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APPROVAL SHEET

Title of Thesis: "Early Phase Endotoxin Tolerance: A Study of the Cellular Mechanisms Which Underlie a State of Acquired Refractoriness to Endotoxin-induced Toxic Manifestations."

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Doctor of Philosophy Degree
December 13, 1985

Thesis and Abstract Approved:

Committee Chairperson	3-26-86 Date
Committee Member	3-26-86 Date
Committee Member	3-26-86 Date
Committee Member	3-26-86 Date
Committee Member	3-27-86 Date

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DEC 1985	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Early Phase Endotoxin Tolerance: A Study of the Cellular Mechanisms which Underlie a State of Acquired Refractoriness to Endotoxin-induced Toxic Manifestations	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences Bethesda, MD 20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**182**	

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ABSTRACT

Title of Dissertation: Early Phase Endotoxin Tolerance: A Study of the Cellular Mechanisms which Underlie a State of Acquired Refractoriness to Endotoxin-Induced Toxic Manifestations

Gary Steven Madonna, M.S.; Candidate, Doctor of Philosophy, 1986

Dissertation directed by: Stefanie N. Vogel, Ph.D., Associate Professor, Department of Microbiology

Endotoxin, the lipopolysaccharide (LPS) cell wall component which is derived from Gram negative bacteria, has been shown to induce a number of toxic manifestations which mimic those seen during systemic Gram negative infection. Hyporesponsiveness to the toxic effects of LPS can be induced for a transient period by pre-exposure of a normally responsive individual to a sublethal dose of LPS. This acquired state of refractoriness to the toxic effects of LPS has been defined as "early endotoxin tolerance". Little is known about the cellular mechanisms that underlie this phenomenon. In this study, an early tolerance system was established by the injection of mice with 25 µg of Escherichia coli K235 LPS. Maximal hyporesponsiveness in response to a challenge injection was observed 3 to 4 days after the initial injection, and

normal responsiveness returned by 8 days after the initial exposure to LPS. Further experiments demonstrate that the acquisition and maintenance of the tolerant state coincides temporally with an increase in the number of macrophage progenitor cells in the bone marrow. Cell sizing profiles of the bone marrow cells from tolerized mice indicate that there is an enrichment of a subpopulation of cells that are significantly larger than the cells in bone marrow preparations from control mice. By density gradient sedimentation, it was shown that the denser population of cells from tolerized mice contained the increased numbers of progenitor cells, which, by cytology, were immature monocytic cell types. The increased numbers of macrophage progenitors were sustained after a second (challenge) injection of LPS, but returned to normal levels after multiple tolerizing injections. These results indicate that early endotoxin tolerance is associated with an increase in a population of bone marrow cells that is enriched for macrophage progenitors and suggests the possibility that the lack of responsiveness observed during the hyporesponsive period is related to a failure of these immature cell types to respond to LPS.

Early endotoxin tolerance was inducible in inbred mice which were genetically athymic (T cell-deficient) and in inbred mice which were deficient in a subpopulation of LPS-responsive B cells. In addition, splenectomized mice were capable of being rendered fully tolerant by the protocol established for normal mice. These findings suggest that T cells, Lyb 5

positive B cells and spleen cells do not contribute significantly to the induction of early endotoxin tolerance.

The endotoxin tolerance protocol developed in this study was highly protective against challenge with heterologous preparations of LPS. However, mice which were rendered tolerant to E. coli LPS exhibited a delayed mean time to death following infection with Pseudomonas aeruginosa. However, the tolerance regimen used in this study did not alter the Ps. aeruginosa LD_{50}.

Lastly, early endotoxin tolerance could be induced with a non-toxic derivative of Lipid A, monophosphoryl Lipid A (MPL). Associated with MPL-induced tolerance were all of the cellular and protective manifestations observed using intact LPS. These findings underscore the potential of non-toxic LPS derivatives to mitigate the toxic effects of endotoxin.

EARLY PHASE ENDOTOXIN TOLERANCE: A STUDY OF THE CELLULAR MECHANISMS WHICH UNDERLIE A STATE OF ACQUIRED REFRACTORINESS TO ENDOTOXIN-INDUCED TOXIC MANIFESTATIONS

by

Gary Steven Madonna

Dissertation submitted to the
Faculty of the Department of Microbiology Graduate Program of
the Uniformed Services University of the Health Sciences
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1986

DEDICATION

To God be the Glory!

Great things He has done:

In my work...

In my family...

In my life...

ACKNOWLEDGEMENTS

I would like to thank Dr. Stefanie N. Vogel for all she has done for me over the last 3 years. She has helped me advance as a scientist. Her resourcefulness and knowledge has broadened my awareness in the field of immunology. Her leadership, help, and understanding at critical times will always be remembered. Thank you for helping me attain this great goal and capture a new one.

Thank you, United States Army, Medical Service Corps and all the "real soldiers" for allowing me to: "Be, all that I can be, in the Army".

Thanks to the faculty and my advisory committee for the great courses, for all your knowledge imparted, and to the graduate students, all great people.

To our special group in the Vogel lab, I couldn't have done it without you.

Great appreciation goes to: Dr. Marion Fultz, for all the help using the FACS II; Eric Kaufman, for his technical help and friendship; Dr. Cathy Barr, for helping me to set up the columns for polysaccharide separation (over and over); Nancy Coles, for assistance in learning the mouse burn model; Robbie Matthai (Litton Bionetics), who taught me to splenectomize mice and have them live; and to the secretaries, for their encouragement and support.

Thanks Mom (and Dad) for "training me up in the way I

should go". Thanks Mom Henry, for your many prayers, special verses to remember and that special talk that showed me the "real adversary".

Lastly, and most dearly, thanks to my family. Elisa, for your smiles, pretty pictures and understanding. Todd, thanks for admiring me; thanks for being my friend. Sharon, see that, you didn't hold me back after all. We did it together. The best is yet to be.

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INTRODUCTION AND HISTORY

A common feature of all Gram negative bacteria is the presence of lipopolysaccharide (LPS) on the bacterial cell surface. Located exclusively in the outer membrane, this glycolipid is one of the major components through which Gram negative bacteria interact with their environment and, as such, represents a major cell surface bacterial antigen. Gram negative lipopolysaccharides share a basic common structure: a polysaccharide moiety bound covalently to a glycolipid (Westphal and Lüderitz, 1954). The polysaccharide component consists of an outermost oligosaccharide polymer or "O-specific chain" which varies among strains of a given genus and is responsible for serotype specificity. This is covalently coupled to an inner "core region" which is composed of a branched oligosaccharide. The composition of the core is common among strains of a given genus, but may vary within different families of bacteria. The core oligosaccharide is attached covalently at a single attachment site (Gmeiner et al. 1971; Sidorczyk et al. 1983; Rosner et al. 1979) to an unusual lipid, Lipid A. In the Enterobacteriaceae, Lipid A consists of a phosphorylated glucosamine disaccharide substituted with ester- and amide-linked long chain fatty acids and represents the least variable region of the LPS molecule.

The synthesis and expression of lipopolysaccharide on the cell surface of Gram negative bacteria has been shown to

play a major role in the pathogenesis of specific enteric diseases. The ability of shigellae to penetrate the epithelial cells of the gut, considered to be the essential step in the pathogenesis of shigellosis, is affected by a number of bacterial surface attributes among which are the chemical composition of the surface lipopolysaccharide O-repeat polymer (Gemski et al., 1972) and the expression of smooth LPS (Gemski and Formal, 1975). LPS expression in Shigella sonnei was shown by Madonna and Allen (1981) to determine the bacterial susceptibility to direct serum bacteriolysis and the opsonification requirements necessary for polymorphonuclear leukocyte-mediated microbicidal action. The loss of sugar components from the O-antigen was also shown to be accompanied by an increased sensitivity to the bactericidal action of antibody and complement in Salmonella (Nelson and Roantree, 1967). Friedberg and Shilo (1970) tested wild-type Salmonella typhimurium and cell wall mutants with deficiencies in their cell wall lipopolysaccharide for sensitivity to the bactericidal action of the lysosomal fraction of polymorphonuclear leukocytes. These investigators found that a complete lipopolysaccharide basal core was essential for resistance to lysosomal bactericidal action, whereas O-specific side chains of the wild type did not enhance resistance. Lastly, Medearis et al. (1968) showed that the presence of lipopolysaccharide side chains in E. coli enabled these organisms to resist phagocytosis.

Endotoxin has also been implicated in the pathogenicity

of the Gram negative diplococcus, Neisseria meningitidis. As in Gram negative rods, the lipopolysaccharide of the meningococcus resides primarily in the outer membrane (Zollinger et al., 1972). Devoe and Gilchrist (1973) showed that during log-phase growth in vitro, this organism over-synthesizes its outer membrane which results in the release of bleblike structures from the surface of the organism. The continuous release of these structures and, hence, endotoxin may play an important role in the severe endotoxic reaction and disease state which ensue. It has also been found that most meningococcal strains express a "rough" LPS, (i.e., no O-antigen; Jennings et al., 1980), although they appear to have the same core and Lipid A components as the Enterobacteriaceae (Jennings et al., 1980). It has been proposed that because meningococcal LPS lacks the high molecular weight O-antigen, it is more likely to bind with platelets and leucocytes which express LPS-binding substances than to remain in body fluids (Springer and Adye, 1975). Although the mechanisms responsible for the production of pathologic changes in meningococcal infection have not been entirely explained, meningococcal endotoxin appears to be responsible for tissue damage, hypotension, vascular collapse and disseminated intravascular coagulation (DIC) seen in fulminant meningococcemia (Beatty, 1983).

Experimentally, investigators have shown that many of the pathophysiologic changes associated with Gram negative sepsis or caused by injection of live or dead Gram negative

bacteria into experimental animals, can also be elicited by injection of purified LPS. Among these are fever (pyrogenicity), hypotension, hypoglycemia, changes in white blood cell counts, disseminated intravascular coagulation, and, if administered in high doses, irreversible (septic) shock and death (Berry, 1977; Galanos et al. 1977; Kadis et al. 1971; Morrison and Ulevitch, 1978; Nowotny, 1969). It has been shown that Lipid A is responsible for many of these biological alterations. Lüderitz et al. (1973) and Weinbaum et al. (1971) showed that incomplete lipopolysaccharides from R mutants, which contain predominantly Lipid A, elicited all the biological activities induced by intact lipopolysaccharides. Direct evidence for the biological activity of Lipid A was provided by Galanos and Lüderitz (1975) using electrodialyzed, triethylamine-neutralized, soluble forms of Lipid A derived from Salmonella minnesota R345. The activity of this Lipid A preparation was shown to be comparable to that of intact lipopolysaccharide as assessed by lethality, local Shwartzman reaction, complement activation, and stimulation of B lymphocyte proliferation (Galanos et al., 1977).

The term "endotoxin" was used to describe the toxic, cell-associated LPS molecule, and to differentiate it from classical bacterial exotoxins. However, not all endotoxin-induced changes are pathologic. Some of the effects of endotoxins are clearly beneficial and include such manifestations as immunogenicity, protection against lethal irradiation, immune adjuvant effects, and enhancement of

nonspecific resistance to infection or to toxic doses of endotoxin (reviewed by Nowotny, 1983).

The cellular mechanisms which underlie the induction of these responses by endotoxin have been investigated using experimental models of LPS-hyporesponsiveness. One such model is the C3H/HeJ mouse strain. As a result of a spontaneous mutation which occurred at Jackson Laboratories sometime between 1960 and 1965 (Watson and Riblet, 1974; Glode and Rosenstreich, 1976), C3H/HeJ mice bear a single autosomal mutation, Lps^{d} (Watson et al., 1978), which renders them highly refractory to the effects of LPS both in vivo and in vitro (reviewed by Morrison and Ryan, 1979). It is interesting to note that a defect in LPS sensitivity also arose spontaneously on another inbred background, C57BL/10ScN (Forni and Coutinho, 1978; Vogel et al., 1979), and by complementation studies, appears to be located at the same genetic locus as the C3H/HeJ defect (Watson et al., 1980).

The fact that C3H/HeJ B cells did not respond to LPS to proliferate in vitro suggested a possible receptor defect. Forni and Coutinho (1978) and Coutinho et al. (1978) described an antiserum which discriminated between responder and nonresponder splenocytes. This antiserum was prepared by injecting rabbits with spleen B cells from C3H/Tif (LPS-responder) mice (two intravenous injections, two weeks apart), collecting the serum one week after the final injection and absorbing the antiserum on C3H/HeJ tissues (pooled liver, thymus, and spleen cells). LPS or free Lipid A, but not two

other B cell mitogens (lipoprotein and purified protein derivative of tuberculin), competed with the antiserum for binding to the B cell surface membrane which suggested a Lipid A receptor defect. However, when Kabir and Rosenstreich (1977) compared the binding of ^{14}C-labeled LPS from Salmonella minnesota to splenic lymphocytes from C3H/HeN (LPS-responsive) and C3H/HeJ (LPS-hyporesponsive) mice, they found that spleen cells from both strains bound LPS equally well. Further, a highly lipophilic, polysaccharide-deficient glycolipid from S. minnesota R595 was shown to bind cells at least 20 times better than did smooth LPS. This binding was enhanced by trypsin or neuraminidase treatment. These data suggested to the authors that LPS binds via a nonspecific interaction between its lipid moiety and some lipid component of the cell membrane.

Jacobs (1984) used a sensitive hapten-sandwich immunofluorescence technique and at the single cell level showed that a positively charged membrane protein is responsible for the initial interaction of LPS with murine lymphocytes. As such, C3H/St (Lps^n) splenic lymphocytes, pre-treated with pronase or the cross-linking reagent paraformaldehyde, lost the ability to bind LPS. Additionally, when the LPS-cell interaction was carried out at pH 4.5 (which increases the number of positively charged imidazole groups) the number of LPS positive cells which were detected was increased. Zimmerman et al. (1977) and Kern (1984) compared the binding of iodinated Lipid A to splenocytes from C3H/HeN and C3H/HeJ mice using an immunofluorescence-autoradiography

procedure. Like Kabir and Rosenstreich (1977), who used intact LPS, Kern found that the strains were indistinguishable on the basis of binding and also noted that spleen cells from the responder strains, but not the non-responder strain, proliferated in the presence of Lipid A. This group postulated that a separation of LPS-binding and LPS-triggering sites could account for the discrepancies between the findings of Coutinho et al. vs. those who had measured binding of LPS to cells directly, and that C3H/HeJ mice had retained the capacity to bind LPS but had lost the capacity to be triggered.

One of the strongest lines of evidence to support the hypothesis that a membrane defect results in a functional defect in C3H/HeJ mice comes from the studies of Jakabovits et al. (1982). Using fusogenic membrane vesicles, these investigators inserted the membrane components from lymphocytes which respond to LPS (Lps^n) into the membranes of C3H/HeJ spleen cells. They found that the latter could now be stimulated by LPS, and suggested that the inability to respond was due to the lack of suitable membrane constituents.

Although the Lps gene defect has not been fully delineated, experiments using C3H/HeJ mice, in conjunction with syngeneic, fully LPS-responsive (Lps^n) strains, have provided strong evidence that the pathophysiologic changes which result from LPS administration are mediated indirectly by a number of immunoregulatory factors [e.g., endogenous pyrogen (Atkins et al., 1967), tumor necrotizing factor (Mannel et al., 1980), colony-stimulating factor (Apte et al., 1976; Apte and Pluznik,

1976; Metcalf, 1971; Quesenberry et al., 1972), glucocorticoid antagonizing factor (Moore et al., 1976), and prostaglandins (Skarnes and Harper, 1972)] produced by macrophages in response to LPS. Furthermore, agents which activate macrophages, either in vivo (Suter et al., 1958; Benacerraf et al., 1959) or in vitro (Moore et al., 1980; Rosenstreich and Vogel, 1980), increase sensitivity to LPS, even in the C3H/HeJ mouse strain (Vogel et al., 1980).

A second model of murine endotoxin hyporesponsiveness is a state of endotoxin refractoriness which is acquired after exposure of a normally sensitive animal to a sublethal injection of endotoxin. The acquisition of such a state of resistance was first encountered nearly 100 years ago when whole bacterial vaccines were used to induce "therapeutic fever" in the treatment of certain diseases. It was found that increasing quantities of typhoid vaccine were required in order to maintain elevated temperatures (Heyman, 1945). Acquired resistance to alcohol-fractionated pyrogenic preparations, initially shown by Centanni (1894), was demonstrated by reduced fever-inducing activity in animals given repeated injections of these preparations. Centanni (1942) also demonstrated that this pyrogenic substance was stable to heat and was not protein in nature. Favorite and Morgan (1942) were the first to use the term "endotoxin tolerance" to describe this phenomenon. They also noted that the presence of anti-LPS antibodies did not correlate with such resistance. These findings led them to postulate that such acquired resistance had a nonimmunologic

basis. The development of tolerance to LPS-induced lethality was also noted by Wharton and Creech (1949). In their studies, tolerance was shown to be induced in mice following a single injection of endotoxin, to peak three days later, and was shown not to correlate with anti-LPS antibody titers. Beeson (1946, 1947a, 1947b) showed that: (1) "endotoxin tolerance" could not be transferred by serum, (2) the acquisition of tolerance was accompanied by enhanced blood clearance of endotoxin and, (3) blockade of the reticuloendothelial system (RES) with thorotrast (thorium dioxide) retarded clearance of endotoxin and abolished tolerance.

Up to this time, endotoxin-induced fever was thought to be the result of a direct effect of LPS on the thermoregulatory center of the brain. In 1948, Beeson provided evidence that endotoxin stimulated "host cells" to release an intermediate factor, endogenous pyrogen (EP), which acted on thermoregulatory centers in the brain to evoke fever. After Beeson (1948) reported the extraction of EP from rabbit polymorphonuclear leukocytes, these cells were considered to be the major source of pyrogen for the next 20 years. Thus, until EP was shown to be a macrophage secretory product by several groups (Atkins et al., 1967; Hahn et al., 1967; Murphy et al., 1980), tolerance to the pyrogenic effects of endotoxin was thought to result from enhanced uptake of endotoxin by the RES which diverted the endotoxin away from EP-producing cells.

In 1965, Greisman and Woodward provided evidence for two distinct mechanisms of endotoxin tolerance which were

described temporally as "early phase" and "late phase" endotoxin tolerance. The concept of an early phase of tolerance was based on experiments in man, in which a refractory state was observed within hours after continuous intravenous infusion of endotoxin and, in rabbits, by 24 hours after a single injection. This type of hyporesponsiveness was shown to be specific for endotoxins as a class (e.g., tolerance was not induced for other pyrogens, such as staphylococcal enterotoxin), was not transferable with serum, and was found to wane over subsequent days. A later phase of tolerance to the pyrogenic effects of endotoxin could be demonstrated if endotoxin were re-administered after 5 days. Unlike early phase tolerance, this late phase was shown to be transferable with serum and exhibited marked O-antigen specificity. Thus, the early phase of pyrogenic tolerance was hypothesized to reflect a decreased capacity of macrophages to release EP, and late tolerance to be mediated by specific humoral antibodies. By passive transfer experiments, Greisman and Woodward (1965) and Greisman et al. (1966) showed that pyrogenic quantities of endotoxin were present in the serum of tolerized animals. These findings indicated that early phase tolerance was not mediated simply by clearance of endotoxin from the circulation.

As with early tolerance to fever induction, an early phase of endotoxin tolerance for the lethal effects of LPS was demonstrated in mice 24 hours after a single intraperitoneal or intravenous injection of endotoxin (Abernathy, 1967; Urbaschek and Nowotny, 1968; Wharton and Creech, 1949; Williams et al.,

1983). Early tolerance to a lethal challenge with LPS also extends to endotoxins of various Gram negative bacteria. Thus, no O-antigen specificity was seen during early tolerance.

As in the case of the genetically LPS-hyporesponsive C3H/HeJ mouse, studies of early endotoxin tolerance in animals have indicated a central role for the macrophage. Dinarello and co-workers (1968) showed that isolated Kupffer cells from rabbits [shown previously by Braude et al. (1955) and Cremer and Watson (1957) to be the primary site of endotoxin localization following i.v. injection] were stimulated by LPS to produce EP. However, isolated hepatic Kupffer cells from rabbits rendered tolerant by a previous LPS injection were found to be refractory to stimulation by LPS to release EP in vitro. Cellular refractoriness to endotoxin was also shown in human blood monocytes. These cells normally respond to LPS with release of large amounts of colony-stimulating activity (Sullivan et al., 1983); however, within 72 hours of a second exposure of endotoxin, a marked reduction in release of this factor was observed. In addition, Rietschel et al. (1982) showed that peritoneal macrophages from tolerant animals secreted substantially lower levels of prostaglandins (PGE_2 and $PGF_{2\alpha}$) than peritoneal cells from control animals. Lastly, Williams et al. (1983) suggested that an interaction of macrophages and splenic nonadherent cells were required for the early tolerant state.

