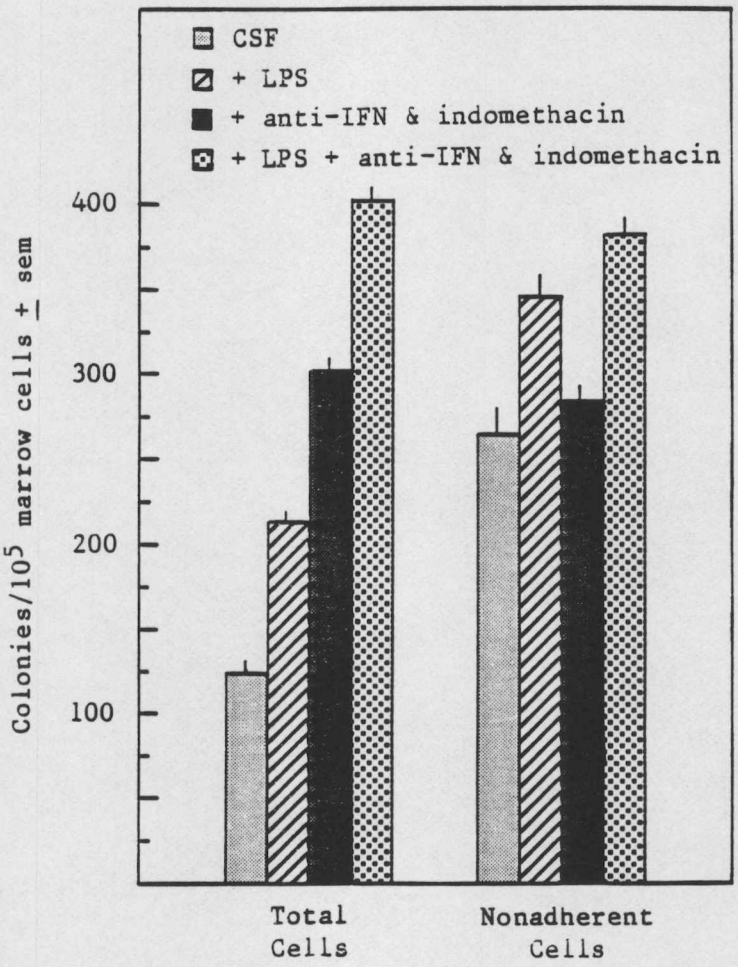

Figure 9. Effect of LPS on the CSF responsiveness of total and nonadherent C3H/AnF marrow cells determined by colony formation in the presence or absence of anti-IFN ($\alpha + \beta$) antiserum and indomethacin.

One-milliliter semisolid agar cultures containing 1×10^5 total nucleated C3H/AnF marrow cells or 1×10^5 nonadherent nucleated C3H/AnF marrow cells harvested after 3 hr incubation of 10^7 total cells/ml were incubated for 7 days with 960 U of CSF/ml with or without 10^{-4} mcg *E. coli* K235 LPS/ml in the presence or absence of both a 1:1000 final dilution of anti-IFN ($\alpha + \beta$) and 0.3 mcM indomethacin. Control cultures containing only CSF are indicated by light-shaded bars (▩). Cultures containing LPS are shown as diagonally striped bars (▨), whereas cultures with anti-IFN ($\alpha + \beta$) and indomethacin are represented by dark shaded bars (■). Cultures receiving LPS, anti-IFN ($\alpha + \beta$), and indomethacin are shown as stippled bars (⊡). Values are the mean ± 1 standard error of triplicate determinations. No colonies were observed in any group without addition of CSF.

via secondary stimulatory products produced by more mature LPS-stimulated adherent cells within the marrow. Most importantly, in both total and nonadherent cultures in the presence or absence of both anti-IFN (α + β) and indomethacin, the addition of 10^{-4} mcg LPS/ml caused a similar enhancement in the number of CSF-responsive marrow cells (all increases in colony formation due to LPS were approximately equivalent [not significantly different at $p \leq 0.05$]). No colonies were observed in any group without the addition of CSF. Based on these results, the LPS appeared to act directly on monocytic bone marrow precursors, stimulating them to become CSF-responsive. A duplicate experiment using LPS-nonresponsive C3H/HeJ marrow cells is presented in Figure 10. Once again removal of adherent cells from the marrow preparation resulted in significantly greater ($p \leq 0.01$) colony formation equal to that seen in cultures containing anti-IFN (α + β) and indomethacin. LPS addition had no effect on colony formation in the C3H/HeJ marrow cultures.

A final experiment was performed to determine if secondary soluble mediators produced by LPS-stimulated marrow cells may enhance CSF-responsiveness of progenitor cells. This was accomplished by incubating 10^5 nonadherent C3H/AnF cells with 10^5 nonadherent C3H/HeJ cells. Results presented in Figure 9 had shown that cells in the adherent population of the marrow were not responsible for the LPS-mediated enhancement of colony formation. The possibility existed however that cells within the nonadherent population could be producing a soluble enhancer of CSF-responsiveness after LPS stimulation. If such a hypothesis were correct, the LPS-responsive C3H/AnF cells would