The goal of this study was to probe further the cellular mechanisms which accompany a state of early endotoxin

tolerance. To this end, a model system, based on that described by Williams *et al.* (1983), was established using highly purified endotoxin derived from *Escherichia coli* K235. Using this model system, a state of early phase endotoxin tolerance was found to be associated with marked alterations in bone-marrow derived macrophage progenitor pools. The cellular mechanisms which underlie the establishment of early tolerance were analyzed in mice which were genetically LPS-hyporesponsive, T cell deficient, B cell deficient, or asplenic. Further experiments were carried out to investigate whether induced hyporesponsiveness to LPS rendered mice refractory to infection by a Gram negative organism, *Pseudomonas aeruginosa*. Lastly, a non-toxic derivative of Lipid A, monophosphoryl Lipid A, was assessed for its efficacy as an inducer of early endotoxin tolerance.

MATERIALS AND METHODS

MICE -- Outbred, female HSD(ICR)BR mice (ICR) were used for the majority of the studies described herein and were obtained from Harlan Sprague Dawley (Indianapolis, IN). For some experiments, inbred C3H/OuJ (Lps^n) and C3H/HeJ (Lps^d) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). For experiments which involved the use of athymic (nude) mice, BALB/c (nu/nu), and their euthymic littermates, BALB/c (nu/+), were obtained from Harlan Sprague Dawley (Indianapolis, IN). T-cell deficiency was verified in BALB/c (nu/nu) mice using fluorescence activated cell sorter (FACS) analysis as described below. Female C3H/HeN mice (Harlan Sprague Dawley, Indianapolis, IN) were age-matched with female, congenic C3.CBA/N (xid/xid) mice. The latter were bred at the Uniformed Services University of the Health Sciences (Bethesda, MD) and were the kind gift of Dr. James Kenny, Dept. of Microbiology, Uniformed Services University of the Health Sciences. In an attempt to minimize potential environmental differences due to the different sources of these two strains, C3H/HeN and C3.CBA/N mice were housed in the same room for 2 weeks prior to treatment.

In one series of experiments, ICR mice were splenectomized. Splenectomies were performed as follows: Mice were given general anesthesia (Metofane (Methoxyflurane), Pitman-Moore, NJ). Each spleen was removed after the arteries and veins which supply the spleen were tied off with 4.0 silk

suture. The peritoneal lining was closed with 4.0 silk suture and the skin brought together and fastened with 9 mm autoclips (Clay Adams, Becton Dickinson, Parsippany, NJ). Sham splenectomies were also carried out in a similar fashion except spleens were gently lifted out of the peritoneal cavity without severing the vessels and were replaced immediately prior to closure. The mice were allowed to recover from surgery for seven days prior to treatment.

All mice were used at 6-8 weeks of age. Mice were housed in cages with Micro-Isolator Unit tops (Lab Products, Rockville, MD) in a negative pressure Horsfall Isolator Unit (Hazelton Systems Inc., Aberdeen, MD), and were fed RMH 3000 mouse chow (Agway, Inc., Syracuse, NY) and acidified water *ad libitum*.

REAGENTS

Endotoxin preparations -- Protein-free, phenol-water extracted LPS, from *Escherichia coli* K235 L+0C+ (serotype O1:K1:HNM, Barry *et al.*, 1962), was prepared by the method of McIntire *et al.* (1967). *Pseudomonas aeruginosa* F-D type I LPS was obtained from List Biological, Inc. (Campbell, CA). Phenol-water extracted *Salmonella typhimurium* LPS and *Bacteroides fragilis* LPS were the kind gifts of Dr. Suzanne Michalek (Univ. of Alabama, Birmingham, AL). Each LPS preparation was prepared in pyrogen-free saline at a final concentration of 1 mg/ml and was put into solution by

sonication (three, 5 sec bursts) using a Sonifier Cell Disrupter, Model W185 (Heat Systems, Ultrasonics, Inc., Plainview, NY) with a microtip and a setting of 3 on the output control.

Detoxified endotoxin (monophosphoryl Lipid A, Lot 375), derived from a heptose-less mutant of S. typhimurium G30/C21 (Qureshi et al., 1982), was obtained from Ribi ImmunoChem Research, Inc. (Hamilton, MT). The monophosphoryl Lipid A (MPL), 1 mg/vial, was solubilized by addition of 0.5 ml sterile distilled water, followed by swirling of the suspension in a 65-70°C water bath for 10-20 seconds, and sonication for 10-20 seconds. After three to four cycles of heating and sonication, the solution clarified to slight opalescence. To this solution was added 0.5 ml of 3.6% NaCl to give a final stock solution of MPL (1 mg/ml) which was subjected to one additional cycle of heating and sonication.

Preparation of lipid-free polysaccharide -- Phenol-water extracted E. coli K235 LPS was subjected to acid hydrolysis [1.0% (approximately 0.17 N) acetic acid, 100°C, 4 h], centrifuged to pellet insoluble Lipid A, and the soluble polysaccharide-rich supernatant removed and dried (Galanos et al., 1971). The polysaccharide fraction was further separated from any remaining LPS by gel chromatography on a Sephadex G-25-300 (Sigma, St. Louis, MO) column (60 x 1.5 cm) which was equilibrated in a solution which consisted of 0.4% (v/v) pyridine (Baker Chemical Co., Phillipsburg, NJ) and 1.0% (v/v) glacial acetic acid (Mallinckrodt Inc., Paris, KY) in water

(Jansson et al., 1981). Those fractions which contained the polysaccharide were identified by detection of glucose using the phenol-sulfuric acid method (Dubois et al., 1956). Fractions which contained polysaccharide were then pooled and dried in vacuo. The final polysaccharide preparation was adjusted by total glucose content to be equivalent to the glucose content of the phenol-water extracted E. coli LPS prepared at a final concentration of 1 mg/ml. Mice were injected with 25 µg/mouse of E. coli K235 LPS or the dilution of purified polysaccharide which was equivalent in glucose content to that of 25 µg E. coli LPS.

MEASUREMENTS OF ENDOTOXIN SENSITIVITY

Measurement of colony stimulating factor (CSF) activity in serum -- After injection of saline or endotoxic preparations into mice, sera were collected. CSF activity in the sera was determined using a bone marrow colony assay in semi-solid agar (Williams et al., 1983). Blood from mice in each experimental group was collected from the orbital plexus 6 h after injection, pooled, and the serum obtained after clot formation and centrifugation (1500 x g, 10 min) in a Beckman Microfuge 12 (Palo Alto, CA). Serial dilutions of serum were prepared in serum-free RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with penicillin/streptomycin (100 IU/ml and 100 µg/ml, respectively; Gibco Laboratories,

Chagrin Falls, OH), 15 mM HEPES buffer (Research Organics Inc., Cleveland, OH), 2 mM glutamine (Gibco Laboratories, Grand Island, NY), and 0.2% sodium bicarbonate (Fisher Scientific Co., Fair Lawn, NJ). Two tenths ml of each serum dilution was added to each of two tissue culture wells (35 mm diameter) in 6 well tissue culture plates (Costar, Cambridge, MA).

LPS has been shown to interfere with the colony assay (Moore et al., 1980). For this reason, bone marrow cells for the CSF colony assay were obtained from endotoxin hyporesponsive C3H/HeJ (Lps^d) mice, to preclude possible effects of endotoxin carried over in serum samples. Femurs and tibias were homogenized in ice cold RPMI medium using a mortar and pestle. Bone fragments and cell aggregates were allowed to settle in a conical centrifuge tube. The crude cell suspension was enriched for mononuclear cells by density gradient centrifugation (400 x g, 20 min, 20°C) in Lymphocyte Separation Medium (LSM; Litton Bionetics, Charleston, SC) using a Sorvall RT 6000 refrigerated centrifuge (Dupont Co., Wilmington, DE). The cells collected from the interface of the LSM gradient were washed twice in RPMI medium supplemented with 10% fetal calf serum and were enumerated using a Coulter Counter Model ZM (Coulter Electronics Ltd., England). Bone marrow cells were diluted to a final concentration of 1×10^{-5} cells per ml in molten colony assay medium (RPMI 1640 supplemented with glutamine, HEPES buffer, sodium bicarbonate, penicillin/streptomycin, 15% fetal calf serum (M.A. Bioproducts), and 0.3% Bacto-agar (Difco Laboratories, Detroit,

MI)). One milliliter of this suspension was then added to each of the wells which contained 0.2 ml of serial serum dilutions, mixed by swirling, and allowed to solidify. The cell cultures were then incubated at 37°C (5% CO_2) for 7 days, at which time bone marrow colonies (>50 cells/colony) were counted under an inverted microscope at 40X magnification. Serum CSF activity is based on the linear part of the dilution curve and is expressed as colony forming units (CFU) per ml of serum.

Interferon production and assay -- To measure the level of interferon in serum after administration of saline or endotoxic preparations, blood samples were collected and processed as described for the collection of serum for CSF activity. Interferon activity was measured by an antiviral assay (Rubinstein et al., 1981) modified as described elsewhere (Vogel et al., 1982). Two-fold serial dilutions of serum samples were prepared in a final volume of 50 µl of assay medium (Minimal Essential Medium Eagle (M.A. Bioproducts, Walkersville, MD) supplemented with antibiotics, HEPES buffer, glutamine and 10% fetal calf serum) in flat-bottomed, 96-well Falcon culture dishes (Becton Dickinson, Oxnard, CA). To each well was added 10^5 L929 fibroblasts in a volume of 50 µl. The supernatants were aspirated after a 24 h incubation at 37° C (5% CO_2) and the cells infected with 100 µl per well of Vesicular Stomatitis Virus (Indiana strain) in medium containing 10% fetal calf serum at a multiplicity of infection of approximately 0.1. At 24-48 h post-infection, complete cytopathic effect was observed microscopically in infected

medium-treated control cells. The cells were then washed with cold Earle's Balanced Salt Solution (Gibco Laboratories, Grand Island, NY) and fixed for 10-30 min with 5% formaldehyde (Fisher Scientific Co., Fair Lawn, NJ). The cells were stained for 5 min with 0.05% crystal violet (Sigma Chemical Co., St. Louis, MO) in 20% ethanol, and subsequently washed with tap water. Optical density was measured at 595 nm using an ELISA reader (Biotek, Inc., Burlington, VT) . Three controls were included for each assay: (i) medium-treated, uninfected L929 cells (cell control); (ii) medium-treated, virus-infected L929 cells (virus control); and (iii) a titration of NIH mouse fibroblast interferon reference standard (G-002-904-511; Research Resources Branch, National Institute of Allergy and Infectious Diseases, NIH) adjusted to 100 U/ml. The first dilution of a sample which exhibited an optical density equal to that of the virus control wells (i.e., no interferon protection) was defined as the endpoint. Reciprocal titers (expressed as units per milliter) were calculated based on the titration of the NIH standard.

Determination of 50% lethal dose (LD_{50}) -- Mice (20 mice/group) were injected intraperitoneally with either saline, E. coli K235 LPS (25 µg/mouse), or MPL (100 µg/mouse) on day 0. Three days later, five mice from each group were challenged i.p. with either 3000, 1500, 750, 375, or 187 µg of E. coli LPS in 0.5 ml of pyrogen-free saline. Deaths were recorded daily for 3 days. The LD_{50} for each experiment was calculated by the

method of Reed and Muench (1939).

Assessment of endotoxin-induced symptoms -- Mice were assessed for visual manifestations of endotoxicity as described by Pier et al. (1981). Accordingly, mice were observed for ruffled fur, conjunctival discharge, and diarrhea 6 h following administration of 25 µg/mouse of endotoxin.

DETERMINATION OF NUMBERS OF MACROPHAGE PROGENITOR CELLS IN THE BONE MARROW AND SPLEEN

Preparation of bone marrow cells -- Groups of 5 mice were injected with either saline, LPS, or MPL as indicated. Five femurs per group (one per mouse) were homogenized using a mortar and pestle with 3 changes of ice cold (4 °C), serum-free RPMI 1640 (supplemented with antibiotics, HEPES buffer, and glutamine). The cell suspension was collected in a 15 ml conical centrifuge tube and brought to final volume of 15 ml. The suspensions were vortexed and were incubated on ice for 10 min to allow for settling of bone fragments and cell aggregates. The top 10 ml of cells were then transferred to a second tube and kept cold (4° C) until cultured. A sample of this cell suspension was used to obtain a nucleated cell count and cell sizing profile using a Coulter Counter Model ZM and Coulter Channelyzer C1000 equipped with a Coulter X-Y Recorder 4 (Coulter Electronics, Inc., Hialeah, FL), which was

calibrated according to the manufacturer's instructions.

Preparation of spleen cells -- Groups of mice were injected with saline or LPS as indicated. Single cell spleen suspensions were prepared from five spleens per experimental group by mascerating spleens on 40 mesh stainless steel screens. The cells were resuspended in ice-cold, serum-free RPMI 1640 (supplemented with antibiotics, HEPES buffer, and glutamine). A sample of each cell suspension was used to obtain a nucleated cell count employing a Coulter Counter Model ZM. These spleen cell suspensions were then used for determination of the number of macrophage progenitor cells.

Assay for macrophage progenitor cells -- The number of macrophage progenitor cells (macrophage colony forming units, M-CFU) in each bone marrow or spleen cell suspension was determined by the method of MacVittie (1979) using a double layer, semi-solid agar colony assay in 60 mm x 15 mm grided tissue culture dishes (Nunc, Roskilde, Denmark). The bottom layer in each culture dish was comprised of approximately 7000 U of macrophage-specific colony stimulating factor (CSF-1) in 2.5 ml of 0.5% Bacto-agar (Difco Laboratories, Detroit, MI) in medium composed of tryptic soy broth and CMRL 1066 supplemented with fetal bovine serum, horse serum, an antibiotic/antimycotic mixture, serine, L-asparagine, sodium pyruvate and sodium bicarbonate as described (MacVittie, 1979). The CSF-1 used was partially purified to insure removal of contaminating

interferon (Warren and Vogel, 1985). The upper layer contained 5×10^4 nucleated bone marrow cells from each cell suspension in 2 ml of 0.33% agar in medium. Duplicate cultures were incubated at 37°C (5% CO_2) for 10 days and colonies (>50 cells) counted.

To test for the presence of more primitive macrophage precursors which exhibit high proliferative potential (HPP), the bottom layer contained CSF-1 and 10% human spleen conditioned medium (HSCM). Human serum (20%) was included in both top and bottom layers. HSCM provides a source of "Synergistic Activity" (SA), shown by Bradley and Hodgson (1979) to be essential for HPP development. HSCM was the kind gift of Dr. Thomas MacVittie (Armed Forces Radiobiology Research Institute, Bethesda, MD), and its preparation has been described elsewhere (Metcalf and Johnson, 1981). HPP colonies fail to develop in the presence of CSF alone (Bradley and Hodgson, 1979), but in the presence of both CSF and SA will produce very large macrophage colonies (>2 mm diameter) which are readily distinguishable from M-CFU by size and are counted after 12 days of incubation.

Separation of bone marrow cells by density gradient sedimentation -- In some experiments, ICR mice were injected with either saline or E. coli LPS and after 3 days their bone marrow cells collected and separated by unit gravity sedimentation (Bertoncello et al., 1981) in a 60 ml separatory funnel. One $\times 10^7$ cells from groups of five mice were

resuspended in 10 ml of RPMI 1640 (supplemented with glutamine, HEPES buffer, sodium bicarbonate, antibiotics and 10% fetal calf serum) and layered onto a 50 ml gradient. The gradient was poured stepwise in four 12.5 ml volumes (which consisted of 30%, 25%, 20%, and 15% fetal calf serum in RPMI medium) layered onto a 10 ml cushion of 50% fetal calf serum in RPMI 1640 supplemented as indicated above. Cells were allowed to sediment at 4 ^{o}C for 3.5 h. Three ml fractions were collected dropwise from the bottom of the gradient, centrifuged, and the cell pellets resuspended in 0.5 ml of 10% fetal calf serum in RPMI 1640 medium supplemented as indicated above. Nucleated cell counts and cell sizing profiles were then obtained from each fraction and the number of M-CFU or HPP in individual or pooled fractions was determined as described above.

INFECTION OF CONTROL AND ENDOTOXIN-TOLERIZED ANIMALS WITH A GRAM NEGATIVE ORGANISM

Bacteria -- Pseudomonas aeruginosa, type I was grown overnight at 32^{o}C in Trypticase Soy Broth (TSB) (Difco Laboratories, Detroit, MI). Four tenths ml of this overnight cell suspension was used to inoculate 65 ml of TSB which was further incubated at 32^{o}C with shaking. Bacterial growth was monitored every 0.5 h using a Spectronic-20 (Bausch and Lomb, Rochester, NY) at OD_{660}. At OD_{660} = 0.25, the late log phase cells were washed and resuspended in saline to OD_{660} = 0.6 (2-5

x 10^8 viable bacteria/ml). Ten-fold dilutions were then made in saline for injections of mice and appropriate dilutions plated on Trypticase Soy Agar (Difco Laboratories, Detroit, MI) to obtain viable cell counts.

Injection protocol -- Mice were injected with saline (control) or LPS (tolerized) on day 0. On day 2, the lower back of each mouse was shaved. On day 3, each mouse was anesthetized with Metofane (Methoxyflurane, Pitman-Moore, NJ) and a third degree burn inflicted by igniting 0.4 ml of 95% alcohol on a 2.5 x 2.5 cm area of exposed bare skin (the area exposed in an asbestos template) (Stieritz and Holder, 1975; Pavlovskis et al., 1977). After 10 sec, the flame was extinguished and 0.5 ml of each 10-fold dilution of bacteria injected subcutaneously at the burn site (5 or 10 mice/bacterial dilution). Deaths were recorded daily for one week.

PREPARATION AND ANALYSIS OF FLUORESCENT ANTIBODY-STAINED CELLS

Individual spleen cell suspensions from five athymic BALB/c (nu/nu) mice and five euthymic BALB/c (nu/+) mice were prepared in Hank's Balanced Salt Solution (without phenol red; Gibco Laboratories, Chagrin Falls, OH) supplemented with 10% newborn calf serum (Gibco Laboratories, Grand Island, NY) and 0.4% (w/v) sodium azide (Matheson Coleman and Bell, Norwood, OH). Following lysis of erythrocytes with an ammonium chloride

lysing solution (AKC-lyse, M.A. Bioproducts, Walkersville, MD), aliquots of each cell suspension were treated with fluoresceinated (FITC) chicken anti-mouse μ polyclonal antibody, FITC-anti-Thy 1.2 antibody (a monoclonal rat IgG2b; Becton Dickinson, Sunnyvale, CA), or a control antibody, FITC-CBPC-101 (a monoclonal IgG2b that does not react with murine cells) at a final concentration of 1 μg antibody/10^6 cells. Both the FITC-chicken anti-mouse μ and FITC-CBPC-101 antibodies were the kind gifts of Dr. Fred Finkelman, Dept. of Medicine, U.S.U.H.S. Fluorescence analysis was carried out using a fluorescence-activated cell sorter (FACS II; Becton Dickinson, Mountain View, CA) in the laboratory of Dr. Lyn Yaffe (Dept. of Pathology, Navy Medical Research Institute, Bethesda, MD). Cells were assigned to fluorescence intensity channels ranging from 1 to 1024 according to the amount of surface-bound fluorescein label detected. For each sample, the percentage of cells which stained with a specific antibody and the median fluorescence intensity (MFI) of the staining were determined by a computer program of curve subtraction and integration as described previously (Mond et al., 1980).

RESULTS

ESTABLISHMENT OF AN EARLY ENDOTOXIN TOLERANCE SYSTEM

Previous studies have shown that injection of LPS into mice resulted in a rapid rise in serum CSF which reached a maximum level at approximately 6 h post-injection (Metcalf, 1971). In a recent report by Williams *et al.* (1983), this manifestation of endotoxin responsiveness was found to be mitigated in mice challenged with LPS 3-4 days after an initial sublethal exposure to LPS. The first series of experiments described herein sought to optimize conditions under which this state of "early endotoxin tolerance" was inducible.