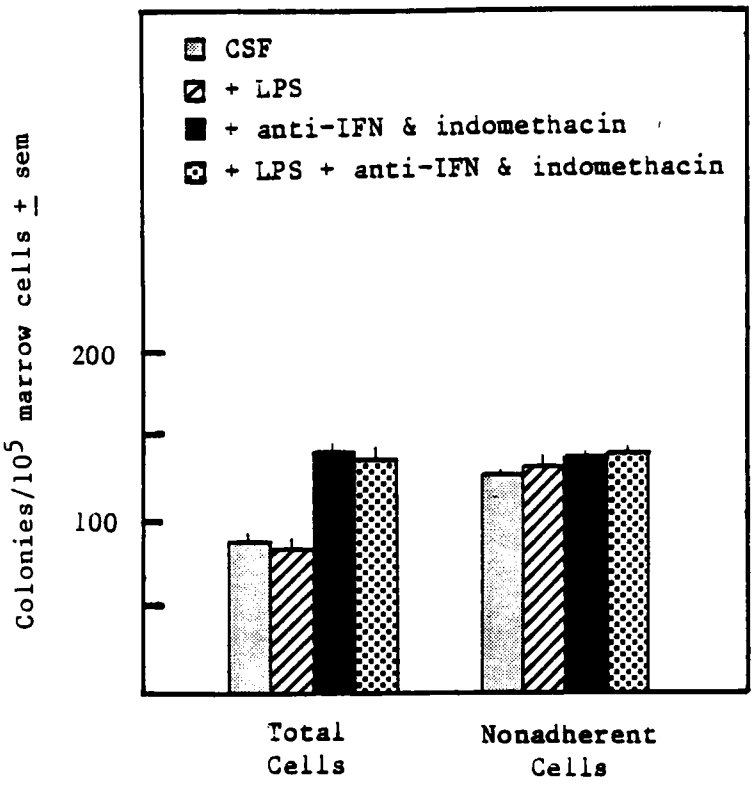

Figure 10. Effect of LPS on the CSF responsiveness of total and nonadherent C3H/HeJ marrow cells determined by colony formation in the presence or absence of anti-IFN ($\alpha + \beta$) antiserum and indomethacin.

One-milliliter semisolid agar cultures containing 1 x 10^5 total nucleated C3H/HeJ marrow cells or 1 x 10^5 nonadherent nucleated C3H/HeJ marrow cells harvested after 3 hr incubation of 10^7 total cells/ml were incubated for 7 days with 960 U of CSF/ml with or without 10^{-4} mcg E. coli K235 LPS/ml in the presence or absence of both a 1:1000 final dilution of anti-IFN ($\alpha + \beta$) and 0.3 mcM indomethacin. Control cultures containing only CSF are indicated by light-shaded bars (▩). Cultures containing LPS are shown as diagonally striped bars (▨), whereas cultures with anti-IFN ($\alpha + \beta$) and indomethacin are represented by dark shaded bars (■). Cultures receiving LPS, anti-IFN ($\alpha + \beta$), and indomethacin are shown as stippled bars (▣). Values are the mean ± 1 standard error of triplicate determinations. No colonies were observed in any group without addition of CSF.

produce enhancing soluble factor(s) which would then be available to the LPS-nonresponsive C3H/HeJ cells, and colony formation in the mixture of cell populations would be significantly more than additive with respect to the individual populations incubated separately. As seen in Table 1, this was not the case. As before, 10^{-4} mcg LPS/ml had no effect on C3H/HeJ colony formation, whereas C3H/AnF colony formation was significantly increased ($p \leq 0.01$). Colony formation in mixed cultures however was no more than additive either in the presence or absence of LPS. Thus, LPS indeed appears to act directly on individual marrow cells, and not via secondary soluble mediators of CSF-responsiveness.

Serum Endotoxin Contamination Influences Bone Marrow Response to CSF

Medium employed in assays measuring the proliferative response of bone marrow cells to colony-stimulating factor requires the addition of 10 to 15 percent serum such as fetal bovine serum (FBS) (55). FBS from Hyclone Sterile Systems (Logan, UT) containing less than 10^{-6} mcg endotoxin/ml had been used in all of the preceding experiments. Therefore, six separate batches of FBS from MA Bioproducts (Walkersville, MD) varying in endotoxin content ($<10^{6}$, 6×10^{-5}, 5.3×10^{-4}, 6.6×10^{-4}, 1.4×10^{-3}, and 1×10^{-1}) in addition to Hyclone FBS were examined in soft agar cloning assays to determine the effect of contaminating serum LPS on CSF-induced colony formation. As seen in Table 2, as serum endotoxin concentrations increased, colony formation in the absence of added LPS increased. At endotoxin levels in the culture medium approximately equal to 10^{-4} to 10^{-5} mcg LPS/ml

Table 1. Colony formation by CSF stimulated nonadherent marrow from C3H/AnF and C3H/HeJ mice in the presence of LPS

Treatment group[a]	LPS[b]	Colonies/culture ± SEM[c]
HeJ	-	98 ± 6
AnF	-	299 ± 10
HeJ + AnF	-	351 ± 10
HeJ	+	103 ± 6
AnF	+	418 ± 7
HeJ + AnF	+	506 ± 9

[a] 10^5 nonadherent nucleated marrow cells of each designated strain in culture for 7 days with 960 U of CSF, 1:1000 final dilution of anti-IFN (α + β), and 0.3 mcM indomethacin.

[b] 10^{-4} mcg *E. coli* K235 LPS/ml.

[c] Mean ± 1 standard error of triplicate determinations.

Table 2. Influence of contaminating serum LPS on CSF-induced colony formation

	Colonies/10^5 marrow cells[b]			
			anti-IFN and indomethacin[e]	
Serum batch[a]	CSF[c]	+LPS[d]	CSF[c]	+LPS[d]
$<10^{-6}$	137 ± 5	206 ± 3[f]	252 ± 5	305 ± 6[g]
$<10^{-6}$	140 ± 4	198 ± 5[f]	248 ± 5	298 ± 6[g]
6×10^{-5}	153 ± 5	191 ± 5[f]	260 ± 6	299 ± 7[g]
5.3×10^{-4}	190 ± 7[f]	191 ± 11[f]	289 ± 10[g]	300 ± 6[g]
6.6×10^{-4}	195 ± 6[f]	203 ± 4[f]	281 ± 6[g]	293 ± 4[g]
1.4×10^{-3}	209 ± 5[f]	194 ± 5[f]	280 ± 9[g]	291 ± 6[g]
1×10^{-1}	121 ± 4	126 ± 5	267 ± 8	268 ± 6

[a]Reported LPS concentration in mcg/ml.

[b]Colonies/10^5 nucleated marrow cells following 7 day incubation (mean ± 1 standard error of triplicate determinations).

[c]960 U of CSF-1/ml.

[d]10^{-4} mcg *E. coli* K235 LPS/ml.

[e]1:1000 final dilution anti-IFN (α + β) + 0.3 mcM indomethacin.

[f]Not significantly different at $p \leq 0.05$.

[g]Not significantly different at $p \leq 0.05$.

(medium endotoxin levels are approximately one log less than the reported serum endotoxin concentrations as the medium contains 15 percent serum), colony formation both in the presence and absence of anti-IFN ($\alpha + \beta$) antiserum and indomethacin is not significantly different ($p \leq 0.05$) to that seen in cultures prepared with medium containing low endotoxin ($<10^{-6}$ mcg LPS/ml) levels supplemented with 10^{-4} mcg E. coli K235 LPS/ml. As seen in earlier figures, at high serum endotoxin levels (1×10^{-1} mcg/ml) colony formation is actually inhibited as compared to low endotoxin cultures.

CHAPTER V

DISCUSSION

Both infection with Gram-negative bacteria (90) and exposure to bacterial LPS (9, 46, 56, 69, 84) at sublethal doses enhance serum CSF levels and numbers of peripheral mononuclear phagocytes and granulocytes. Additionally, endotoxin increases the number of myeloid stem cells in cell cycle in treated animals (14), as well as increasing the mass of organs rich in reticuloendothelial cells. While it is implied that LPS stimulates peripheral CSF production, thereby enhancing proliferation of myeloid progenitor cells within the bone marrow, available evidence fails to support this hypothesis. Work with long term bone marrow cultures (13, 29) has provided evidence that marrow cells produce and consume their own CSF. Other researchers (78) have shown that repeated injections of anti-CSF antisera in mice have no suppressive effect on numbers of marrow committed granulocyte/ macrophage colony forming cells (CFUc). Therefore, whereas CSF produced exogenously to the bone marrow may affect extramedullary myelopoiesis, cells of the marrow appear to produce and consume their own CSF.

The present investigation provides evidence for a direct effect of LPS on myeloid progenitor cells. Very low doses of LPS (10^{-4} mcg *E. coli* K235 LPS/ml) were found to significantly enhance both ^{3}H-TdR incorporation and soft-agar colony formation by bone marrow macrophage progenitor cells exposed to suboptimal and optimal CSF doses. This enhancement was not due to LPS-stimulated production of CSF, as

the addition of LPS alone did not result in any colony formation. Moore, et al. (56) have recently described a similar LPS effect on peritoneal exudate cells, resulting in enhanced colony formation by macrophage progenitor cells at lower doses of CSF. These data contrast with the present report in that enhanced CSF-responsiveness of bone marrow myeloid precursors after LPS exposure occurred not only at suboptimal doses of CSF, but additionally at optimal and supraoptimal CSF concentrations as well. The importance of these differences is discussed later.

Lipopolysaccharide-mediated enhancement of both CSF-induced ^{3}H-TdR incorporation and soft-agar colony formation by marrow cells occurred above and beyond the enhanced response seen with the addition of indomethacin and anti-IFN (α + β) antisera. Dose-dependent stimulation of prostaglandin E synthesis by macrophages occurs in response to increasing concentrations of CSF, and this system acts as a negative feedback mechanism limiting excessive monocytopoiesis (40, 42). CSF-stimulated macrophages also produce small amounts of interferon (4, 54) which also act to inhibit CSF-induced proliferation. Our laboratory has recently shown (54) that the addition of indomethacin, a potent inhibitor of the cyclo-oxygenase pathway responsible for prostaglandin synthesis, and anti-IFN (α + β) antiserum to bone marrow cultures results in significantly enhanced colony formation. In the present study, indomethacin and anti-IFN (α + β) antisera significantly enhanced both ^{3}H-TdR incorporation and soft-agar colony formation by bone marrow cells. An important finding presented in Figure 9 (page 37), but not previously reported, is that

the removal of adherent cells from bone marrow preparations resulted in enhanced colony formation equal to that seen in cultures receiving indomethacin and anti-IFN (α + β) antisera. Nonadherent cell cultures did not show enhanced CSF-induced colony formation in the presence of indomethacin and anti-IFN (α + β) antisera. Thus, CSF-stimulated adherent macrophages indeed appear to be the source of inhibitory PGE and IFN within the marrow cultures.

Since mature adherent macrophages are known to produce a variety of soluble mediators upon LPS stimulation such as interleukin-1 (91), interferons (81), E prostaglandin (42), and tumor necrosis factor (79), the possibility that a secondary soluble mediator produced by LPS stimulated macrophages within the marrow being responsible for the enhanced CSF-responsiveness of myeloid progenitor was examined. Removal of adherent cells within the marrow (Figure 9, page 37) did not abrogate the enhancement of CSF-induced colony formation seen after LPS-stimulation. To exclude the possibility of nonadherent cells producing such a secondary mediator in response to LPS, nonadherent cells from LPS-responsive C3H/AnF mice were co-incubated with nonadherent cells from LPS-nonresponsive C3H/HeJ mice in soft-agar assays and were stimulated with CSF and LPS (Table 1, page 41). Since total colony formation in the mixed cultures was no more than additive with respect to cultures of each cell type incubated separately, it is probable that a secondary soluble mediator is not involved. Thus, LPS appears to have a direct stimulatory effect on myeloid precursors within the marrow. Such a direct effect of LPS is not without precedent in the literature. Doe, et al. (16) have shown that the

lack of differentiation and activation of LPS-nonresponder C3H/HeJ peritoneal macrophages in response to LPS is not overcome by co-culture of responder spleen cells with the C3H/HeJ macrophages, indicating that LPS stimulates these cells directly and not indirectly through some secondary soluble mediator.