Establishment of early endotoxin tolerance in outbred ICR mice with E. coli K235 LPS -- An 8 day time course experiment was carried out in which groups of outbred ICR mice were injected intraperitoneally with either *E. coli* K235 LPS (25 μg/mouse) or saline. This dose of LPS was chosen based on previous studies by Williams *et al.* (1983) in which *E.coli* O55:B5 LPS was used to induce early endotoxin tolerance in this mouse strain. On successive days following an initial injection, individual groups of mice were challenged with *E. coli* K235 LPS (25 μg/mouse) and bled 6 h later for serum which was subsequently tested for the presence of CSF. The ability of mice to respond to a second (challenge) injection of LPS was

most suppressed at 3 and 4 days after the initial exposure to LPS (Figure 1). After this time, tolerance (hyporesponsiveness to LPS) gradually waned, as indicated by the return of near-normal CSF production by day 8. Groups of mice injected with saline on day 0 responded to an injection of E. coli LPS with high levels of CSF throughout the 8 day time period. These results confirmed those of Williams et al. (1983).

Maintaining a 3 day period between the initial and challenge doses of LPS, the concentration of LPS used for the initial injection was varied to determine the optimal amount of LPS which rendered the mice most hyporesponsive to a subsequent LPS challenge. Figure 2 illustrates that tolerance, as assessed by a decreased capacity to produce serum CSF, was induced over a wide dose range of E. coli K235 LPS (6.25 µg - 100 µg/mouse) but optimally at 50 µg/mouse. However, 50 µg/mouse was occasionally lethal, so a lower dose of 25 µg/mouse was used in all subsequent experiments. The level of CSF induced by one injection of LPS (25 µg/mouse) was the same if administered intraperitoneally or intravenously (i.p. = 4,320 ± 565 vs. 4,520 ± 201 CFU/ml serum). With respect to tolerance induction (as assessed by suppressed CSF activity), little difference in the degree of suppression induced was observed if the LPS were administered intraperitoneally (89% suppression) or intravenously (82% suppression). This confirmed findings which were previously reported by Williams et al. (1983).

To insure that the 6 h time point used in initial

Figure 1. Time course of the induction of early endotoxin tolerance as assessed by LPS-induced CSF -- ICR mice were injected i.p. with either saline or E. coli LPS (25 µg/mouse). At intervals of 1 to 8 days, mice (5 mice/group) were challenged i.p. with a second injection of E. coli LPS (25 µg/mouse). Six hours later, the mice were bled and their sera pooled and assayed for CSF activity as described in the Materials and Methods section. Each point represents the arithmetic mean ± one standard deviation of duplicate determinations.

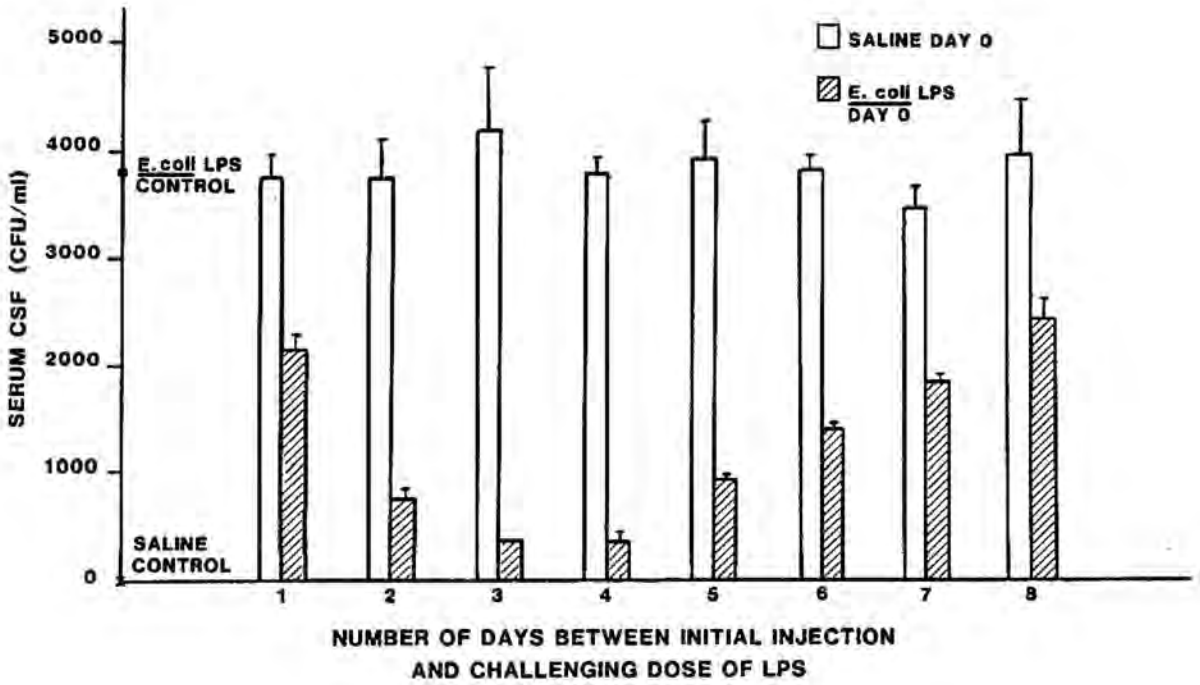
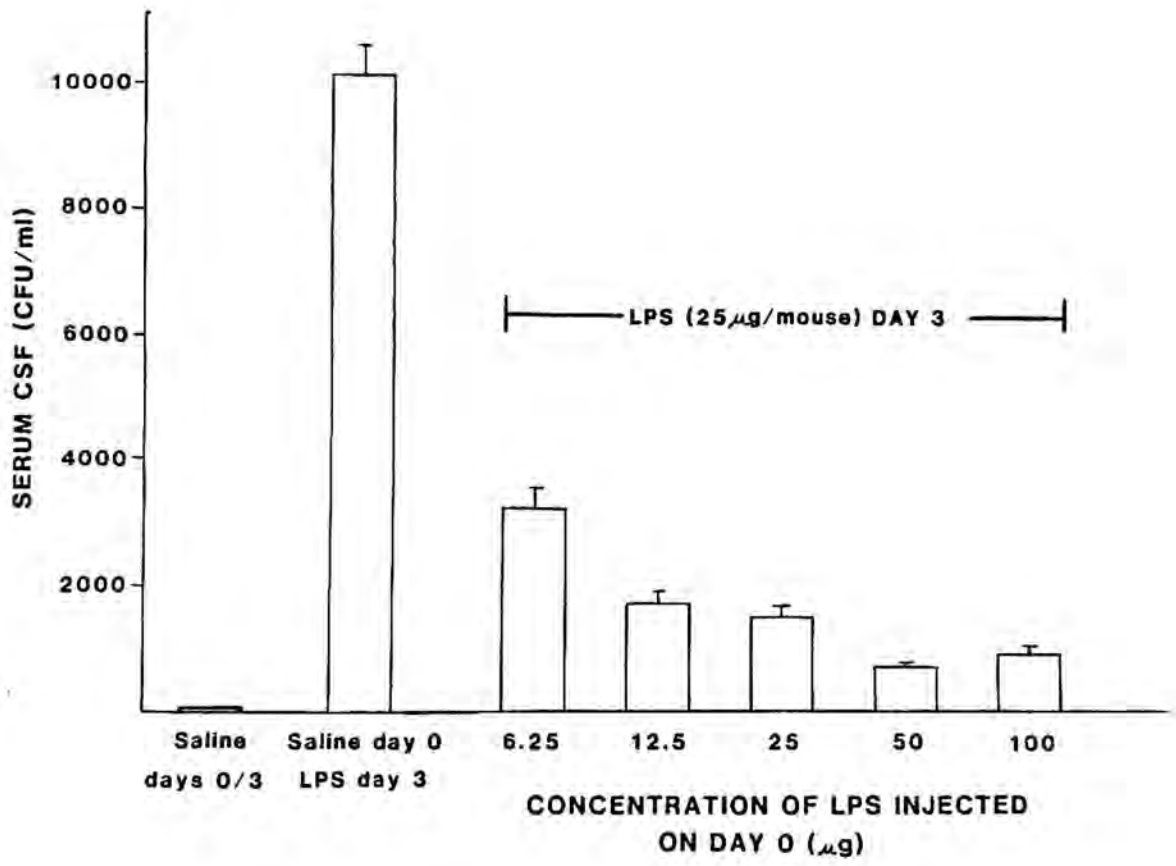

SERUM CSF (CFU/ml)
5000
4000
3000
2000
1000
0
E.coli LPS CONTROL
SALINE CONTROL
SALINE DAY 0
E. coli LPS DAY 0
1
2
3
4
5
6
7
8
NUMBER OF DAYS BETWEEN INITIAL INJECTION AND CHALLENGING DOSE OF LPS

Figure 2. Determination of the optimal LPS concentration for induction of early endotoxin tolerance -- ICR mice were injected i.p. with saline or varying concentrations of E. coli LPS. At 3 days post-injection, mice (5 mice/group) were challenged i.p. with a second injection of E. coli LPS (25 µg/mouse). Six hours later, the mice were bled and their sera pooled and assayed for CSF activity as described in the Materials and Methods section. Each point represents the arithmetic mean ± one standard deviation of duplicate determinations.


SERUM CSF (CFU/ml)
10000
8000
6000
4000
2000
LPS (25μg/mouse) DAY 3
Saline days 0/3
Saline day 0 LPS day 3
6.25
12.5
25
50
100
CONCENTRATION OF LPS INJECTED ON DAY 0 (μg)

experiments was indeed optimal for the production of serum CSF in response to the particular preparation of E. coli K235 LPS used in this study, a 24 h time course was additionally performed. Groups of 5 mice were injected on day 0 with saline or E. coli LPS (25 μg/mouse) i.p. Three days later, these mice were challenged with 25 μg LPS and were bled at various times (0-24 h) thereafter. The results in Figure 3 confirmed the previous findings of Metcalf (1971) that 6 h post-injection of LPS was optimal for detection of serum CSF in control (saline pre-treated) mice. In tolerized mice, i.e., those which had received LPS on day 0, the CSF response was reduced throughout the 24 h time period following the challenge (day 3) injection.

The experiments described thus far utilized CSF production as an indicator of endotoxin responsiveness in vivo. To insure that the apparent induction of endotoxin tolerance was not unique or specific for this particular manifestation of endotoxin sensitivity, the serum samples assayed in Figure 3 for CSF activity were also assayed for the presence of interferon (IFN). Previous studies have shown that administration of LPS to endotoxin-responsive animals results in a sharp rise in serum IFN which reaches maximum levels at approximately 2 h post-administration (Youngner and Stinebring, 1965). The results in Figure 4 illustrate that control mice (i.e., those that received saline on day 0) responded normally to LPS with peak IFN levels at 2 h post-injection of LPS. Serum from tolerized mice, however, yielded no detectable levels of IFN throughout the 24 h time period following

Figure 3. 24-hour time course for serum CSF production in non-tolerized and tolerized mice -- ICR mice were injected i.p. with saline or E. coli LPS (25 μg/mouse). After 3 days, the mice (5 mice/group) were challenged with E. coli LPS (25 μg/mouse) and bled at the indicated times. Sera from each group were pooled and assayed for CSF activity as described in the Materials and Methods section. Each point represents the arithmetic mean ± one standard deviation of duplicate determinations.

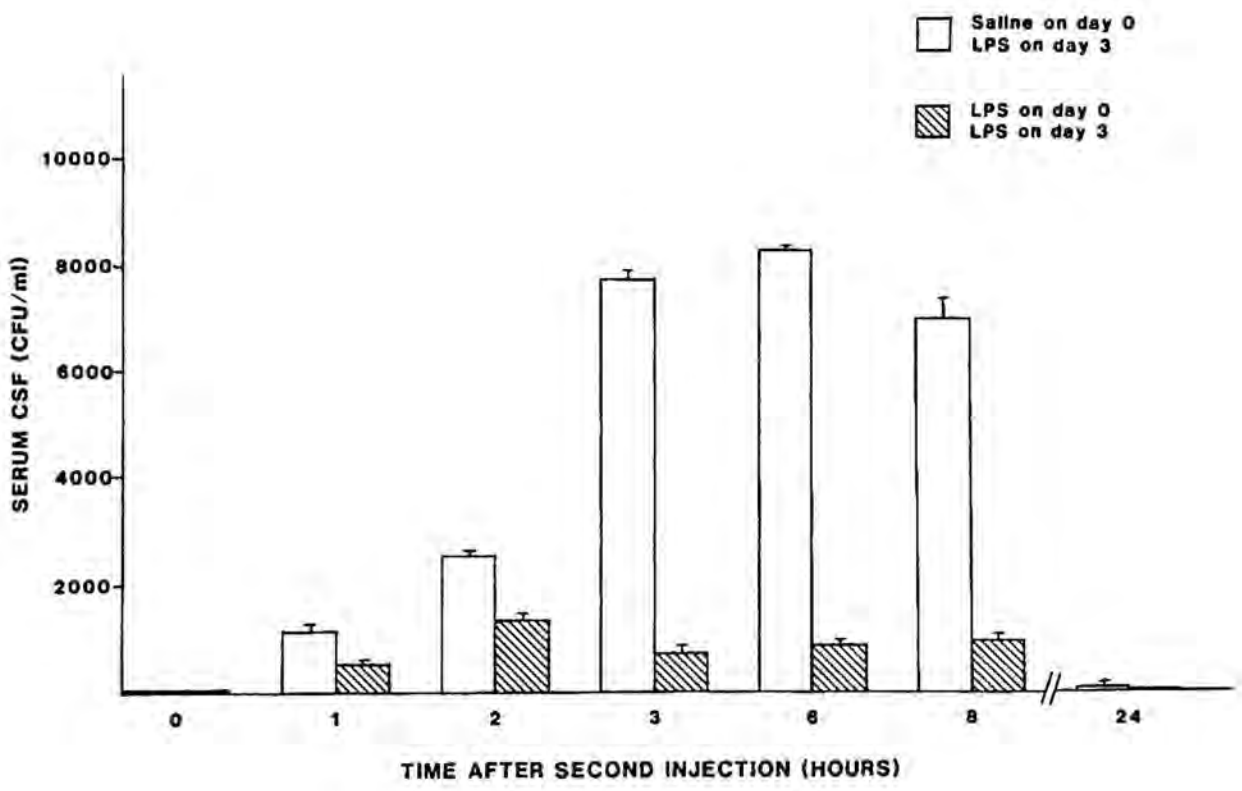
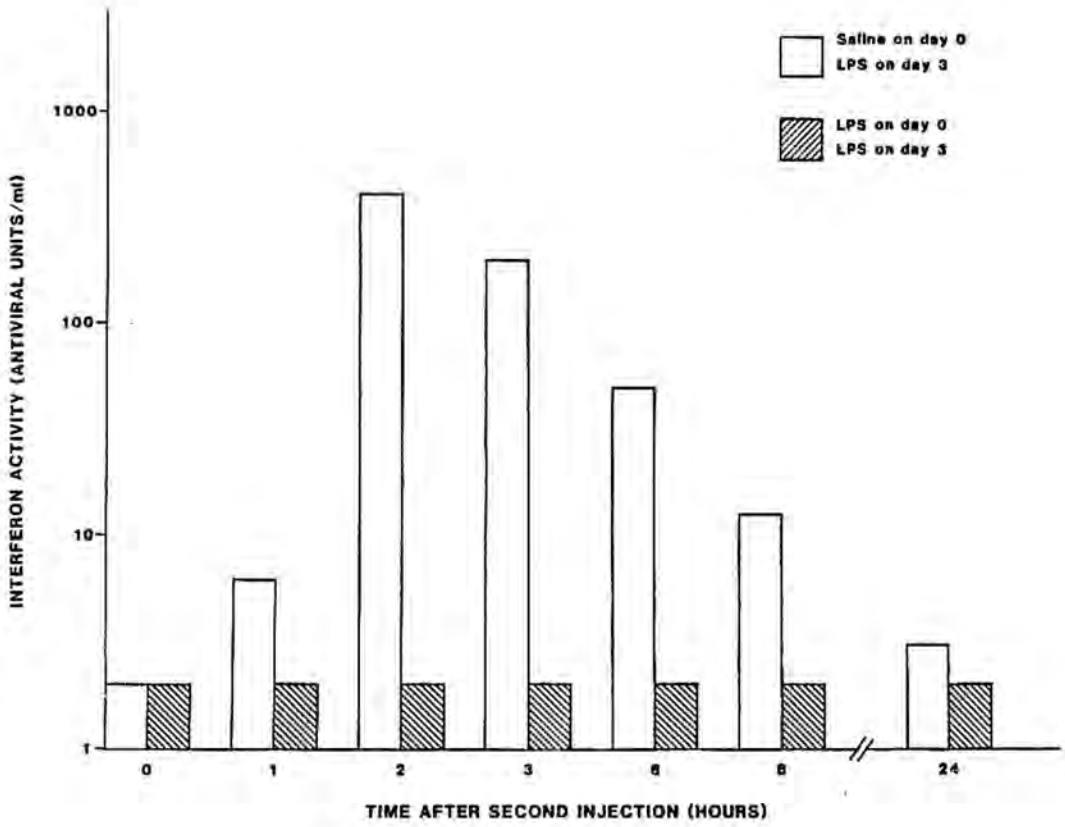

Saline on day 0
LPS on day 3
LPS on day 0
LPS on day 3
SERUM CSF (CFU/ml)
10000
8000
6000
4000
2000
0
1
2
3
6
8
24
TIME AFTER SECOND INJECTION (HOURS)

Figure 4. 24-hour time course for interferon induction in non-tolerized and tolerized mice -- Pooled serum samples, obtained from mice treated as described in the legend of Figure 3, were assayed for the presence of interferon as described in the Materials and Methods section. Results represent the antiviral activity contained in serum pools obtained from 5 mice per treatment group.


Saline on day 0
LPS on day 3
LPS on day 0
LPS on day 3
INTERFERON ACTIVITY (ANTIVIRAL UNITS/ml)
1000
100
10
1
0
1
2
3
6
8
24
TIME AFTER SECOND INJECTION (HOURS)

challenge.

Further confirmation of the efficacy of this tolerizing regimen was established using LPS-induced lethality and symptoms as a measure of endotoxin sensitivity. Exposure to an initial dose of 25 µg LPS/mouse more than doubled the LD_{50} (414 µg non-tolerized vs. 994 µg tolerized) and essentially eliminated overt symptoms of endotoxicity (i.e., diarrhea, ruffled fur, and conjunctival discharge) which are normally observed by 6 h after challenge with a sublethal dose of LPS (25 µg/mouse). These data are shown in Table I.

Since one of the characteristics of early endotoxin tolerance is that it is inducible with unrelated LPS preparations (i.e., it is not O-antigen-specific; Greisman and Hornick, 1976), groups of mice were injected with saline or E. coli LPS (25 µg/mouse) on day 0, but challenged on day 3 with 25 µg LPS/mouse of either homologous E. coli LPS or heterologous LPS preparations derived from Pseudomonas aeruginosa, Salmonella typhimurium or Bacteroides fragilis. Table II shows that an initial exposure on day 0 to E. coli LPS markedly reduced the level of CSF which was induced by challenge with any of the LPS preparations 3 days later.

Failure of the purified polysaccharide derived from E. coli K235 LPS to induce early endotoxin tolerance -- Although previous studies had indicated that Lipid A, the biologically active moiety of the LPS molecule, was capable of inducing a state of endotoxin tolerance (Staber, 1980), it had not been

Table I

Effect of early phase endotoxin tolerance on clinical endotoxic manifestations

Experimental group[a]	LD_{50} (µg)[b]	Symptoms[c]		
		Diarrhea	Ruffled fur	Conjunctival discharge
Non-tolerized	414±55	9/16	13/16	11/16
Tolerized	994±92	0/16	0/16	0/16

[a] Mice were injected i.p. on day 0 with saline (non-tolerized) or E. coli K235 LPS (25 µg/mouse; tolerized). Three days later, the mice were challenged with E. coli LPS for LD_{50} determination and assessment of symptoms as described in the Materials and Methods section.

[b] The LD_{50} was calculated by the method of Reed and Muench (1939). Results represent the arithmetic mean ± one standard deviation of two separate LD_{50} experiments.

[c] The proportion of mice which clearly exhibited the indicated symptom 6 h after the injection of 25 µg LPS on day 3.