Important work on lymphocyte stimulation by Eda, et al. (19) also provides evidence of direct stimulatory effects of LPS on immune cells. Immature B lymphocytes are mitogenically stimulated by LPS to proliferate and differentiate into more mature immunoglobulin-secreting cells; LPS-hyporesponsive C3H/HeJ B cells, however, will not respond to LPS stimulation. Eda extracted a 100,000 mw, membrane free factor from the cytosol of LPS-stimulated C3H/HeN LPS-responsive B cells, sensitive to protease treatment and resistant to treatment with RNase, that upon microinjection into C3H/HeJ B cells would render these cells capable of proliferating and differentiating upon subsequent LPS exposure. Little is known about the process of cytoplasm-mediated signal transmission to the nucleus from the cell membrane occurring in mitogen-stimulated cells, but it appears this internalized factor produced by LPS-stimulated B cells is at least one component involved in such signaling. The defect in the C3H/HeJ cells appears then to be the inability to produce this early signal after LPS-receptor stimulation.

Similar experiments could be performed with myeloid cells from the bone marrow to test this thesis' suggestion that LPS directly affects these cells. Cytosol extracted from LPS-stimulated C3H/AnF myeloid cells could be micro-injected into C3H/HeJ cells, and proliferation measured following LPS-stimulation. An even more intriguing

possibility is to insert membrane fragments from LPS-responsive myeloid cells into the membranes of C3H/HeJ cells, quickly exposing these cells to LPS and hoping competent LPS-stimulated signal transmission occurs. Experiments such as these have been performed by Jakobovits et al. (33), in which T cells fused with B cell membrane components were made responsive to LPS. Harris, et al. (27) was even able to transfer the membrane-associated antigen-specific T cell receptor and the cytotoxic T cell (CTL) lytic machinery to recipient lymphocytes. The difficulty in performing such experiments would be related to the difficulty in obtaining a pure population of myeloid precursor cells from the marrow.

There are a number of other experiments that could test for secondary soluble mediators produced in response to LPS that may be responsible for enhancement for CSF-stimulated proliferation by marrow cells. An obvious one is to separate C3H/AnF and C3H/HeJ cultures by a semi-permeable membrane, stimulate the AnF culture with LPS and measure the proliferative response of the HeJ cells via ^{3}H-TdR incorporation to see if enhancement occurs. A soft-agar analog of this experiment would be to set up bilayer cultures similar to the long-term bone marrow cultures described in the literature survey. A dense layer of agar would be placed over LPS-stimulated C3H/AnF adherent cells, with a second agar layer containing C3H/HeJ cells on top. Enhancement of CSF-stimulated colony formation in the upper layer could be compared to similar cultures in the absence of LPS. Finally, C3H/AnF cells could be irradiated such that the cells would no longer proliferate although possible factor production would continue in response to LPS

stimulation; mixtures of these and C3H/HeJ cells could be stimulated with CSF and LPS, with proliferation compared to cultures receiving CSF alone. This experiment was attempted repeatedly with inconclusive results probably related to technical difficulties.

It remains possible in spite of the possible confirmation of the results presented in this paper even by these proposed experiments that LPS may indeed produce extracellular mediators that act to enhance CSF-stimulated monocytopoiesis. The lack of evidence of such factors could be due to their extremely short half-life in culture, or to the presence of enzymes within the culture that destroy the factors upon release from the cells. However, if such a case were true, the enhanced colony formation seen in the C3H/AnF cultures would be prevented as readily as enhanced colony formation by the C3H/HeJ cells in culture with the C3H/AnF cells. Since this was not the case, it appears then that LPS stimulation indeed occurs by direct action of LPS on the precursor cell, and any release of such an intracellular factor to the cell's exterior is of secondary importance.

That LPS enhances colony formation by CSF-stimulated bone marrow cells even at optimum doses suggests that within the bone marrow a certain proportion of myeloid progenitors will not proliferate and differentiate in response to CSF without additional LPS activation. As seen in Figure 6 (page 32), at optimal CSF doses, approximately 30 to 40 percent (around 150 to 200 cells/10^5 marrow cells) of marrow cells capable of ultimately responding to CSF (approximately 500 cells/10^5 marrow cells) do so only with concurrent LPS stimulation. This could represent an important immune mechanism developed in response to

elevated serum LPS levels due to infection with Gram-negative organisms. Thus, expansion of the mononuclear phagocyte population seen in Gram-negative infection (90) and exposure to bacterial LPS (9, 45, 56, 69, 84) is not only due to enhanced CSF-responsiveness of marrow progenitors, but also recruitment of myeloid progenitors unresponsive to CSF alone.

Although LPS appears to act directly on the myeloid precursors, the exact mechanism of LPS enhancement of CSF-induced ^{3}H-TdR incorporation and colony formation by bone marrow cells is unclear. Since LPS is known to inhibit CSF binding to cell membranes, with subsequent CSF-binding recovery susceptible to inhibition by cyclohexamide (10), one possible mechanism of LPS-mediated enhanced CSF-responsiveness is simply increased CSF receptor insertion after LPS-enhanced membrane turnover. This same mechanism, LPS-induced membrane turnover, could account for the suppression of CSF-induced proliferation seen in the experiments by higher doses (1 mcg/ml) of LPS. Rapid membrane turnover could result in net loss of CSF-receptors if those receptors are lost at a faster rate than they are inserted in the membrane. Another explanation is that LPS pushes committed myeloid precursors into cell cycle, a known effect of LPS (14). It is possible that LPS provides the necessary stimulus for these cells to enter the cell cycle, while CSF provides stimulation for sustained proliferation.

The absence of previous reference to the LPS-enhancing effect of marrow myeloid CSF-responsiveness may well be due to the subnanogram quantities of LPS required to observe the effect. Just as Moore, et

al. (56) have shown that 10^{-4} to 10^{-7} mcg LPS/ml enhance the CSF-responsiveness of peritoneal exudate mononuclear cells while LPS levels above 10^{-2} mcg/ml actually inhibit colony formation, data presented in this report show that the stimulatory effect of LPS for CSF-induced marrow cell colony formation is not only lost at LPS concentrations above 10^{-1} mcg/ml colony formation under such conditions is actually inhibited. Contaminating endotoxin in the various serum lots, even at very low levels (Table 2, page 42), masks the stimulatory effect of low levels of experimentally administered lipopolysaccharide.

These results suggest that LPS plays an important, direct role in monocytopoiesis in the bone marrow. Mononuclear phagocyte production within the marrow is not only enhanced by LPS presence, maximum myeloid expansion appears to depend on LPS presence.

LIST OF REFERENCES

LIST OF REFERENCES

1. Austin, P. E., McCulloch, E. A., and Till, J. E. 1971. Characterization of the factor in L-cell conditioned medium capable of stimulating colony formation by mouse marrow cells in culture. J. Cell. Phys. 77:121-128.

2. Bagby, G. C., McCall, E., Bergstrom, K. A., and Bruger, D. 1983. A monokine regulates colony-stimulating activity production by vascular endothelial cells. Blood 62:663-668.

3. Broxmeyer, H. E., Bognacki, J., Ralph, P., Dorner, M. H., Li, L., and Castro-Malaspina, H. 1982. Monocyte-macrophage-derived acidic isoferritins: Normal feedback regulators of granulocytes-macrophage progenitor cells in vitro. Blood 60:595-607.

4. Broxmeyer, H. E., Lu, L., Platzer, E., Feit, C., Juliano, L., and Rubin, B. Y. 1983. Comparative analyses of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitor cells. J. Immunol. 131:1300-1307.

5. Broxmeyer, H. E., Smithyman, A., Eger, R. R., Meyers, P. A., and de Sousa, M. 1978. Identification of lactoferrin as the granulocyte-derived inhibitor of colony-stimulating activity production. J. Exp. Med. 148:1052-1067.

6. Bull, F. G., and Rosendaal, M. 1978. Macrophage colony development: Properties of colony stimulating factors from murine embryo and pregnant uterus. Immunology 34:479-486.

7. Burgess, A. W. 1984. The complex mediators of cell growth and differentiation. Immunol. Today 5:155-158.

8. Burgess, A. W., Camakaris, J., and Metcalf, D. 1977. Purification and properties of colony-stimulating factor from mouse lung conditioned medium. J. Biol. Chem. 252:1998-2003.

9. Butler, R. C., Abdelnoor, A. M., and Nowotny. 1978. Bone marrow CSF and tumor resistance-enhancing activity of postendotoxin mouse sera. PNAS 75:2893-2896.

10. Chen, B. D. M., Lin, H. S., and Hsu, S. 1983. Lipopolysaccharide inhibits the binding of colony-stimulating factor (CSF-1) to murine peritoneal exudate macrophages. J. Immunol. 130:2256-2260.

11. Chervenick, P. A. 1972. Effect of endotoxin and postendotoxin plasma on in vitro granulopoiesis. J. Lab. Clin. Med. 79:1014-1020.

12. Cline, M. J., Rothman, B., and Golde, D. W. 1974. Effect of endotoxin on the production of colony-stimulating factor by human monocytes and macrophages. J. Cell. Phys. 84:193-196.

13. Dexter, T. M., Spooncer, E., Toksoz, D., and Lajtha, L. G. 1980. The role of cells and their products in the regulation of in vitro stem cell proliferation and granulocyte development. Jour. Supramolecular Struc. 13:513-524.

14. Dienstman, S. R., and Defendi, V. 1978. Necessary and sufficient conditions for recruitment of macrophages into the cell cycle. Exp. Cell. Res. 115:191-199.

15. Doe, W. F., and Henson, P. M. 1978. Macrophage stimulation by bacterial lipopolysaccharides. I. Cytolytic effect on tumor target cells. J. Exp. Med. 148:544-556.

16. Doe, W. F., and Henson, P. M. 1979. Macrophage stimulation by bacterial lipopolysaccharides. III. Selective unresponsiveness of C3H/HeJ macrophages to the lipid A differentiation signal. J. Immunol. 123:2304-2310.

17. Duncan, R. L., and Morrison, D. C. 1984. The fate of E. coli lipopolysaccharide after uptake of E. coli by murine macrophages in vitro. Inf. Imm. 132:1416-1424.

18. Eaves, A. C., and Bruce, W. R. 1974. In vitro production of CSA. I. Exposure of mouse peritoneal cells to endotoxin. Cell Tissue Kinet. 7:19-30.

19. Eda, Y., Ohara, J., and Watanabe, T. 1983. Restoration of LPS responsiveness of C3H/HeJ mouse lymphocytes by microinjection of cytoplasmic factors from LPS-stimulated normal lymphocytes. J. Immunol. 131:1294-1299.

20. Ezekowitz, R. A. B., Austyn, J., Stahl, P. D., and Gordon, S. 1981. Surface properties of BCG-activated mouse macrophages. J. Exp. Med. 154:60-76.

21. Gery, I., Kruger, J., and Spiesel, S. Z. 1972. Stimulation of B-lymphocytes by endotoxin. J. Immunol. 108:1088-1091.

22. Girard, R., Chaby, R., and Bordenave, G. 1981. Mitogenic response of C3H/HeJ mouse lymphocytes to polyanionic polysaccharides obtained from Bordetella pertussis endotoxin and from other bacterial species. Inf. Imm. 31:122-128.

23. Golde, D. W., Finley, T. N., and Cline, M. J. 1972. Production of colony-stimulating factor by human macrophages. Lancet 2:1397-1399.

24. Gordon, S., Unkeless, J. C., and Cohn, Z. A. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis. J. Exp. Med. 140:995-1010.