Table II

Effect of initial exposure to E. coli LPS on CSF induced by subsequent challenge with homologous and heterologous LPS preparations[a]

Treatment on day 0	Serum CSF (CFU/ml) Species of LPS Injected on Day 3			
	E.coli	P.aeruginosa	S.typhimurium	B.fragilis
Saline	3,003 ± 450	3,050 ± 361	2,975 ± 136	820 ± 113
E. coli LPS	450 ± 14	380 ± 74	190 ± 70	0

[a]Groups of 5 ICR mice were injected i.p. with either E. coli LPS (25 µg/mouse) or saline on day 0 and again on day 3 with either homologous E. coli LPS or a heterologous LPS preparation (25 µg/mouse) as indicated. Six hours after the day 3 injection, the mice were bled and sera assayed for CSF. The results represent the arithmetic mean ± one standard deviation of duplicate determinations.

demonstrated previously that the polysaccharide moiety was inactive as a tolerogen. This issue was particularly relevant since "purified" polysaccharide derived from LPS using the procedure of Freeman (1942) had been used to induce certain LPS-mediated responses, including the induction of CSF (Urbaschek et al., 1980). In this study, the polysaccharide was prepared by mild acid hydrolysis of the phenol-water extracted E. coli K235 LPS and was separated from residual LPS by gel chromatography, as described in the Materials and Methods section. The concentration of polysaccharide was adjusted by total glucose content to be equivalent to the glucose content of 25 µg E. coli K235 LPS (70 nmoles). When administered to mice on day 0, it was found that the polysaccharide moiety was non-toxic, unable to induce serum CSF, and failed to induce tolerance to LPS as assessed by serum CSF or symptoms of endotoxicity (Table III).

EARLY ENDOTOXIN TOLERANCE IS ASSOCIATED WITH ALTERATIONS IN BONE MARROW-DERIVED MACROPHAGE PRECURSOR POOLS

Effect of early endotoxin tolerance on the elicitation of peritoneal exudate macrophages -- Previous studies have indicated that macrophages play a central role in endotoxic reactions through the production of soluble immunoregulatory mediators such as Interleukin 1 and CSF (reviewed in Rosenstreich and Vogel, 1980; Vogel and Mergenhagen, 1982). Since it had been shown that: (1) relative LPS sensitivity

Table III

Effect of purified polysaccharide derived from E. coli LPS on induction of CSF or early endotoxin tolerance

Treatment[a]		Serum CSF (CFU/ml)[b]
Day 0	Day 3	
Saline	Saline	0
Saline	LPS	3,490 ± 199
LPS	LPS	600 ± 187
Saline	PS	0
PS	PS	0
PS	LPS	4,100 ± 891

[a]Groups of 5 ICR mice were injected i.p. on day 0 and day 3 with either saline, E. coli LPS (25 µg/mouse), or polysaccharide (PS) derived from E. coli LPS. LPS and PS were equilibrated by total glucose content as described in the Materials and Methods section.

[b]Six hours after the day 3 injection, the mice were bled and the sera assayed for CSF activity. The results represent the arithmetic mean ± one standard deviation of duplicate determinations.

correlated with the differentiation state of the macrophage (Suter et al., 1958; Benacerraf et al., 1959; Moore et al., 1980; Rosenstreich and Vogel, 1980; Vogel et al., 1980) and (2) macrophages from endotoxin-tolerized mice were refractory to LPS stimulation in vitro (Dinarello et al., 1968; Rietschel et al., 1980), we had originally planned to examine the differentiation state of peritoneal exudate macrophages from endotoxin-tolerized mice. When thioglycollate was administered to mice between the initial and challenge LPS injections (Williams et al., 1983), the peritoneal exudate cells recovered from mice which had been injected twice with LPS were strikingly reduced in the proportion of macrophages which were elicited in control mice (Table IV).

Changes in the number of bone marrow-derived macrophage progenitor cells after a single injection of LPS -- It has been established that mononuclear phagocytes originate from precursors in the bone marrow (Volkman and Gowans, 1965). Therefore, the reduction in peritoneal exudate macrophages in mice injected twice with LPS suggested that such mice might be blockaded in macrophage development. A blockade in development could result in an accumulation of immature precursors in the bone marrow. To test this, an 8 day time course was carried out. Groups of mice were injected with either saline or E. coli LPS on day 0, and, on successive days, the pooled bone marrow cells from the femurs of each group were enumerated and plated in an excess of CSF-1. CSF-1 has been shown to be

Table IV

Effect of LPS administration on recovery of thioglycollate-induced peritoneal exudate cells

Experimental Group[a]	Percent of White Blood Cells Recovered[b]			
	Macrophages	Neutrophils	Lymphocytes	Other
Saline control (Saline/Saline)	84	10	5	1
Non-tolerized (Saline/LPS)	76	22	1	1
Tolerized (LPS/LPS)	26	72	1	1

[a] Mice were treated on day 0 and on day 3 as indicated by the order of treatments in parentheses. Thioglycollate was administered on day 1 and peritoneal exudate cells collected by lavage on day 3, 3 h after the second injection as described by Williams et al. (1983).

[b] Cytospin preparations were fixed in methanol and were stained using a modified Wright's stain. Differential counts were performed on 200 cells per slide under oil immersion. Results

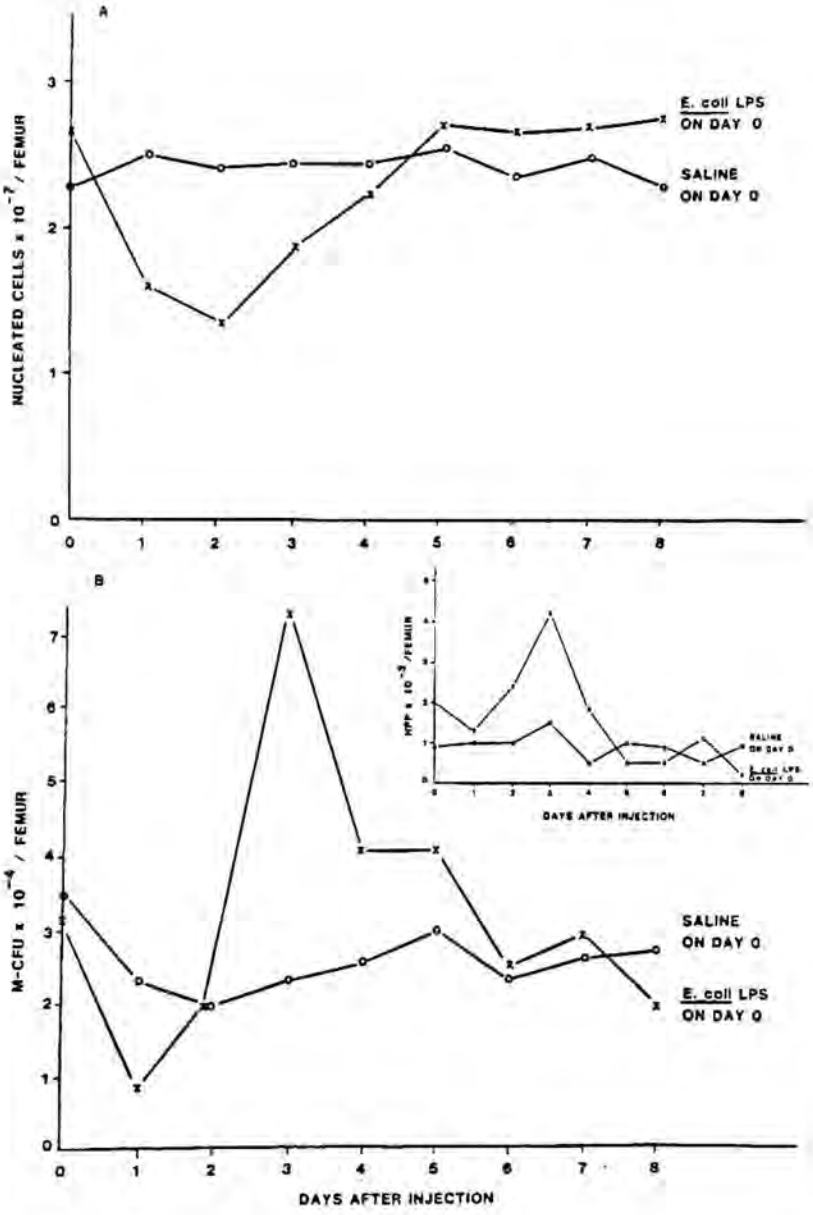
represent the arithmetic mean of differential counts derived from two separate experiments.

specific for cells of macrophage lineage and to give rise exclusively to macrophage colonies in culture (Stanley and Guilbert, 1980). Those precursors which respond to CSF-1 to form colonies are referred to as macrophage colony forming units (M-CFU).

A single injection of LPS on day 0 resulted in an initial decrease in the total number of nucleated cells per femur on days 1 and 2 post-injection, followed by a return to normal levels over the next 3 days (Figure 5A). Figure 5B shows that the number of macrophage progenitors (M-CFU), adjusted for the total nucleated cell count from each group, was greatly increased by the third day after LPS injection and returned to normal by day 8. Injection of saline on day 0 produced no significant change in the number of M-CFU per femur over the 8 day period. Additional studies in which mice were injected i.p. with 3 ml/mouse of the sterile inflammatory agent, thioglycollate, and the bone marrow cells cultured for M-CFU on day 3, showed no such increase in the number of macrophage progenitors when compared to saline-injected mice (saline-injected = $2.1 \times 10^4 \pm 3.9 \times 10^3$ M-CFU/femur vs. thioglycollate-injected = $2.7 \times 10^4 \pm 4.8 \times 10^3$ M-CFU/femur).

A more primitive population of macrophage progenitors with a higher proliferative potential (HPP) than M-CFU, responds to CSF-1 only in the presence of a synergistic co-factor, referred to as "Synergistic Activity" (Bradley and Hodgson, 1975). The insert in Figure 5B shows that, like the more mature progenitor population (M-CFU), the number of HPP

Figure 5. Effect of a tolerizing injection of LPS on the number of nucleated cells and macrophage precursors in the bone marrow -- ICR mice (5 mice/group) were injected i.p. on day 0 with either saline or E. coli LPS (25 μg/mouse) and the bone marrow cells from each group were collected and pooled at the indicated times thereafter. Nucleated cell counts for each bone marrow cell suspension were obtained using a Coulter Counter (Figure 5A). Five X 10^4 cells from each cell suspension were then plated in duplicate in an excess of CSF-1 and the number of M-CFU determined (Figure 5B) as described in the Materials and Methods section. Simultaneously, 5 X 10^4 cells were plated in an excess of CSF-1 plus human spleen conditioned medium (as a source of synergistic activity) for determination of the number of HPP colonies (Figure 5B, insert).


A
NUCLEATED CELLS x 10^{-7} / FEMUR
E. coli LPS
ON DAY 0
SALINE
ON DAY 0
B
M-CFU x 10^{-4} / FEMUR
HPP x 10^{-3} /FEMUR
DAYS AFTER INJECTION
SALINE
ON DAY 0
E. coli LPS
ON DAY 0
DAYS AFTER INJECTION

precursors was also increased by day 3 after injection of LPS and the kinetics of their appearance (and disappearance) was similar to the observed alterations in M-CFU numbers.

The number of M-CFU in spleens from tolerized mice was also greatly increased. In the spleens of mice injected 3 days previously with E.coli LPS, the number of M-CFU was approximately 14-fold greater than in control mice (saline-injected, 4×10^3 M-CFU/spleen; LPS-injected, 5.7×10^4 M-CFU/spleen).

Changes in bone marrow cell sizing profiles following a single injection of LPS -- Cell sizing profiles of the various bone marrow cell suspensions were obtained using a Coulter Channelyzer. When control bone marrow cells (day 3, post-saline) were analyzed, a bimodal distribution of cells was observed (Figure 6). Control Peak 1 accounted for 35-40% of the total cells analyzed and consisted of smaller cells (average diameter = 7.5 μ) relative to those in Peak 2 (average diameter = 10 μ) which accounted for the remaining 55-60% of cells. In contrast, a marked reduction in the relative number of bone marrow cells in Peak 1 (20% of total) occurred in tolerized mice (day 3, post-LPS) and there was a compensatory increase in the number of cells within Peak 2 (80%). In addition, the Peak 2 cells from tolerized mice were larger than those in the Control Peak 2 (average diameter = 11.4 μ; as indicated by the shift to the right on the sizing profile). Figure 7 indicates that the acquisition and loss of this cell

Figure 6. Effect of a tolerizing injection of LPS on bone marrow cell sizing profiles -- ICR mice (5 mice/group) were injected i.p. on day 0 with either saline or E. coli LPS (25 µg/mouse). At 3 days post-injection, the mice were re-injected with saline or E. coli LPS (25 µg/mouse) and sacrificed immediately. Bone marrow cells were then pooled from each group and cell sizing profiles obtained using a Coulter Channelyzer.

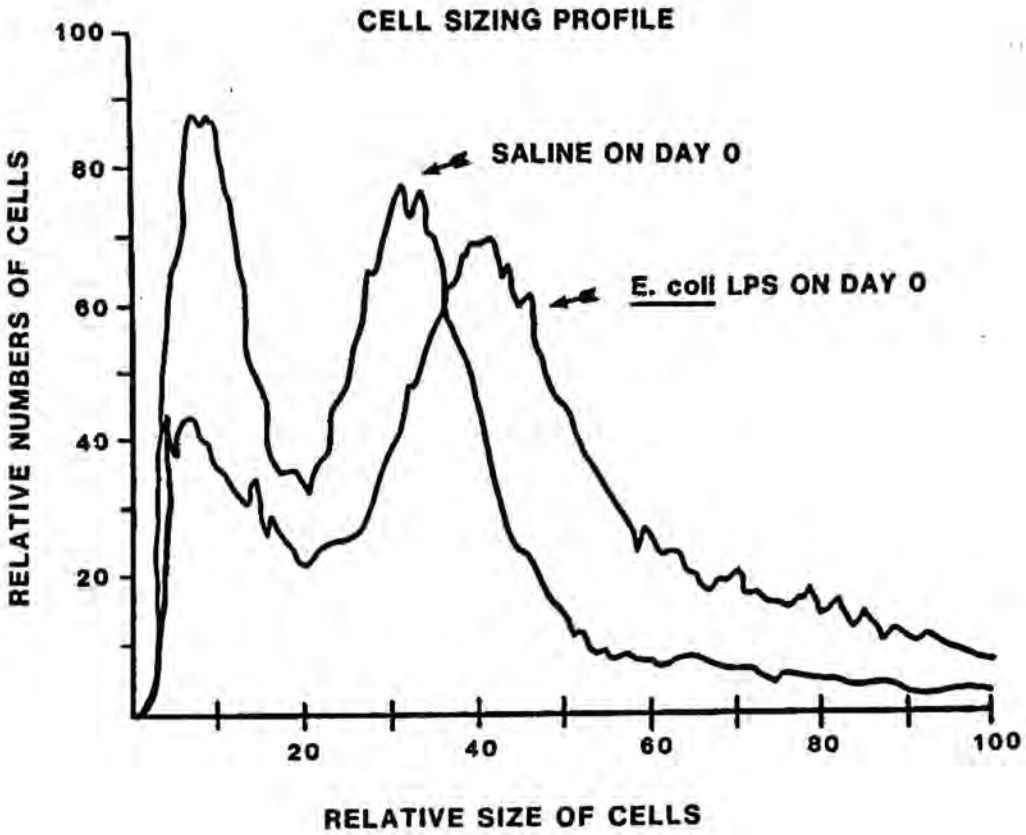
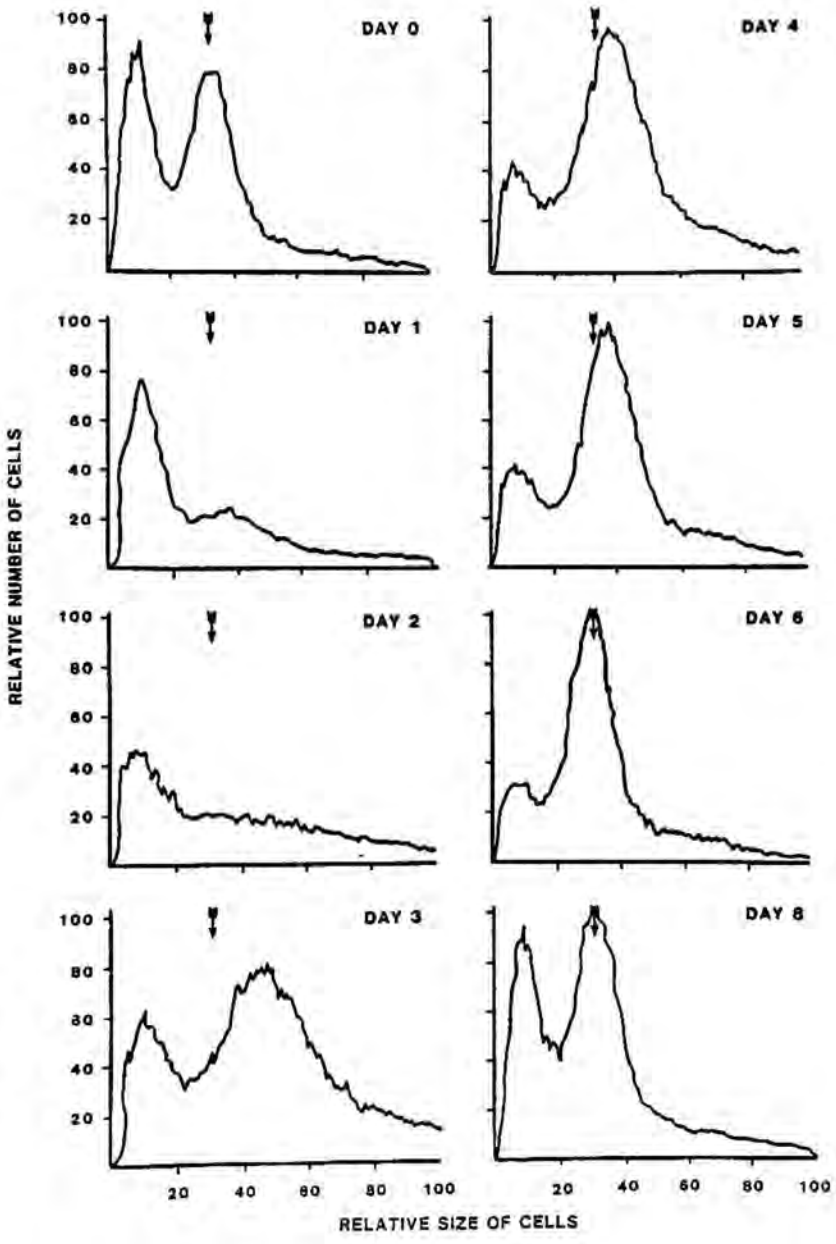

CELL SIZING PROFILE
SALINE ON DAY 0
E. coli LPS ON DAY 0
RELATIVE NUMBERS OF CELLS
RELATIVE SIZE OF CELLS
100
80
60
40
20
20
40
60
80
100

Figure 7. Cell sizing profiles of bone marrow cells obtained at various times after LPS injection -- ICR mice (5 mice/group) were injected i.p. with E. coli LPS (25 μg/mouse) and sacrificed at the indicated times after injection. Cell sizing profiles of bone marrow cells pooled from each group were obtained using a Coulter Channelyzer. The arrow indicates the initial position of Peak 2.


DAY 0
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 8
RELATIVE NUMBER OF CELLS
RELATIVE SIZE OF CELLS
100
80
60
40
20
20
40
60
80
100

sizing profile correlated temporally with the acquisition, maintenance, and loss of tolerance (see Figure 1).

Density gradient separation of mouse bone marrow cells -- To determine whether a relationship existed between the cell sizing profile in tolerized mice (day 3, post-LPS) and the increased number of M-CFU also seen at this time, Peak 1 was separated from Peak 2 by density gradient sedimentation and the numbers of M-CFU in individual fractions determined (Figure 8). All of the M-CFU activity was restricted to the dense fractions (i.e., Peak 2) in both saline- and LPS-injected mice. Based on two separate experiments, the total number of M-CFU (obtained by adding the M-CFU values determined for individual density gradient fractions) from tolerized mice was >2.5 times greater than obtained from control mice. To verify the relative positions of Peak 1 and Peak 2 after density gradient sedimentation, pooled fractions were re-analyzed by Coulter Channelyzer (Figure 9). Thus, the results of Figures 8 and 9 indicate that all of the M-CFU activity was found in Peak 2, and that the increase observed in unfractionated, tolerized bone marrow cells was reflected in the separated fractions. In addition, the distribution of HPP precursors was also measured in individual density gradient fractions and also correlated positionally with Peak 2 as well as with M-CFU activity (Figure 8).