25. Gregory, S. H., Zimmerman, D. H., and Kern, M. 1980. The lipid A moiety of lipopolysaccharide specifically bound to B-cell subpopulations of responder and nonresponder animals. J. Immunol. 125:102-107.

26. Guilbert, L. J., and Stanley, E. R. 1980. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J. Cell. Biol. 85:153-159.

27. Harris, D. T., MacDonald, H. R., and Cerottini, J. C. 1984. Direct transfer of antigen-specific cytolytic activity to noncytolytic cells upon fusion with liposomes derived from cytolytic T cell clones. J. Exp. Med. 159:261-275.

28. Haeffner-Cavaillon, N., Chaby, R., Cavaillon, J. M., and Szabo, L. 1982. Lipopolysaccharide receptor on rabbit peritoneal macrophages. I. Binding characteristics. Inf. Imm. 128:1950-1954.

29. Heard, J. M., Fichelson, S., and Varet, B. 1982. Role of colony stimulating factor activity in long-term bone marrow cultures: Evidence for its production and consumption by the adherent cells. Blood 59:761-767.

30. Ho, M. 1980. Cellular sources of endotoxin-induced interferons. Microbiology 1980, ASM:128-130.

31. Hook, W. A., Snyderman, R., and Mergenhagen, S. E. 1972. Further characterization of a factor from endotoxin-treated serum which releases histamine and heparin from mast cells. Inf. Imm. 5:909-914.

32. Hultner, L., Staber, F. G., Mergenthaler, H. G., and Dormer, P. 1982. Production of murine granulocyte-macrophage colony-stimulating factors (GM-CSF) by bone marrow derived and non-hemopoietic cells in vivo. Exp. Hematol. 10:798-808.

33. Jakobovits, A., Sharon, N., and Zan-Bar, I. 1982. Acquisition of mitogenic responsiveness by nonresponding lymphocytes upon insertion of appropriate membrane components. J. Exp. Med. 156:1274-1279.

34. Kabin, S., and Rosenstreich, D. L. 1977. Binding of bacterial endotoxin to murine spleen lymphocytes. Inf. Imm. 15:156-164.

35. Kampschmidt, R. F., Pulliam, L. A., and Upchurch, H. F. 1980. The activity of partially purified leukocytic endogenous mediator in endotoxin-resistant C3H/HeJ mice. Jour. Lab. Clin. Med. 95:616-623.

36. Kampschmidt, R. F., and Upchurch, H. F. 1977. Possible involvement of leukocytic endogenous mediator in granulopoiesis. Proc. Soc. Exp. Bio. Med. 155:89-93.

37. Kampschmidt, R. F., and Upchurch, H. F. 1980. Neutrophil release after injections of endotoxin or leukocytic endogenous mediator into rats. Jour. Reticulo. Soc. 28:191-201.

38. Kimura, N., Niho, Y., and Yanase, T. 1982. A high level of colony-stimulating activity in a lung cancer patient with extensive leucocytosis, and the establishment of a CSA producing cell line (KONT). Scand. Jour. Haematol. 28:417-424.

39. Kriegler, A. B., Bradley, T. R., Januszewicz, E., Hodgens, G. S., and Elms, E. F. 1982. Partial purification and characterization of a growth factor for macrophage progenitor cells with high proliferative potential in mouse bone marrow. Blood 60:503-508.

40. Kurland, J. I., Broxmeyer, H. E., Pelus, L. M., Bockman, R. S., and Moore, M. A. S. 1978. Role for monocyte-macrophage-derived CSF and PGE in the positive and negative feedback control of myeloid stem cell proliferation. Blood 52:388-407.

41. Kurland, J. I., Hadden, J. W., and Moore, M. A. S. 1977. Role of cyclic nucleotides in the proliferation of committed granulocyte-macrophage progenitor cells. Cancer Research 37:4534-4538.

42. Kurland, J., and Moore, M. A. S. 1977. Modulation of hemopoiesis by prostaglandins. Exp. Hematol. 5:357-373.

43. Kurland, J. I., Pelus, L. M., Bockman, R. P., and Moore, M. A. S. 1979. Induction of PGE synthesis in normal and neoplastic macrophages: Role for CSF(s) distinct from effects on myeloid progenitor cell proliferation. PNAS 76:2326-2330.

44. Lehrer, S., and Nowotny, A. 1972. Isolation and purification of endotoxin by hydrolytic enzymes. Inf. Imm. 6:928-933.

45. Luderitz, D., Tanamoto, K., Galanos, C., McKenzie, G. R., Brade, H., Zahringer, U., Rietschel, E. Th., Kusumoto, S., and Shiba, T. 1984. Lipopolysaccharides: Structural principles and biologic activities. Rev. Inf. Dis. 6:428-438.

46. MacVittie, T. J., and Walker, R. I. 1978. Endotoxin induced alterations in canine granulopoiesis: CSF, colony forming cells in culture and growth of cells in diffusion chambers. Exp. Hematol. 6:613-618.

47. McAdam, K. P. W. J., and Sipe, J. D. 1976. Murine model for human secondary amyloidosis: Genetic variability of the acute-phase serum protein SAA response to endotoxins and casein. J. Exp. Med. 144:1121-1127.

48. McIntire, F. C., Sievert, H. W., Barlow, G. H., Finlay, R. A., and Lee, A. Y. 1967. Chemical, physical, and biological properties of a lipopolysaccharide from E. coli K235. Biochemistry 6:2363-2372.

49. Mergenthaler, H. G., Dormer, P., Staber, F. G., and Hultner, L. 1982. The effect of two different types of colony-stimulating factor on the expression of aminopeptidase on marrow-derived murine macrophages. Exp. Haematol. 10:789-797.

50. Michalek, S. M., Moore, R. N., McGhee, J. R., Rosenstreich, D. L., and Mergenhagen, S. E. 1980. The primary role of lymphoreticular cells in the mediation of host responses to bacterial endotoxin. J. Inf. Dis. 141:55-63.

51. Moore, R. N., Deana, D. G., Horohov, D. W., and Rouse, B. T. 1983. Enhanced responsiveness to colony-stimulating factor induced in vitro with murine interferon and lymphokine preparations. Intracellular Communication in Leucocyte Function, J. Wiley and Sons, Ltd.: 55-58.

52. Moore, R. N., Hoffeld, J. T., Farrar, J. J., Mergenhagen, S. E., Oppenheim, J. J., and Shadduck, R. K. 1981. Role of colony-stimulating factors as primary regulators of macrophage functions. Lymphokines, Vol. 3, Academic Press, Inc.: 119-148.

53. Moore, R. N., Larsen, H. S., Horohov, D. W., and Rouse, B. T. 1984. Endogenous regulation of macrophage proliferative expansion by colony-stimulating factor-induced interferon. Science 223:178-181.

54. Moore, R. N., Pitruzzello, F. J., Larsen, H. S., and Rouse, B. T. 1984. Feedback regulation of colony-stimulating factor (CSF-1)-induced macrophage proliferation by endogenous E prostaglandins and interferon-α/β. J. Immunol. 133:541-543.

55. Moore, R. N., and Rouse, B. T. 1983. Enhanced responsiveness of committed macrophage precursors to macrophage-type colony-stimulating factor (CSF-1) induced in vitro by interferons $\alpha + \beta$. J. Immunol. 131:2374-2378.

56. Moore, R. N., Steeg, P. S., Mannel, D. N., and Mergenhagen, S. E. 1980. Role of lipopolysaccharide in regulating CSF-dependent macrophage proliferation in vitro. Inf. Imm. 30:797-804.

57. Moore, R. N., Vogel, S. N., Wahl, L. M., and Mergenhagen, S. E. 1980. Factors influencing lipopolysaccharide-induced interferon production. Microbiology 1980, ASM: 131-134.

58. Morrison, D. C., and Ryan, J. L. 1979. Bacterial endotoxins and host immune responses. Adv. Immunol. 28:293-450.

59. Morrison, D. C., and Ulevitch, R. J. 1978. The effect of bacterial endotoxins on host mediation systems. A review. Am. J. Pathol. 93:527-533.

60. Motoyoshi, K., Takaku, F., and Miura, Y. 1983. High serum colony-stimulating activity of leukocytopenic patients after intravenous infusions of human urinary colony-stimulating-factor. Blood 62:685-688.

61. Naughton, B. A., Gamba-Vitalo, C., Naughton, G. K., Liu, P., and Gordon, A. S. 1982. Granulopoiesis and colony stimulating factor production in regenerating liver. Exp. Haematol. 10:451-458.

62. Nicola, N. A., Metcalf, D., Johnson, G. R., and Burgess, A. W. 1978. Preparations of colony stimulating factors from human placental conditioned medium. Leuk. Res. 2:313-322.

63. Nicola, N. A., and Vadas, M. 1984. Hemopoietic colony-stimulating factors. Immunol. Today 5:76-80.

64. Ninnemann, J. L. 1984. Prostaglandin structure and chemistry: a primer. Immunol. Today 5:170-173.

65. Nozawa, R. T., Sekiguchi, R., and Yokota, T. 1980. Stimulation by conditioned medium of L-929 fibroblasts, E. coli lipopolysaccharide, and muramyl dipeptide of candidacidal activity of mouse macrophages. Cellular Immunol. 53:116-124.

66. Pabst, M. J., and Johnston, R. B., Jr. 1980. Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. J. Exp. Med. 151:101-114.

67. Parker, J. W., and Metcalf, D. 1974. Production of colony-stimulating factor in mitogen stimulated lymphocyte cultures. J. Immunol. 112:502-510.

68. Quesenberry, P. J., and Gimbrone, M. A. 1980. Vascular endothelium as a regulator of granulopoiesis: Production of colony-stimulating activity by cultured human endothelial cells. Blood 56:1060-1067.

69. Quesenberry, P., Morley, A., Stohman, F., Richard, K., Howard, D., and Smith, M. 1972. Effect of endotoxin on granulopoiesis and CSF. New Eng. J. Med. 286:227-232.

70. Ralph, P., Broxmeyer, H. E., Moore, M. A. S., and Nakoinz. 1978. Induction of myeloid colony-stimulating activity in murine monocyte tumor cell lines by macrophage activators and in a T-cell line by concanavalin A. Cancer Research 38:1414-1419.

71. Ramadoii, G., Hopf, U., and Meyer-zum-Buschenfelde, K. H. 1979. Binding sites for endotoxic lipopolysaccharide on the plasma membrane of rabbit hepatocytes. Acta Hepatogastroenterol. 26:368-372.

72. Rosenstreich, D. L., Vogel, S. N., Jacques, A. R., Wahl, L. M., and Oppenheim, J. J. 1978. Macrophage sensitivity to endotoxin: Genetic control by a single co-dominant gene. J. Immunol. 121:1664-1670.

73. Ruco, L. P., and Meltzer, M. S. 1978. Defective tumoricidal capacity of macrophages from C3H/HeJ mice. J. Immunol. 120:329-334.

74. Ruco, L. P., and Meltzer, M. S. 1978. Macrophage activation for tumor cytotoxicity: Development of macrophage cytotoxic activity requires completion of a sequence of short lived intermediary reactions. J. Immunol. 121:2035-2042.

75. Ruco, L. P., Meltzer, M. S., and Rosenstreich, D. L. 1978. Macrophage activation for tumor cytotoxicity: Control of macrophage tumoricidal activity by the LPS gene. J. Immunol. 121:543-548.

76. Ruscetti, F. W., and Chervenick, P. A. 1975. Release of colony-stimulating activity from thymus-derived lymphocytes. J. Clin. Invest. 55:520-526.