When differential counts of cytospin preparations of the pooled, density gradient-separated Peak 2 cells were

Figure 8. Density gradient separation and measurement of M-CFU and HPP activity -- ICR mice (5 mice/group) were injected i.p on day 0 with either saline or E. coli LPS (25 μg/mouse). On day 3, bone marrow cells were collected and 1 X 10^7 cells from each group were separated by density gradient sedimentation as described in the Materials and Methods section. Fractions (3 ml/fraction) were collected from the bottom of the gradient after 3.5 h. Cells from each fraction were enumerated and then cultured in the presence of CSF-1 or CSF-1 plus human spleen conditioned medium (as a source of synergistic activity) to determine the numbers of M-CFU and HPP, respectively. The first fractions collected had to be pooled in order to obtain adequate numbers of cells for culture. M-CFU and HPP determinations were carried out in duplicate as described in the Materials and Methods section.

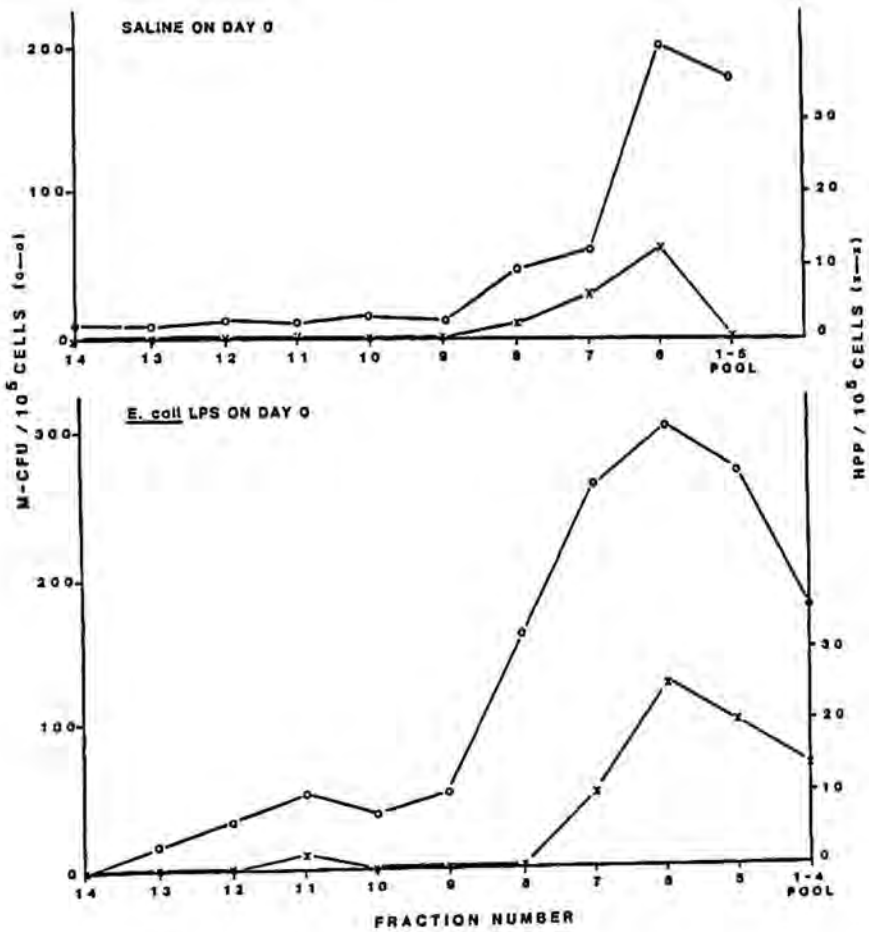
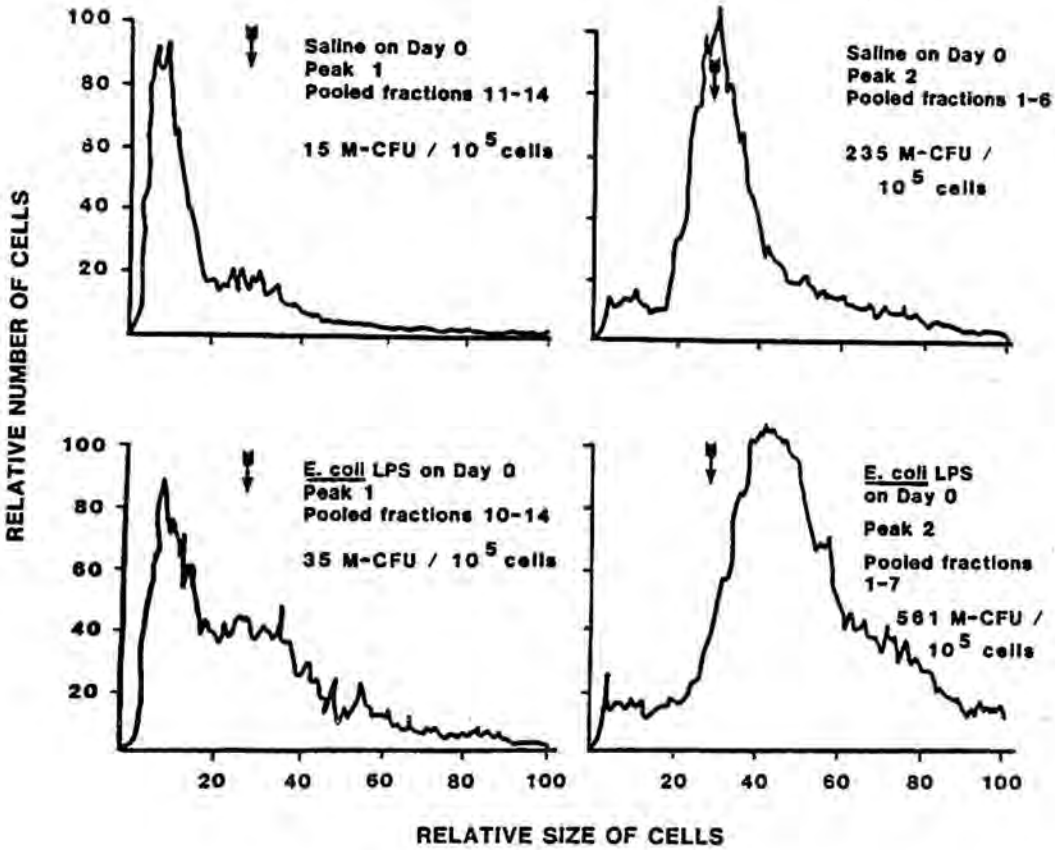

SALINE ON DAY 0
E. coli LPS ON DAY 0
M-CFU / 10^5 CELLS (o—o)
HPP / 10^5 CELLS (x—x)
FRACTION NUMBER
POOL
1-5
1-4

Figure 9. Cell sizing profiles of pooled density gradient-separated bone marrow cells -- Cells from each fraction (see Figure 8) were sized using a Coulter Channelyzer and then pooled according to profile similarities with unseparated Peak 1 vs. Peak 2. Cell sizing profiles were then obtained on the pooled fractions. The arrow indicates the position of control Peak 2. Cells from the pooled fractions were enumerated and cultured in the presence of CSF-1 and the number of M-CFU determined in duplicate as described in the Materials and Methods section.


RELATIVE NUMBER OF CELLS
100
80
60
40
20
Saline on Day 0
Peak 1
Pooled fractions 11-14
15 M-CFU / 10^5 cells
Saline on Day 0
Peak 2
Pooled fractions 1-6
235 M-CFU / 10^5 cells
E. coli LPS on Day 0
Peak 1
Pooled fractions 10-14
35 M-CFU / 10^5 cells
E. coli LPS on Day 0
Peak 2
Pooled fractions 1-7
561 M-CFU / 10^5 cells
20
40
60
80
100
RELATIVE SIZE OF CELLS

performed (Table V), cells derived from tolerized mice were enriched for the number of large mononuclear cell types. These latter cells contained a large nucleus (nuclear:cytoplasmic ratio >1) and the cytoplasm was strongly basophilic, suggesting the monoblast-promonocyte stage of macrophage development (Sumner et al., 1972; Goud et al., 1975).

Changes in the number of M-CFU after a second injection of LPS -- Based on the observation that a single injection of E. coli LPS resulted in profound changes in the numbers of bone marrow-derived macrophage progenitors which correlated temporally with maximum tolerance induction, the effect of a second (challenge) injection of LPS on M-CFU levels was also investigated. Groups of mice were injected with either saline or LPS on day 0 and again on day 3. After the second injection, bone marrow cells from each group were collected at 0, 3, and 24 h post-injection. Figure 10 demonstrates that in mice which received LPS on day 3 for the first time, a marked decrease was observed in both the number of nucleated cells (data not shown; refer to Figure 5A) and M-CFU within 24 h post-LPS (refer back to Figure 5B). In mice which received LPS for a second time on day 3, only a slight decrease in the number of nucleated cells was observed (from 9.8 to 7.5 x 10^6 cells/femur at 24 h); however, the already elevated level of M-CFU/femur was maintained (Figure 10).

Effect of repeated injections of E. coli LPS -- Since

Table V

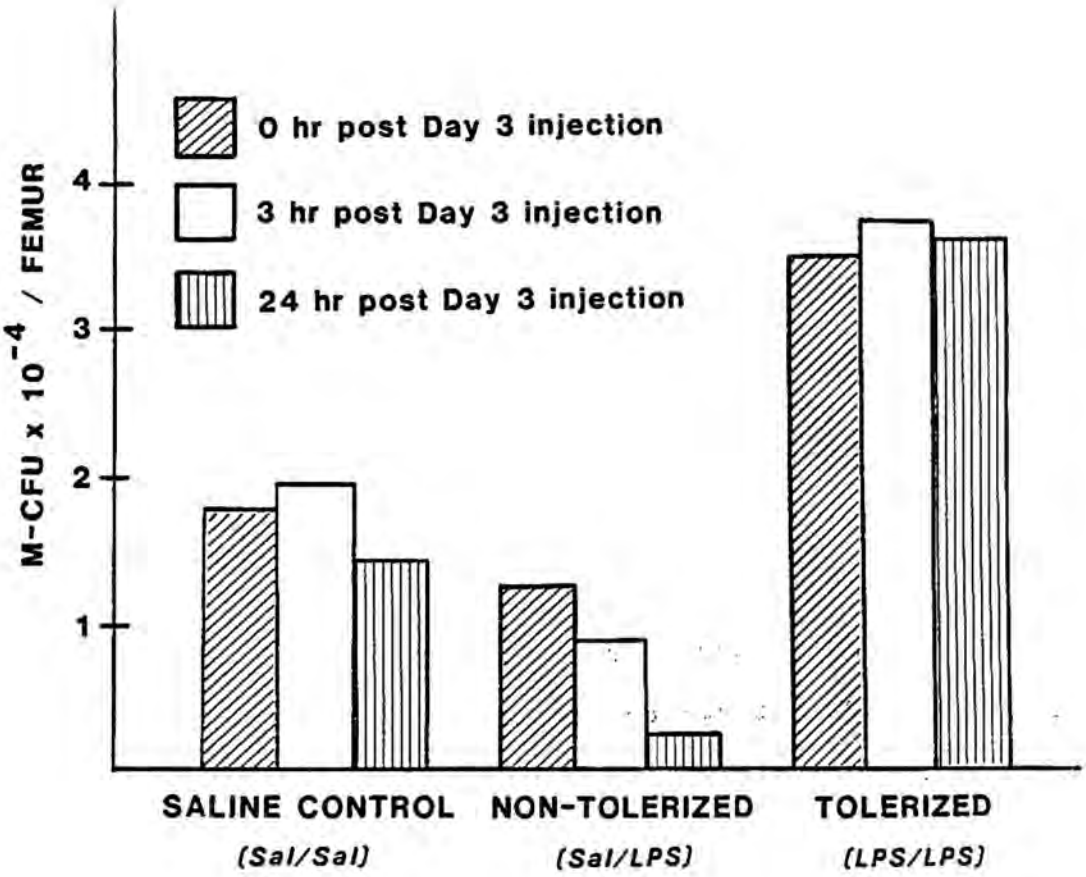
Differential counts of cytospin preparations of density gradient - separated Peak 2 cells[a]

	Treatment Group	
	Control	Tolerized
Polymorphonuclear cells	48.5%[b]	16.0%
Small mononuclear cells (10-15 μ diameter)	50.5%	43.3%
Large mononuclear cells (15-21 μ diameter)	1.0%	41.0%

[a]Peak 2 bone marrow cells were isolated and pooled by density gradient sedimentation on day 3 after the injection of saline (control) or 25 μg/mouse E. coli LPS (tolerized) on day 0.

[b]Cytospin preparations of pooled Peak 2 cells were fixed in methanol and stained using a modified Wright's stain. Differential counts (200 cells per slide) were obtained under oil immersion.

Figure 10. Effect of a second injection of E. coli LPS on the number of bone marrow-derived M-CFU -- ICR mice (5 mice/group) were injected i.p. on day 0 with either saline or E. coli LPS (25 μg/mouse). On day 3, the mice injected with saline on day 0 were injected again with either saline (control) or LPS (non-tolerized) and those injected initially with LPS were re-injected with LPS (tolerized). At 0, 3 and 24 h after the day 3 injection, the mice were sacrificed and the number of M-CFU determined in duplicate as described in Materials and Methods section.


0 hr post Day 3 injection
3 hr post Day 3 injection
24 hr post Day 3 injection
M-CFU x 10^{-4} / FEMUR
4
3
2
1
SALINE CONTROL
(Sal/Sal)
NON-TOLERIZED
(Sal/LPS)
TOLERIZED
(LPS/LPS)

early endotoxin tolerance was found to be optimal 3 days after initial exposure to LPS and the increased number of bone marrow M-CFU, also observed on day 3, was maintained for at least 24 h following a second injection of LPS (Figure 10), the effect of multiple LPS injections was investigated next. Groups of mice were injected with either saline or LPS one time (on day 0 only), twice (on days 0 and 3), or three times (on days 0, 3, and 6). To assess the effect of repeated injections of LPS on tolerance, mice were challenged with 25 μg E. coli K235 LPS 3 days after their last injection, and their serum collected 6 h post-challenge for measurement of CSF activity. The results in Figure 11A indicate that, with respect to serum CSF, endotoxin tolerance was maintained at the same level, regardless of whether the mice received one, two, or three "tolerizing" injections. Control mice injected with saline one, two, or three times responded normally to a single challenge injection of LPS to produce high levels of serum CSF.

In order to assess the effect of repeated LPS injections on the number of bone marrow M-CFU, mice injected with either saline or LPS were sacrificed 3 days after their last "tolerizing" injection and their bone marrow cells cultured in an excess of CSF-1. Figure 11B demonstrates that mice which received one previous injection of LPS responded with a dramatic increase in M-CFU 3 days later (compare with Figure 5B). However, with repeated injections of LPS, the number of M-CFU was diminished so that after 3 injections, M-CFU numbers were equivalent to saline-injected controls.

Figure 11A. Effect of repeated injections of E. coli LPS on the level of serum CSF following LPS challenge -- ICR mice (5 mice/group) were injected once, twice, or three times at three day intervals with saline or E. coli LPS (25 µg/mouse) and were challenged with 25 µg E. coli LPS 3 days after the last injection. Six hours later, the mice were bled, the sera from each group pooled and assayed for CSF activity. The data represent the arithmetic mean ± one standard deviation of duplicate determinations.

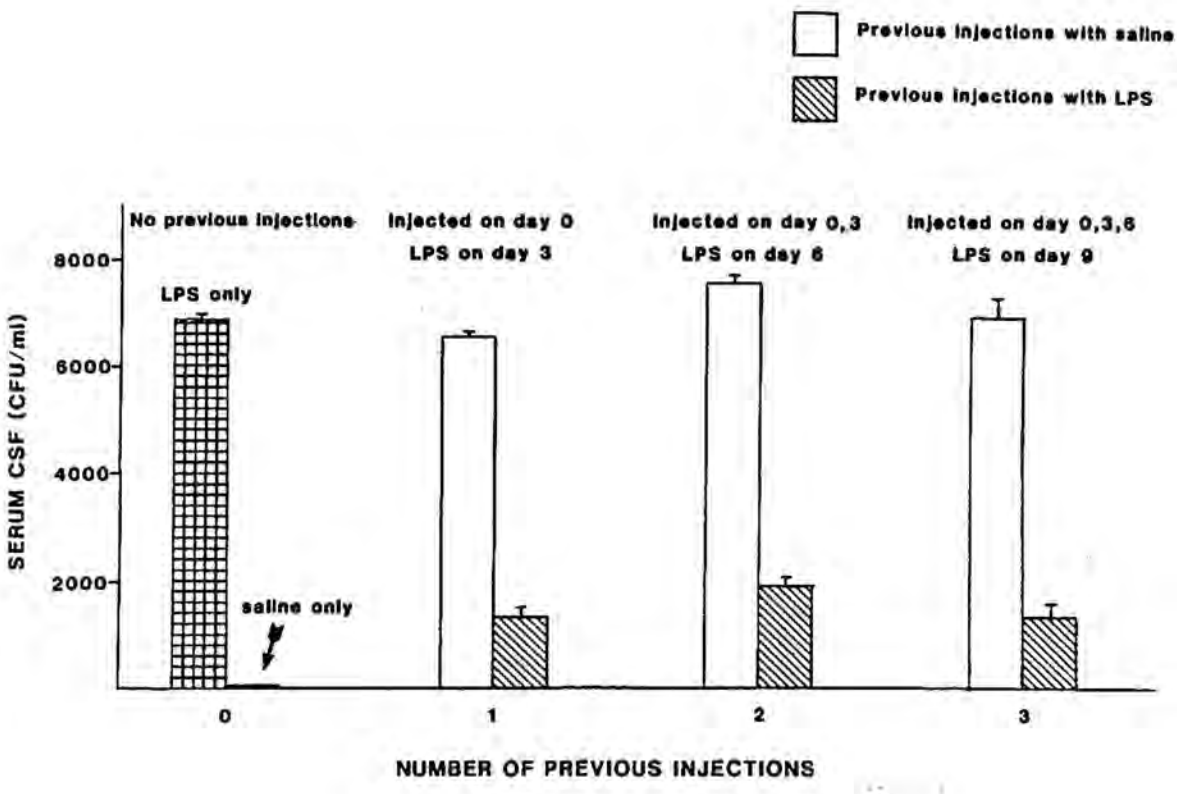
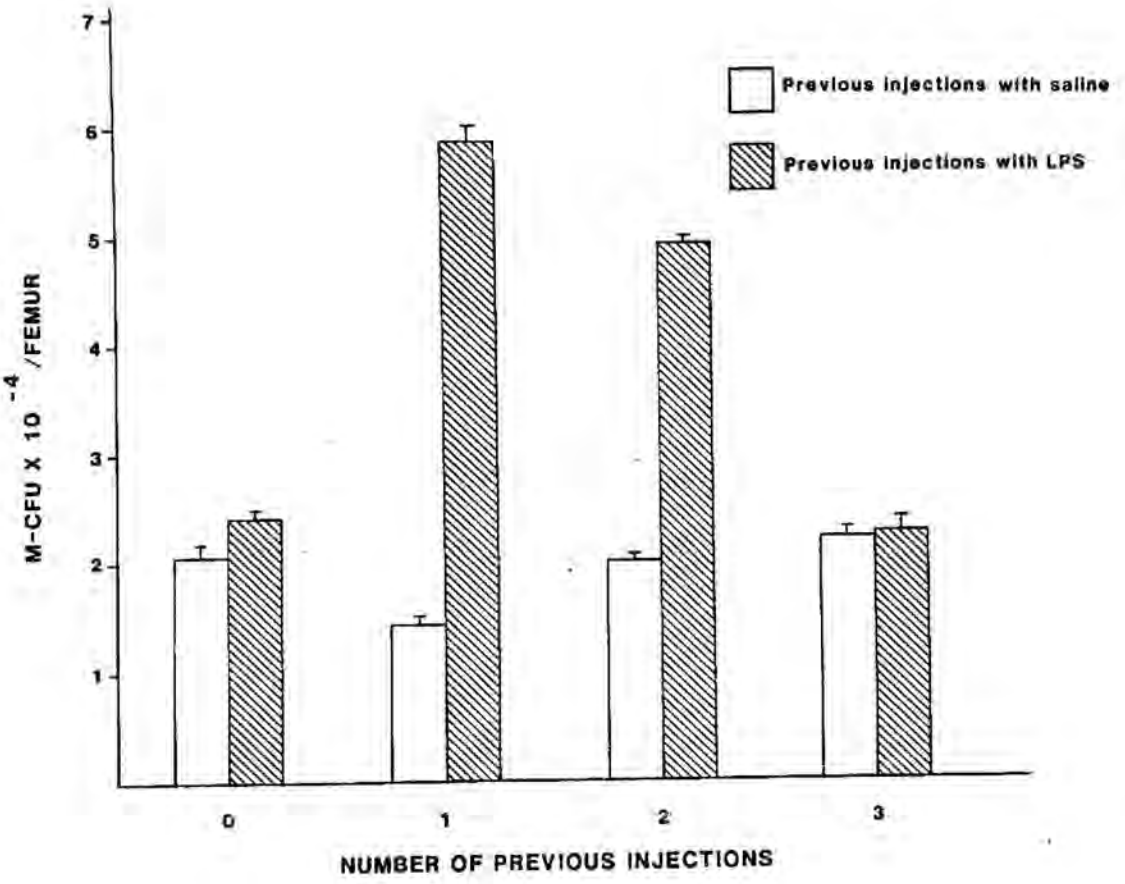

Previous injections with saline
Previous injections with LPS
No previous injections
LPS only
saline only
Injected on day 0
LPS on day 3
Injected on day 0,3
LPS on day 6
Injected on day 0,3,6
LPS on day 9
SERUM CSF (CFU/ml)
8000
6000
4000
2000
0
1
2
3
NUMBER OF PREVIOUS INJECTIONS

Figure 11B. Effect of repeated injections of E. coli LPS on the number of bone marrow-derived M-CFU -- ICR mice (5 mice/group) were injected once, twice, or three times at three day intervals with saline or E. coli LPS (25 μg/mouse). Mice were sacrificed 3 days after their last injection and bone marrow cells from each group pooled, enumerated, and the number of M-CFU determined as described in the Materials and Methods section. The data represent the arithmetic mean ± one standard deviation of duplicate determinations.


Previous injections with saline
Previous injections with LPS
M-CFU X 10 -4 /FEMUR
7
6
5
4
3
2
1
0
1
2
3
NUMBER OF PREVIOUS INJECTIONS

Cell sizing profiles of the various bone marrow suspensions were also compared using a Coulter Channelyzer. As can be seen in Figure 11C, one injection of LPS induced a marked reduction in the relative number of cells in Peak 1 (saline-injected = 40% of total cells; LPS-injected = 20% of total cells), and, a compensatory increase in the number of cells within Peak 2 (saline-injected = 60% of total cells; LPS-injected = 80% of total cells). This was accompanied by an increase in size in Peak 2. Repeated injections of LPS resulted in a marked decline in the average size of cells in Peak 2 (see Figure 6).

Effect of repeated injections of Pseudomonas aeruginosa LPS on serum CSF induction by E. coli K235 LPS -- Because early phase endotoxin tolerance is not O-antigen specific (Greisman and Hornick, 1976; see Table II), the previous series of experiments was modified such that groups of mice were injected once, twice, or three times with saline or Ps. aeruginosa LPS (25 µg/mouse). Three days after the last injection, groups of mice were either challenged with E. coli K235 LPS (for serum CSF) or sacrificed and their bone marrow cells analyzed for alterations in sizing profiles. Figure 12A illustrates that heterologous tolerance for E. coli LPS was induced by multiple injections of Ps. aeruginosa LPS, but on the basis of percent suppression of the control response, appeared to be somewhat less efficacious (Table VI). The bone marrow profiles in Figure 12B indicate that: (1) the "shift" in

Figure 11C. Effect of repeated injections of E. coli LPS on bone marrow cell sizing profiles -- ICR mice (5 mice/group) were repeatedly injected once, twice, or three times at 3 day intervals with saline or E. coli LPS (25 μg/mouse). Three days following the last injection, the mice were sacrificed, bone marrow cells obtained, and cell sizing profiles determined using a Coulter Channelyzer. The arrow indicates the relative position of Peak 2 bone marrow cells from control ICR mice.

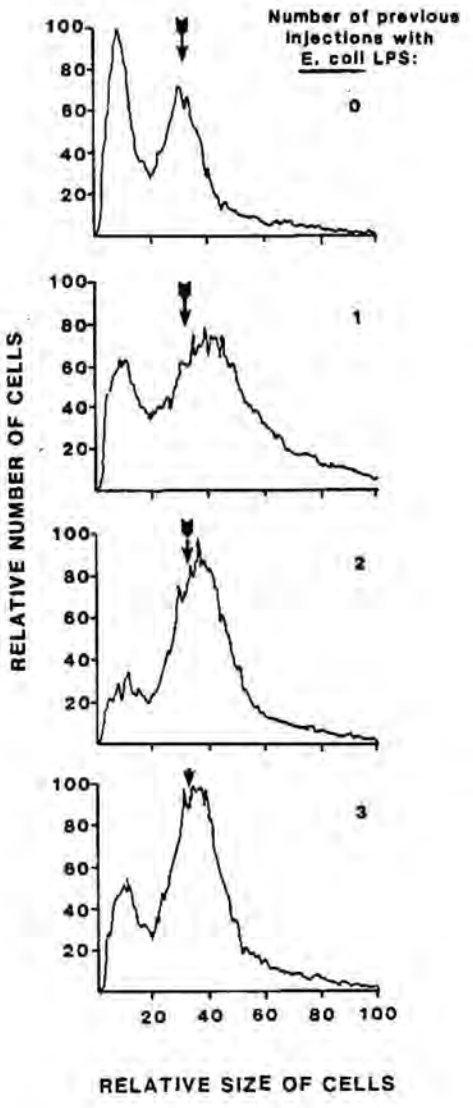

Number of previous injections with E. coli LPS:
0
1
2
3
100
80
60
40
20
RELATIVE NUMBER OF CELLS
20 40 60 80 100
RELATIVE SIZE OF CELLS

Figure 12A. Effect of repeated injections of Pseudomonas aeruginosa LPS on serum CSF induction by E. coli LPS -- ICR mice (5 mice/group) were injected with saline or Pseudomonas aeruginosa LPS (25 µg/mouse) once, twice, or three times at three day intervals and were challenged with E. coli LPS (25 µg/mouse) 3 days after the last injection. Six hours later, the mice were bled and the sera assayed for CSF activity. Each point represents the arithmetic mean ± one standard deviation of duplicate determinations.

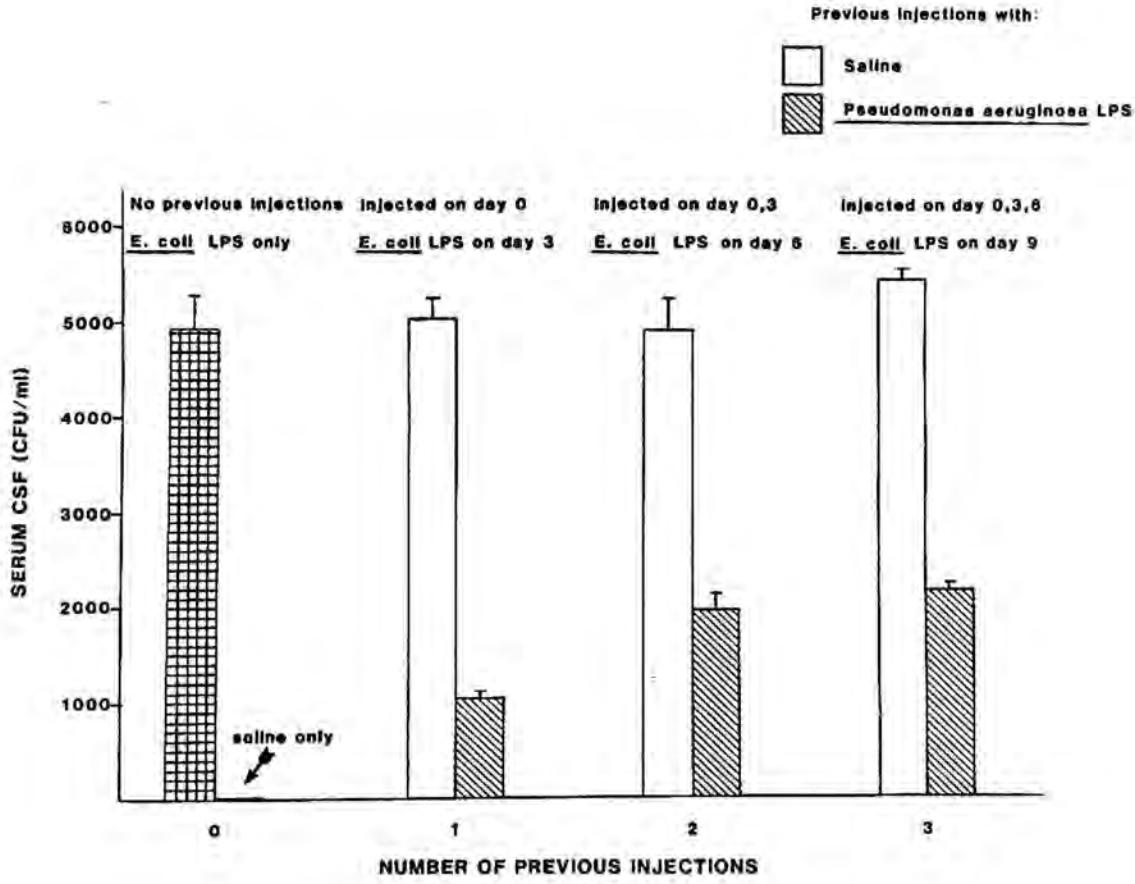

Previous injections with:
Saline
Pseudomonas aeruginosa LPS
No previous injections
E. coli LPS only
Injected on day 0
E. coli LPS on day 3
Injected on day 0,3
E. coli LPS on day 6
Injected on day 0,3,6
E. coli LPS on day 9
SERUM CSF (CFU/ml)
8000
5000
4000
3000
2000
1000
saline only
0
1
2
3
NUMBER OF PREVIOUS INJECTIONS

Table VI

Percent suppression of control serum CSF response in mice tolerized with homologous or heterologous preparations of LPS[a]

Treatment Group[b]	No. of LPS Injections Prior to Challenge with E.coli LPS		
	1	2	3
Homologous tolerance system (*E. coli* LPS)	80[c]	75	82
Heterologous tolerance system (*Ps. aeruginosa* LPS)	80	60	60

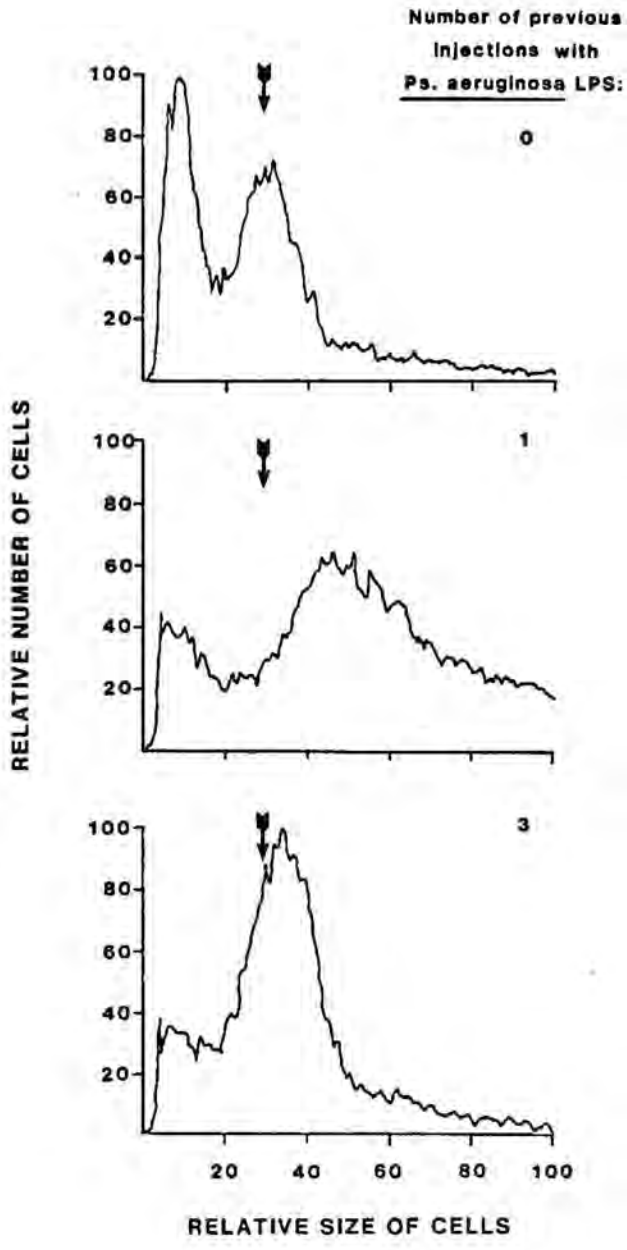
[a] This table is a summary of the data presented in Figures 12A (homologous endotoxin tolerance) and 13A (heterologous endotoxin tolerance).

[b] Mice were injected once, twice, or three times at 3 day intervals with saline, *E. coli* LPS, or *Ps. aeruginosa* LPS. Three days after the last injection, all mice were challenged with *E. coli* LPS.

[c] The data represent the percent suppression of the control CSF

response (saline-injected 1 to 3 times followed by challenge with *E. coli* LPS).

Figure 12B. Effect of repeated injections of Pseudomonas aeruginosa LPS on bone marrow cell sizing profiles -- ICR mice (5 mice/group) were injected with saline or Ps. aeruginosa LPS once, twice, or three times at three day intervals. Three days following the last injection, the mice were sacrificed, the bone marrow cells obtained, and cell sizing profiles determined using a Coulter Channelyzer. The arrow indicates the relative position of Peak 2 bone marrow cells from control (saline-injected) ICR mice.


Number of previous injections with Ps. aeruginosa LPS:
0
1
3
RELATIVE NUMBER OF CELLS
RELATIVE SIZE OF CELLS
100
80
60
40
20
20
40
60
80
100

Peak 2 seen three days after one injection of E. coli LPS was also seen after a single injection of Ps. aeruginosa LPS, and (2) the reduction in the average size of cells in Peak 2 observed after repeated injections of E. coli LPS was also observed in mice injected repeatedly with Ps. aeruginosa LPS (compare Figure 12B with Figure 11C).

INDUCTION OF EARLY ENDOTOXIN TOLERANCE IN OTHER MURINE MODELS

In order to gain a better understanding of the cellular mechanisms which underlie early phase endotoxin tolerance, several additional murine models were studied with respect to serum CSF induction, bone marrow M-CFU, or bone marrow cell sizing profiles after prior exposure to LPS.

Effect of tolerizing regimen on inbred mice which differ in their inherited sensitivity to LPS -- C3H/HeJ mice bear a single autosomal mutation, Lps^{d} (Watson et al., 1978), which renders this strain highly refractory to the effects of LPS both in vivo and in vitro (reviewed by Morrison and Ryan, 1979). Use of this naturally LPS-hyporesponsive strain in conjunction with syngeneic, fully LPS-responsive strains (Lps^{n}), has provided evidence that macrophages figure centrally in the induction of LPS-induced pathophysiologic changes associated with endotoxicity (reviewed by Rosenstreich and Vogel, 1980). We next sought to determine if any of these

changes were present in the LPS-hyporesponsive C3H/HeJ strain. C3H/HeJ (LPS-hyporesponsive) and syngeneic, LPS-responsive C3H/OuJ mice were subjected to the tolerizing regimen which had been established for ICR mice. Each mouse strain received either saline (control) or E. coli K235 LPS (25 µg/mouse) i.p. on day 0. On day 3, mice were challenged with E. coli LPS (25 µg/mouse), bled 6 h later, and sera assayed for CSF activity. The results in Table VII show that C3H/OuJ mice responded to LPS-challenge similarly to outbred ICR mice, i.e., a dramatic increase in serum CSF from control (saline-pretreated) mice and a mitigated CSF response in tolerized mice (i.e., those pretreated with LPS). In contrast, C3H/HeJ mice did not respond with detectable levels of serum CSF after either treatment.

Bone marrow cell sizing profiles of LPS-responsive (C3H/OuJ) and LPS-hyporesponsive (C3H/HeJ) mice were compared 3 days after injection of saline or LPS (Figure 13). Cell profiles from LPS-responsive C3H/OuJ mice, injected on day 0 with 25 µg LPS, showed a cell sizing profile similar to that seen in LPS-injected, outbred ICR mice, i.e., a dramatic increase in the average size and number of cells in Peak 2. In contrast, the cell sizing profile from LPS-hyporesponsive C3H/HeJ mice was not altered. One mg LPS per mouse was required to cause any significant alteration in the cell profile. Specifically, in response to saline, 25 µg or 100 µg LPS, Peak 1 comprised approximately 40% of the total cell population. In response to 1 mg LPS, Peak 1 comprised 33% of

Table VII

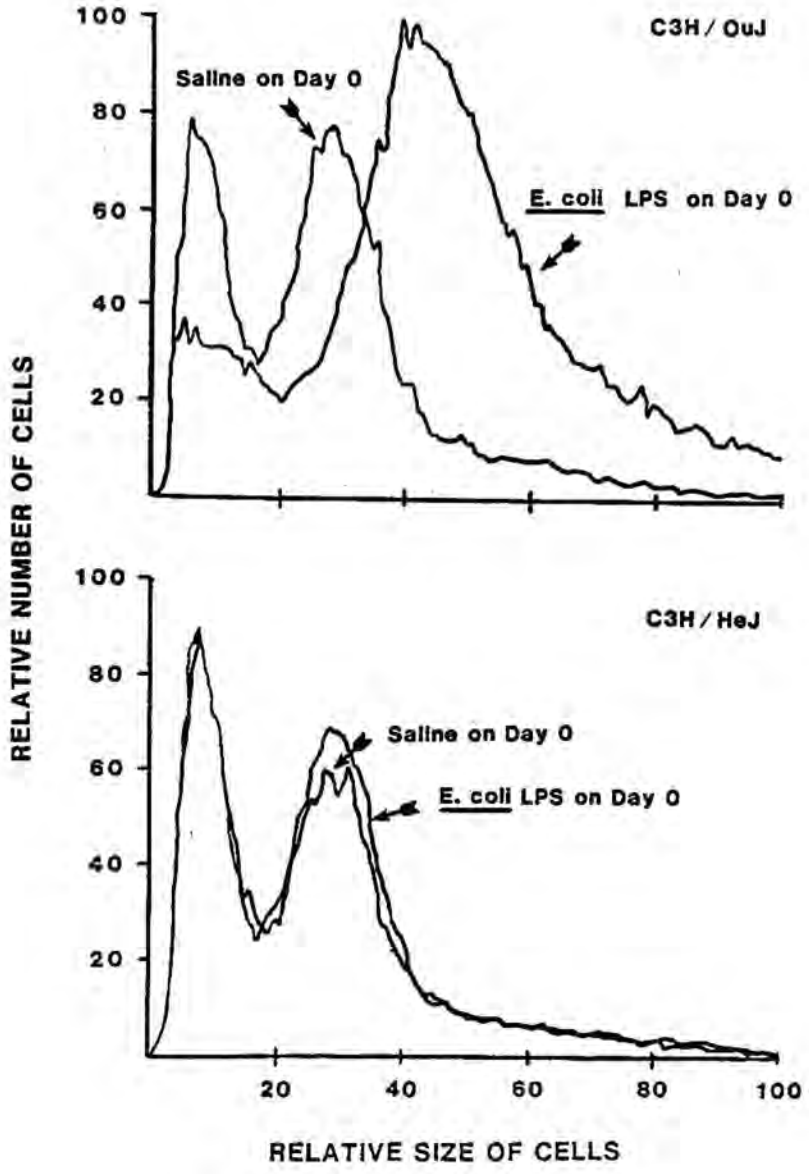
LPS-induced serum CSF response in LPS-responsive (C3H/OuJ) and LPS-hyporesponsive (C3H/HeJ) mice

Expermental group	Treatment[a]		CSF (CFU/ml)[b]
	Day 0	Day 3	
C3H/OuJ (Lps^n)	Saline	Saline	0
	Saline	LPS	7,000 ± 250
	LPS	LPS	1,300 ± 110
C3H/HeJ (Lps^d)	Saline	Saline	0
	Saline	LPS	0
	LPS	LPS	0

[a]Groups of 5 C3H/OuJ or C3H/HeJ mice were injected i.p. with either saline or E. coli LPS (25 µg/mouse) on day 0. Three days later, mice were injected with saline or challenged with E. coli LPS (25 µg/mouse).