77. Scibiensky, R. J. 1980. Immunologic properties of protein-lipopolysaccharide complexes. III. Role of carbohydrate in the LPS adjuvant effect. Mol. Immunol. 17:21-27.

78. Shadduck, R. K., Carsten, A. L., Chikkappa, G., Cronkite, E. P., and Gerard, E. 1978. Inhibition of diffusion chamber (DC) granulopoiesis by anti-CSF antiserum. Proc. Soc. Exp. Bio. Med. 158:542-549.

79. Shah, R. G., Green, S., and Moore, M. A. S. 1978. Colony-stimulating and inhibiting activities in mouse serum after Corynebacterium parvum-endotoxin treatment. J. Reticulo. Soc. 23:29-41.

80. Skidmore, B. J., Chiller, J. M., Morrison, D. C., and Weigle, W. O. 1975. Immunologic properties of bacterial lipopolysaccharide (LPS): Correlation between the mitogenic, adjuvant, and immunogenic activities. J. Immunol. 114:770-775.

81. Smith, T. J., and Wagner, R. R. 1967. Rabbit macrophage interferons: Conditions for biosynthesis by virus-infected and uninfected cells. J. Exp. Med. 125:559-577.

82. Springer, G. F., and Adye, J. C. 1975. Endotoxin binding substances from human leucocytes and platelets. Inf. Imm. 12:978-986.

83. Springer, G. F., Adye, J. C., Bezkorovainey, A., and Murtzy, J. R. 1973. Functional aspects and nature of the lipopolysaccharide-receptor of human erythrocytes. J. Infect. Dis. 128:5202-5211.

84. Staber, F. G., Gisler, R. H., Schumann, G., Tarcsay, L., Schafli, E., and Dukor, P. 1978. Modulation of myelopoiesis by different bacterial cell-wall components. Cellular Immunol. 37:174-187.

85. Stanley, E. R. 1981. Colony stimulating factors. The Lymphokines: Biochemistry and Biological Activity. Humana Press; Clifton, New Jersey: 101-132.

86. Stanley, E. R., and Guilbert, L. J. 1981. Methods for the purification, assay, characterization, and target cell binding of a colony stimulating factor. J. Immunol. Methods 42:253-265.

87. Stanley, E. R., and Heard, P. M. 1977. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. J. Biol. Chem. 252:4305-4311.

88. Sullivan, R., Gans, P. J., and McCarroll, L. A. 1983. The synthesis and secretion of GM-CSA by isolated human monocytes: Kinetics of the response to bacterial endotoxin. J. Immunol. 130:800-807.

89. Sultzer, B. M. 1972. Genetic control of host responses to endotoxin. Inf. Imm. 5:107-113.

90. Trudgett, A., McNeill, T. A., and Killen, M. 1973. Granulocyte-macrophage precursor cell and CSF responses of mice infected with Salmonella typhimurium. Inf. Imm. 8:450-455.

91. Unanue, E. R., Kiely, J. M., and Calderon, J. 1976. The modulation of lymphocyte functions by molecules secreted by macrophages. II. Conditions leading to increased secretion. J. Exp. Med. 144:155-166.

92. Vadas, M. A., Nicola, N. A., and Metcalf, D. 1983. Activation of ADCC of human neutrophils and eosinophils by separate CSFs. J. Immunol. 130:795-799.

93. Verret, G. R., Rosner, M. R., and Khorana, H. G. 1982. Fatty acyl amidases from Dictyostelium discoideum that act on lipopolysaccharide and derivatives. J. Biol. Chem. 257:10222-10227.

94. Waheed, A., and Shadduck, R. K. 1979. Purification and properties of L cell-derived colony-stimulating factor. J. Lab. Clin. Med. 94:180-194.

95. Watson, J. 1977. Differentiation of B lymphocytes in C3H/HeJ mice: The induction of Ia antigens by lipopolysaccharide. J. Immunol. 118:1103-1108.

96. Watson, J., Largen, M., and McAdam, K. P. W. J. 1978. Genetic control of endotoxic responses in mice. J. Exp. Med. 147:39-49.

97. Watson, J., Riblet, R., and Taylor, B. A. 1977. The response of recombinant inbred strains of mice to bacterial lipopolysaccharides. J. Immunol. 118:2088-2093.

98. Williams, N. 1979. Preferential inhibition of murine macrophage colony formation by prostaglandin E. Blood 53:1089-1094.

99. Wing, E. J., Waheed, A., Shadduck, R. K., Nagle, L. S., and Stephenson, K. 1982. Effect of colony stimulating factor on murine macrophages: Induction of anti-tumor activity. J. Clin. Invest. 69:270-276.

100. Wu, M. C., Cini, J. K., and Yunis, A. A. 1979. Purification of a colony-stimulating factor from cultured pancreatic carcinoma cells. J. Biol. Chem. 254:6226-6228.

VITA

Daniel Gregorio Deana was born in North Tarrytown, New York on July 24, 1959. His family moved to Cleveland, Tennessee where in 1977 he graduated from Cleveland High School as a National Merit student.

He attended Tennessee Technological University on a P. R. Mallory Foundation Scholarship and graduated in 1981 with a Bachelor of Science degree, Magna Cum Laude. At Tech, he was elected to Phi Kappa Phi, and served as president of Beta Beta Beta Biological Honor Society.

After graduation, he entered postgraduate training at The University of Tennessee in Knoxville and will obtain the Master of Science degree in Microbiology in December 1984. At U.T.K., he has held a graduate teaching assistantship for the Biology and Microbiology programs, and a graduate research assistantship. He also worked for an orthopaedic surgeon as a surgical assistant.

Dan will begin medical classes in the fall of 1984 at The University of Tennessee Center for the Health Sciences where he expects to receive the Doctor of Medicine degree in June of 1988.