[b]CSF levels were determined on pooled serum samples collected 6 h after the injections on day 3. Results represent the arithmetic mean ± one standard deviation of duplicate determinations.

Figure 13. Effect of the tolerizing regimen on the bone marrow cell sizing profile of inbred LPS-responsive and LPS-hyporesponsive mice -- LPS-responsive (C3H/OuJ) and LPS-hyporesponsive (C3H/HeJ) mice (5 mice/group) were injected i.p. on day 0 with either saline or E. coli LPS (25 µg/mouse). At 3 days post-injection, bone marrow cells were pooled and sizing profiles obtained using a Coulter Channelyzer. Bone marrow cells obtained 3 days post-injection were also plated in an excess of CSF-1 for determination of the numbers of M-CFU as described in the Materials and Methods section. Based on two separate experiments, the number of M-CFU/10^5 nucleated bone marrow cells were as follows: saline-treated, C3H/OuJ = 101 ± 11.9; LPS-treated, C3H/OuJ = 233 ± 9.8; saline-treated, C3H/HeJ = 92.6 ± 14; LPS-treated, C3H/HeJ = 102 ± 2.8.


C3H / OuJ
Saline on Day 0
E. coli LPS on Day 0
C3H / HeJ
Saline on Day 0
E. coli LPS on Day 0
RELATIVE NUMBER OF CELLS
RELATIVE SIZE OF CELLS
100
80
60
40
20
20
40
60
80
100

the total cell population with a compensatory increase in the number of cells in Peak 2 (67%). The number of M-CFU was also increased in bone marrow cells derived from C3H/OuJ mice exposed on day 0 to LPS. No such increase was observed in bone marrow cells derived from endotoxin-treated C3H/HeJ mice (indicated in the figure legend, Figure 13).

Induction of early phase endotoxin tolerance in congenitally athymic, T-cell deficient (nude) mice -- Since the initial description of a hairless, mutant (nude) mouse by Flanagan (1966) and further observations by Pantelouris (1968) which showed that such mice were athymic, T cell-deficient, nude mice have been used to study the role of T cells in antibody- and cell-mediated immune responses (reviewed by Kindred, 1981). Homozygous expression of the "nude" allele (nu/nu) results in congenitally athymic mice that possess extremely low levels of functional T cells. Several recent reports suggest the existence of LPS-responsive T cells (Vogel et al., 1983; Mita et al., 1982; McGhee et al., 1979; Koenig et al., 1977; Scheid et al., 1973). Therefore, to determine if T cells were involved in early endotoxin tolerance induction, nude mice were studied.

BALB/c athymic nude (nu/nu) mice and their euthymic (nu/+) littermates were injected with either saline or E. coli K235 LPS (25 µg/mouse) i.p. on day 0 and challenged 3 days later with E. coli LPS (25 µg/mouse). Mice were bled 6 h following challenge and sera tested for CSF activity. When

athymic and euthymic mice were injected with saline on day 0, both responded to an LPS challenge on day 3 with the production of serum CSF (Figure 14). Exposure to LPS on day 0 tolerized both athymic and euthymic mice as evidenced by a greatly reduced production of serum CSF when challenged on day 3 with LPS.

Recent studies have suggested that certain athymic mouse strains contain substantial numbers of T cells (Ikehras et al., 1984; Habu and Okumura, 1984). The spleens of the nude mice used in this study were analyzed for the presence of a T-cell marker, Thy 1.2, by analysis with a fluorescence activated cell sorter (FACS). Single cell spleen cell suspensions from individual BALB/c (nu/nu) and (nu/+) mice were prepared. Aliquots of each suspension were treated with fluoresceinated anti-mouse IgM (μ heavy chain specific) to detect B cells, anti-Thy 1.2 (to detect T cells), or control antibody, as described in the Materials and Methods section, and fluorescence analysis was carried out. The BALB/c (nu/nu) mice used in this study were very deficient in the number of cells which stained specifically for Thy 1.2 (3%) when compared with spleen cells derived from the euthymic controls (24.4%) (Table VIII). The percentage of Thy 1.2 positive cells also differed with respect to median fluorescence intensity (MFI). Thus, the few spleen cells from athymic mice which stained positively for Thy 1.2 expressed far less of this surface antigen than spleen cells from euthymic mice. The percentage of spleen cells expressing μ was as expected higher in the

Figure 14. Effect of the tolerizing regimen on LPS-induced serum CSF response in BALB/c euthymic (nu/+) and BALB/c athymic (nu/nu) mice -- BALB/c euthymic and athymic mice (5 mice/group), were injected on day 0 with either saline or E. coli LPS (25 μg/mouse) and challenged 3 days later with 25 μg E. coli LPS. Six hours later, serum was collected and assayed for CSF activity as described in the Materials and Methods section. The results represent the arithmetic mean ± one standard deviation of duplicate determinations.

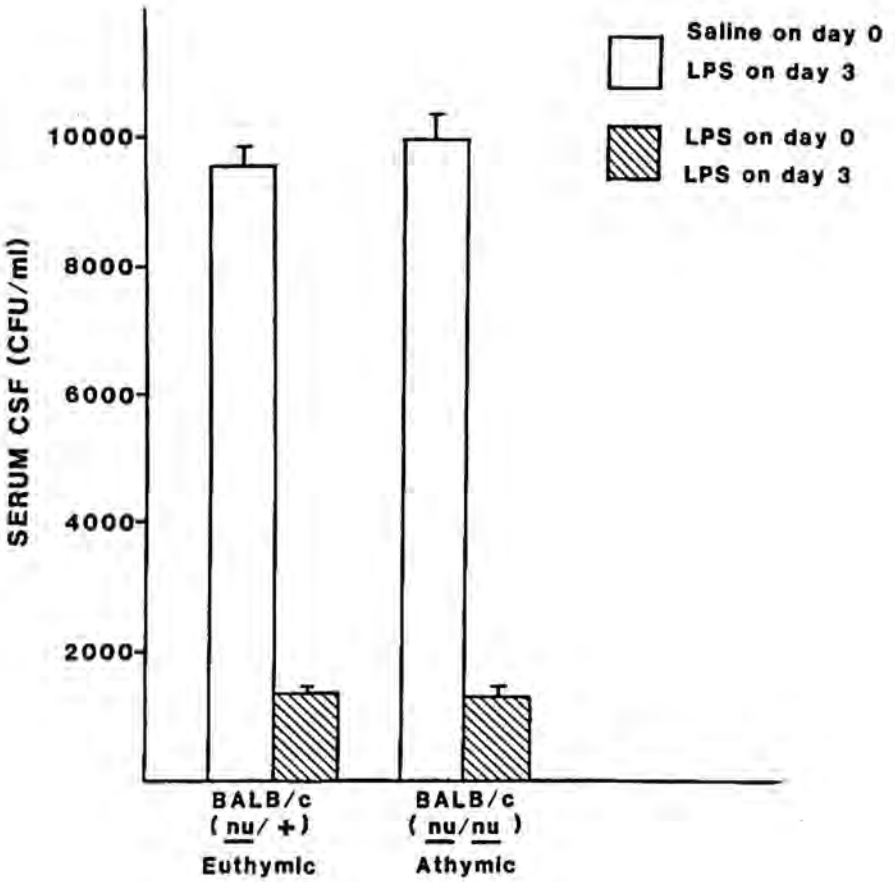

Saline on day 0
LPS on day 3
LPS on day 0
LPS on day 3
SERUM CSF (CFU/ml)
10000
8000
6000
4000
2000
BALB/c
(nu/+)
Euthymic
BALB/c
(nu/nu)
Athymic

Table VIII

FACS analysis of Thy 1.2 positive and IgM positive cells obtained from BALB/c euthymic (nu/+) or BALB/c athymic (nu/nu) mice

Spleen cell source[a]	Percent Positive Cells (MFI)[b]	
	Thy 1.2	IgM
BALB/c euthymic (nu/+)	24.4±1.5 (473±28)	58.2±2.8 (352±36)
BALB/c athymic (nu/nu)	3.0±0.7 (290±34)	71.0±4.6 (392±5.4)

[a] Individual spleen cell suspensions from 5 athymic and 5 euthymic BALB/c mice were treated with fluoresceinated antibody as indicated in the Materials and Methods section.

[b] Fluorescence analysis was carried out on each sample using a fluorescence-activated cell sorter, and the percentage of cells which stained with the indicated antibody and median fluorescence intensity (MFI) determined. Results, expressed as percent positive cells, represent the arithmetic mean ± one standard deviation of measurements made on individual spleen

cell suspensions.

athymic mouse strain due to the anticipated T-cell deficiency.

Induction of early endotoxin tolerance in the C3.CBA/N (xid) mouse strain -- CBA/N mice possess a defective X-linked gene, xid, which interferes with the development of cells that express the Lyb'5 surface antigen (Ahmed et al., 1977). These mice are unable to respond to a number of B cell mitogens, including purified polysaccharides and LPS (Amsbaugh et al., 1974; Scher, 1982). Although spleen cells from CBA/N mice are deficient with respect to LPS-induced mitogenesis (Glode and Rosenstreich, 1976), these mice are fully responsive to the toxic effects of LPS and possess macrophages which respond normally to LPS in vitro to produce Interleukin 1 (Rosenstreich et al., 1978). Zaldivar and Scher (1979) have compared immune-defective (xid) F_1 male mice with immunologically normal F_1 female mice derived from the crosses of the CBA/N strain with DBA/2 mice. Their results indicate that although the immune-defective male mice were unable to make a specfic immune response to E. coli LPS, this did not influence either their natural resistance to LPS lethal challenge or the development of late phase tolerance (antibody-mediated) to LPS after pretreatment with a sublethal amount of LPS and administration of LPS 10 days later.

In 1983, Mond et al. showed that spleen cells from the C3.CBA/N mice (which possess the xid gene defect on a fully LPS-responsive C3H/HeN background) fail to respond in vitro to B-cell mitogens, including LPS. In the following series of

experiments, C3.CBA/N mice were utilized to determine whether the population of LPS-responsive B cells which is deficient in these mice were involved in the induction of early endotoxin tolerance. Consequently, C3.CBA/N female mice, homozygous for the xid defect (xid/xid), were age-matched with C3H/HeN female mice and were subjected to the tolerizing regimen. Mice from each strain were injected with either saline or E. coli K235 LPS on day 0 and either challenged 3 days later with LPS and bled to obtain serum for assessment of CSF activity, or sacrificed on day 3 to obtain bone marrow cells for culture of M-CFU and cell sizing profiles. The xid defect of C3.CBA/N mice had no effect on the induction of serum CSF after LPS challenge of mice injected on day 0 with saline (Figure 15). Moreover, pre-exposure on day 0 to LPS resulted in early endotoxin tolerance as assessed by a reduction in serum CSF following LPS challenge.

Bone marrow cell sizing profiles from control (saline-injected) C3.CBA/N and C3H/HeN mice revealed a characteristic bimodal distribution of cells. The pattern was altered in both the normal and defective mice by exposure to LPS 3 days earlier. Both strains also responded to prior LPS exposure with an increase (approximately 3-fold) in the number of bone marrow-derived macrophage progenitor cells (Table IX).

Induction of early endotoxin tolerance in splenectomized ICR mice -- The LPS-tolerance system in ICR mice was recently established by Williams et al. (1983) to

Figure 15. Effect of the tolerizing regimen on LPS-induced serum CSF response in C3H/HeN and C3.CBA/N (xid) mice -- C3H/HeN and C3.CBA/N mice (5 mice/group) were injected on day 0 with either saline or E. coli LPS (25 µg/mouse) and challenged 3 days later with 25 µg E. coli LPS. Six hours later, serum was collected and assayed for CSF activity as described in the Materials and Methods section. The results represent the arithmetic mean ± one standard deviation of duplicate determinations.

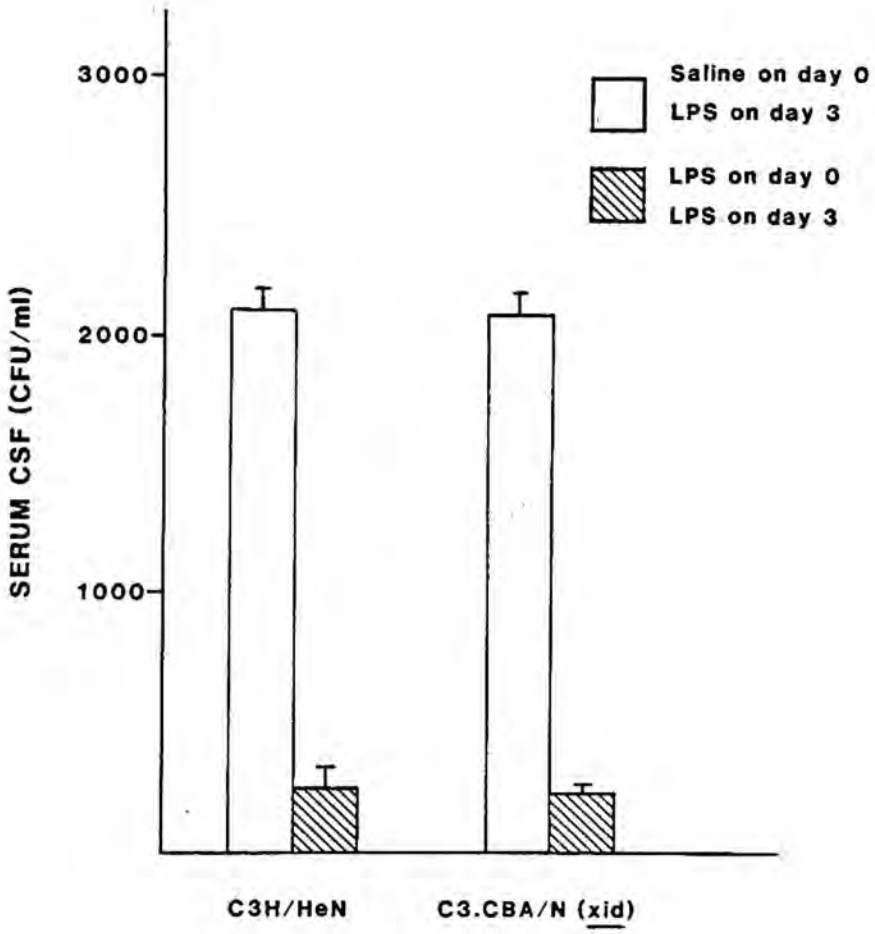

3000
2000
1000
SERUM CSF (CFU/ml)
Saline on day 0
LPS on day 3
LPS on day 0
LPS on day 3
C3H/HeN
C3.CBA/N (xid)

Table IX

LPS-induced alterations in bone marrow cell sizing profiles and M-CFU numbers in C3H/HeN and C3.CBA/N (xid) mice[a]

Mouse Strain	Treatment on day 0	M-CFU/femur[b]	% of cells in Peak 2[c]	Diameter of Peak-2 cells (μ)[c]
C3H/HeN	Saline	13,260 ± 1,441	59	10
	LPS	26,345 ± 233	77	11.4
C3.CBA/N	Saline	11,050 ± 919	64	10
(xid)	LPS	29,325 ± 487	84	11.4

[a] Mice (5 mice/group) were injected i.p. with saline or *E. coli* LPS (25 μg/mouse) on day 0 and sacrificed on day 3. Bone marrow cells from each group were then pooled and sizing profiles obtained using a Coulter Channelyzer.

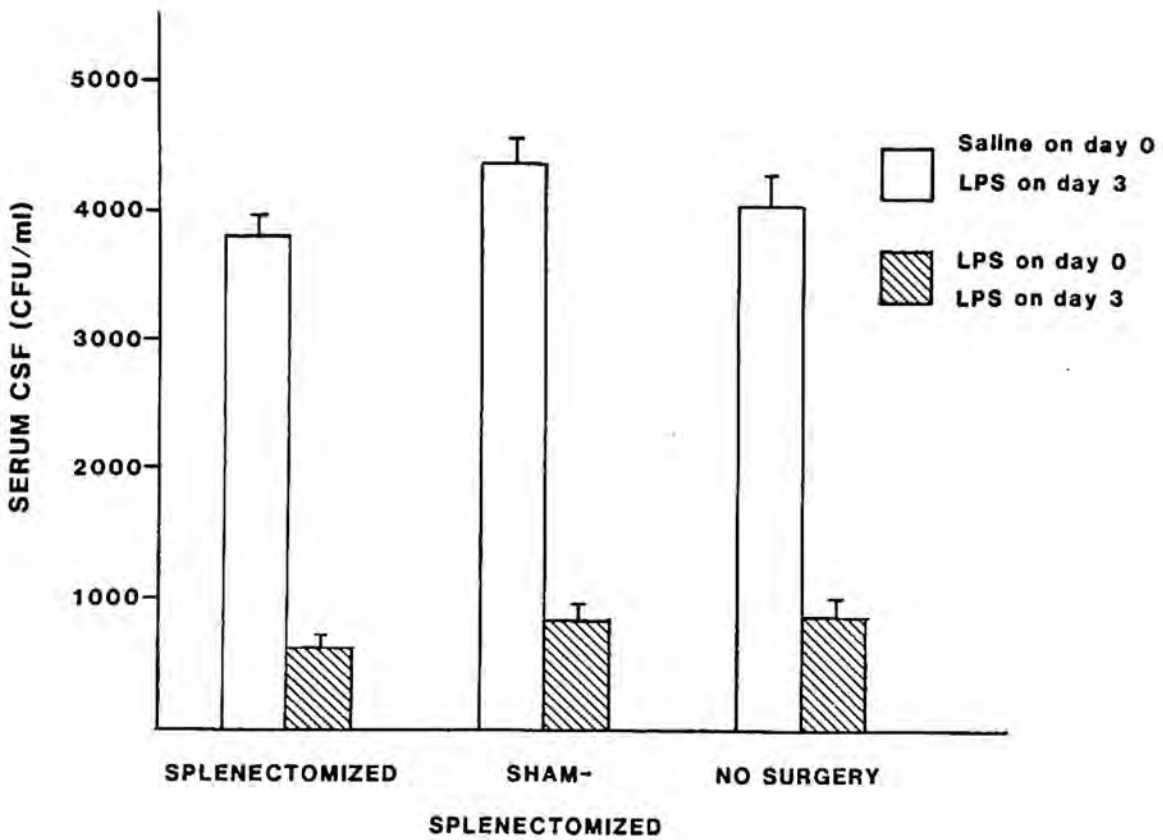
[b] Five X 10^4 pooled bone marrow cells from each group were plated in an excess of CSF-1 and the number of M-CFU per femur determined as described in the Materials and Methods section.

[c] Calculated from integration data which was obtained using the Coulter Channelyzer which was calibrated according to the manufacturer's instructions.

identify those cells which produce CSF in response to LPS. In that study, it was demonstrated that early endotoxin tolerance could be reversed by injection of tolerized mice with spleen cells or a mixture of splenic lymphocytes (macrophage depleted) plus thioglycollate-elicited peritoneal macrophages from control mice 24 h prior to LPS challenge. The authors, therefore, suggested that spleen cells contribute significantly in the production of circulating CSF. To assess further the importance of the spleen and its resident cells in the induction of early phase endotoxin tolerance, a different approach was employed. ICR mice were divided into three main experimental groups, i.e., splenectomized, sham-splenectomized, and untreated (no surgery). Mice which had surgery were allowed to recover completely and were then injected on day 0 with either saline or E. coli K235 LPS. Three days later, the respective groups were either challenged with LPS and bled 6 h later (for measurement of serum CSF activity), or sacrificed without challenge for the collection of bone marrow cells (for determination of the numbers of M-CFU colonies and cell sizing profiles). Results in Figure 16A illustrate that: (1) high levels of LPS-induced serum CSF were found in all three experimental groups, regardless of treatment and, (2) splenectomized mice had a reduced capacity to generate CSF in response to LPS challenge (i.e., they could be tolerized to the same extent as controls).

The effect of the tolerizing regimen on bone marrow cell sizing profiles and M-CFU numbers was also assessed. Bone

Figure 16A. Effect of the tolerizing regimen on LPS-induced serum CSF in control and splenectomized mice -- Groups of 5 ICR mice from each experimental group, i.e., splenectomized, sham-splenectomized, and untreated (no surgery), were injected on day 0 with either saline or E. coli LPS (25 µg/mouse) and challenged 3 days later with 25 µg E. coli LPS. Six hours later, serum was collected and assayed for CSF activity as described in the Materials and Methods section. The results represent the arithmetic mean ± one standard deviation of duplicate determinations.

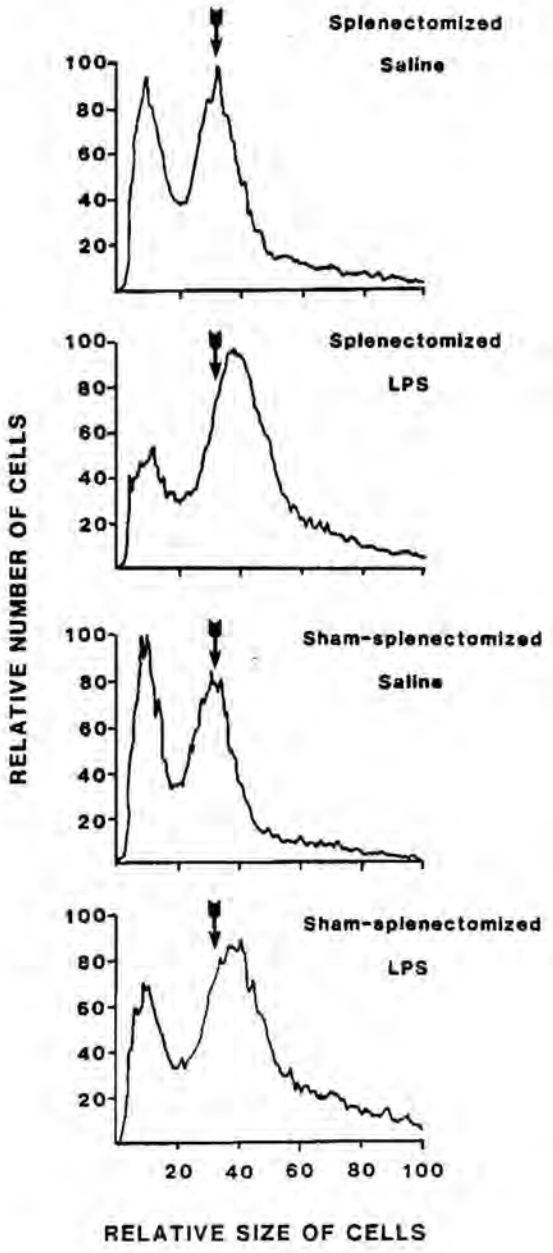

5000
4000
3000
2000
1000
SERUM CSF (CFU/ml)
Saline on day 0
LPS on day 3
LPS on day 0
LPS on day 3
SPLENECTOMIZED
SHAM-
SPLENECTOMIZED
NO SURGERY

marrow cell sizing profiles from splenectomized and sham-splenectomized mice were obtained 3 days after injection of saline or LPS. The bone marrow profiles shown in Figure 16B illustrate that removal of spleens has little or no effect on the LPS-induced bone marrow cell sizing profile, i.e., both splenectomized and sham-splenectomized bone marrow cells exhibited a reduction in Peak 1 and increase in cell size and number in Peak 2 following injection with LPS when compared to the profiles obtained from saline-treated mice. The number of bone marrow M-CFU was also shown to be increased in both groups three days following administration of LPS (indicated in figure legend, Figure 16B).

INFECTION OF CONTROL AND LPS-TOLERIZED ICR MICE WITH A GRAM NEGATIVE ORGANISM

Bacterial endotoxins affect the pathogenicity of a wide variety of organisms including bacteria, fungi, parasites, and viruses (reviewed by Cluff, 1971). Endotoxins induce resistance to infection over approximately the same time interval as they induce LPS-hyporesponsiveness, (Beeson, 1947a; Beeson, 1947b). Endotoxin-induced resistance to infection occurs independently of the generation of O-specific antibodies (Milner, 1973). However, the correlation of endotoxin tolerance with resistance to infection varies enormously. This variation has been attributed to differences in the dose of

Figure 16B. Bone marrow cell sizing profiles from splenectomized and sham-splenectomized ICR mice following LPS administration -- On day 0, splenectomized or sham-splenectomized ICR mice (5 mice/group) were injected with saline or E. coli LPS (25 µg/mouse). Three days later the mice were sacrificed, the bone marrow cells obtained, and the cell sizing profiles determined using a Coulter Channelyzer. The arrow indicates the relative position of Peak 2 bone marrow cells from control (saline-injected) ICR mice. The number of M-CFU/10^5 nucleated bone marrow cells were as follows: saline-treated, splenectomized = 110 ± 3; LPS-treated, splenectomized = 192 ±9; saline-treated, sham-splenectomized = 98 ± 4; LPS-treated, sham-splenectomized = 234 ± 21.


Splenectomized
Saline
Splenectomized
LPS
Sham-splenectomized
Saline
Sham-splenectomized
LPS
100
80
60
40
20
RELATIVE NUMBER OF CELLS
20
40
60
80
100
RELATIVE SIZE OF CELLS

endotoxin, the route of administration, the time interval prior to infectious challenge, and the pathogenic course of the agent.

To assess the potential of the early endotoxin tolerance regimen established in this study to protect against a Gram negative infection, it was deemed important to choose an infectious model which coincided temporally with the transiency of the tolerance system (see Figure 1). A well-defined model for Ps. aeruginosa infection was established by Stieritz and Holder (1975) and modified by Pavlovskis et al. (1977). With this system, mice are first administered a third-degree burn and then are immediately infected subcutaneously with Ps. aeruginosa at the burn site. Deaths are recorded for a seven day period.

Groups of 5 or 10 mice were injected on day 0 with either saline or 25 µg E. coli K235 LPS i.p. Three days later, the mice were burned and injected subcutaneously at the burn site with varying concentrations of Ps. aeruginosa. The results shown in Table X indicate that mice tolerized with E. coli LPS exhibit a higher degree of resistance to infection (compared with control saline-injected mice), particularly at the higher doses (3×10^{7} - 3×10^{8} bacteria/mouse). This resistance, as assessed by mean time to death (MTD), however, was transient, waned within 2 days post-challenge (day 5 post-LPS), and was not observed with lower challenge inocula. There was no statistical difference in the LD_{50}'s calculated from control and tolerized experimental groups.

Table X

Infection of control and tolerized ICR mice with Pseudomonas aeruginosa

Treatment Group[a]	Inoculum	Percent Cumulative Deaths[b] Day post-bacterial challenge 1	2	3	4
Control	3×10^8	60	100	100	100
Tolerized	3×10^8	27[c]	87[c]	93	93
Control	3×10^7	53	100	100	100
Tolerized	3×10^7	0[c]	87[c]	100	100
Control	3×10^6	0	47	67	73
Tolerized	3×10^6	0	60	62	62
Control	3×10^5	0	20	33	40
Tolerized	3×10^5	0	20	40	40
Control	3×10^4	0	0	0	0
Tolerized	3×10^4	0	0	7	7
Control	3×10^3	0	0	0	0
Tolerized	3×10^3	0	0	7	7

[a] ICR mice were treated on day 0 with either saline or E. coli LPS (25 µg/mouse). On day 3, mice were injected subcutaneously with Pseudomonas aeruginosa at a burn site with the indicated number of bacteria (inoculum).

[b] The results are presented as the cumulative percentage of deaths for each day after bacterial injection and represent the combined data from two separate experiments in which a total of 15 mice per experimental group were treated. The calculated LD_{50} (based on two separate experiments) for control and tolerized mice were 8.8×10^5 and 7.6×10^5 bacteria, respectively.

[c] $p < .05$ by a one-tailed, Kolmogorov-Smirnov two-sample test (Siegel, 1956).

INDUCTION OF EARLY PHASE ENDOTOXIN TOLERANCE BY A DETOXIFIED LIPID A DERIVATIVE, MONOPHOSPHORYL LIPID A (MPL)

Although endotoxin has been shown to induce many of the harmful manifestations seen in patients with Gram negative sepsis, some of the effects of endotoxin are beneficial (reviewed by Nowotny, 1983). However, therapeutic approaches which use endotoxin to induce beneficial effects have been limited because man is very sensitive to the toxic effects of endotoxin. Investigators have explored the possibility of using chemical modification techniques to reduce the toxicity and pyrogenicity of endotoxin while retaining beneficial effects such as adjuvanticity (Nowotny, 1963; Johnson et al., 1964; Ribi et al., 1979). Takayama et al. (1984) have recently described a detoxified Lipid A derivative, monophosphoryl Lipid A (MPL). Therefore, in this final series of experiments, we have studied the capacity of the nontoxic, monophosphoryl Lipid A derivative to induce early endotoxin tolerance.

Induction of serum CSF by MPL -- Injection of E. coli K235 LPS into mice was followed by a rapid rise of serum CSF which reached a maximum level at approximately 6 h post-injection. To test whether the non-toxic Lipid A derivative, MPL, was also able to induce this manifestation of endotoxin responsiveness, groups of 5 mice were injected

intravenously with varying doses of MPL. At 6 h following injection, the mice were bled and the serum subsequently tested for the presence of CSF. The results in Figure 17A show that serum CSF was induced over a wide dose range (1.0 μg - 100 μg/mouse) and was induced optimally with 50 μg MPL/mouse. Additionally, MPL induced a similar (although somewhat reduced) dose-dependent response in mice injected intraperitoneally. These data are shown in Table XI.

Induction of early endotoxin tolerance by MPL to challenge with intact E. coli K235 LPS -- The findings of Williams et al. (1983) and those presented in Figure 1 indicated that early endotoxin tolerance was optimal 3-4 days after initial exposure to either a homologous or heterologous preparation of LPS. To test whether an initial exposure to MPL would also render animals refractory to LPS, we injected groups of mice with varying concentrations of MPL, and 3 days later challenged these same mice with E coli LPS (25 μg/mouse). Six hours after challenge, blood was collected and the serum assayed for CSF activity. As can be seen in Figure 17B, increasing the dose of MPL given on day 0 from 1 μg to 100 μg reduced significantly the LPS-induced serum CSF response upon challenge with 25 μg E. coli LPS on day 3. This degree of LPS-hyporesponsiveness was equivalent to that seen when 25 μg E. coli LPS was used as the tolerogen (Figure 17B).

Another indicator of LPS responsiveness in vivo is the induction of serum interferon (IFN) which usually peaks 2-3 h following intravenous administration of intact LPS or Lipid A

Figure 17A. MPL-induction of serum CSF -- ICR mice (5 mice/group) were injected with either saline, E. coli LPS (25 µg/mouse), or MPL (1 - 100 µg/mouse) i.v. Six hours later, the mice were bled, sera collected, pooled, and assayed for CSF activity as described in the Materials and Methods section. The results represent the arithmetic mean ± one standard deviation of duplicate samples of serum pools from three separate experiments in which 5 mice per treatment per experiment were pooled.

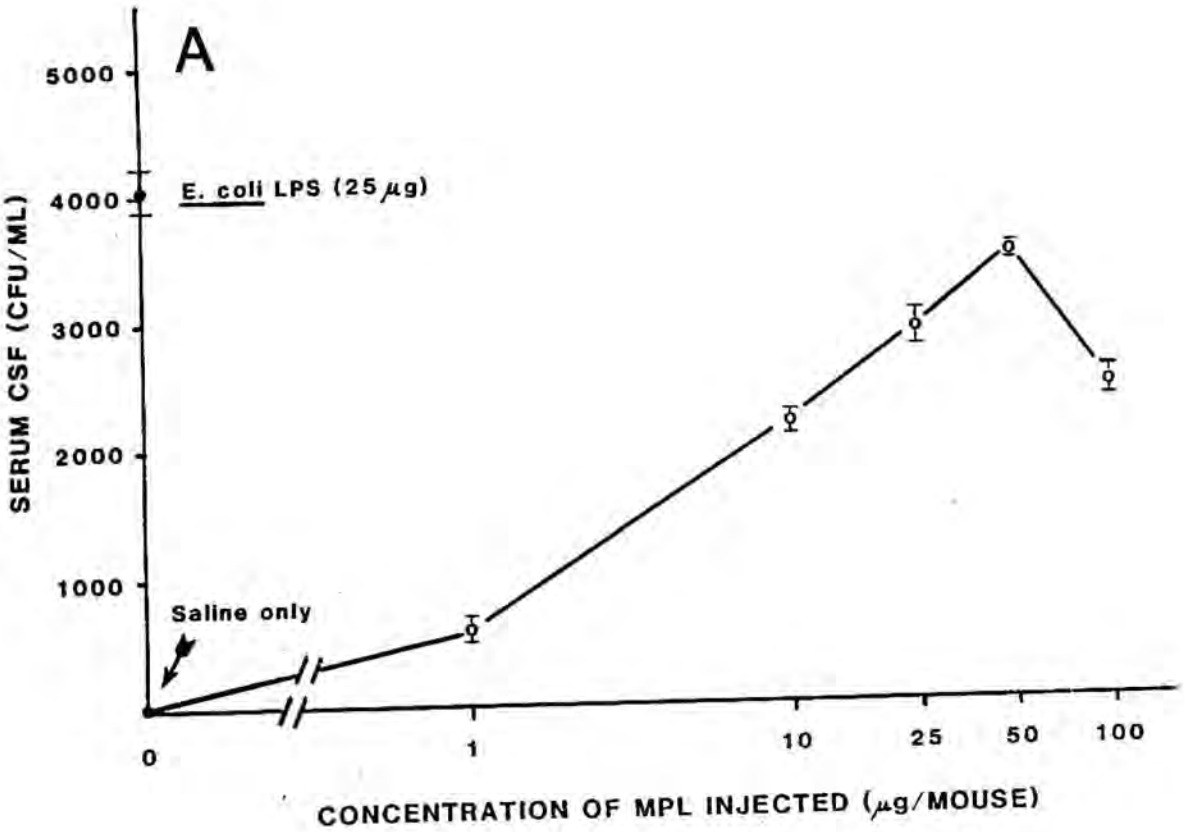

A
5000
4000
3000
2000
1000
E. coli LPS (25 μg)
Saline only
SERUM CSF (CFU/ML)
0
1
10
25
50
100
CONCENTRATION OF MPL INJECTED (μg/MOUSE)

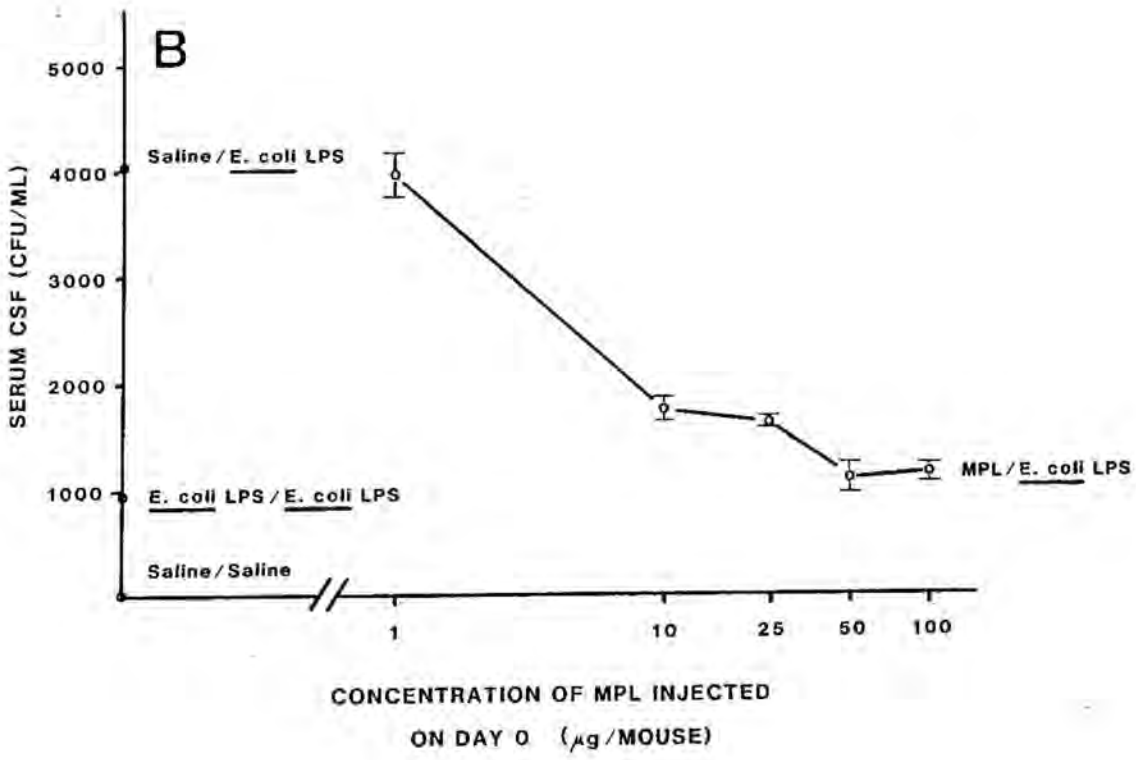
Table XI

Induction of serum CSF in ICR mice injected intravenously or intraperitoneally with MPL [a]

Concentration of MPL injected (µg/mouse)	Route of MPL Administration	
	Intravenous	Intraperitoneal
	Serum CSF (CFU/ml)	
0	0	0
1	560 ± 37	NT
10	2,170 ± 186	640 ± 130
25	2,900 ± 365	1,900 ± 254
50	3,500 ± 174	2,560 ± 488
100	2,400 ± 218	1,210 ± 285

[a] ICR mice (5 mice/group) were injected with either saline or MPL (1-100 µg) i.v. or i.p. Six hours later, the mice were bled, sera collected, pooled, and assayed for CSF activity as described in the Materials and Methods section. The results represent the arithmetic mean ± one standard deviation of duplicate samples of serum pools from three separate experiments in which 5 mice per treatment per experiment were pooled.

Figure 17B. MPL-induction of early phase tolerance to E. coli LPS -- ICR mice (5 mice/group) were injected on day 0 with either saline, E. coli LPS (25 µg/mouse), or the indicated concentration of MPL (1-100 µg/mouse). On day 3, mice were challenged with E. coli K235 LPS (25 µg/mouse). Six hours later sera were collected and assayed for CSF activity as described in the Materials and Methods section. The results represent the arithmetic mean ± one standard deviation of duplicate samples of serum pools from three separate experiments in which 5 mice per treatment per experiment were pooled. The treatment designation in Figure 17B indicates the treatments which were administered on day 0/day 3, respectively.


B
5000
4000
3000
2000
1000
SERUM CSF (CFU/ML)
Saline / E. coli LPS
E. coli LPS / E. coli LPS
Saline / Saline
MPL / E. coli LPS
1
10
25
50
100
CONCENTRATION OF MPL INJECTED
ON DAY 0 (μg / MOUSE)

(Youngner and Stinebring, 1965). Figure 18 illustrates that the level of serum IFN induced by MPL was comparable to that stimulated by E. coli LPS; however, the tolerance induced by MPL for IFN was less than the tolerance induced by the intact LPS for IFN (30% vs. 99% suppression of the IFN response) or the MPL-induced tolerance for CSF (see Figure 17B).

It was also found that prior exposure to an initial dose of MPL (100 µg/mouse) increased significantly the LD_{50} (approximately 5-fold) and essentially eliminated overt symptoms of endotoxicity, i.e., ruffled fur, conjunctival discharge, and diarrhea in response to a sublethal challenge of LPS. These data are shown in Table XII.

MPL-induced changes in the number of bone marrow-derived macrophage progenitor cells -- To test the efficacy of MPL to increase bone marrow M-CFU (as shown earlier for E coli LPS; see Figure 5B), groups of 5 mice were injected with saline, E coli LPS (25 µg/mouse), or varying concentrations of MPL, and on the third day following injection, bone marrow cells from the femurs of each group were enumerated and cultured in an excess of CSF-1 to determine the number of M-CFU. The results in Figure 19 indicate that MPL increased the number of M-CFU in the bone marrow in a dose-dependent fashion. When mice were given 100 µg MPL per mouse, the level of M-CFU per femur was equal to that induced by 25 µg E. coli LPS.

These findings were further supported by changes in

Figure 18. Interferon induction by MPL and effect of MPL-treatment on LPS-induced interferon production in vivo -- ICR mice (5 mice/group) were injected with saline, E. coli LPS (25 µg/mouse), or MPL (50 or 100 µg/mouse) i.v. on day 0. Three days later, the various groups of mice were injected i.v. with either saline, LPS, or MPL, as indicated. Serum samples were collected 2 h later and assayed for interferon (antiviral) activity as described in the Materials and Methods section. Results represent the arithmetic mean ± standard error of the arithmetic mean of antiviral activities of individual serum samples. Along the abscissa are indicated the treatments on days 0 and 3, respectively.

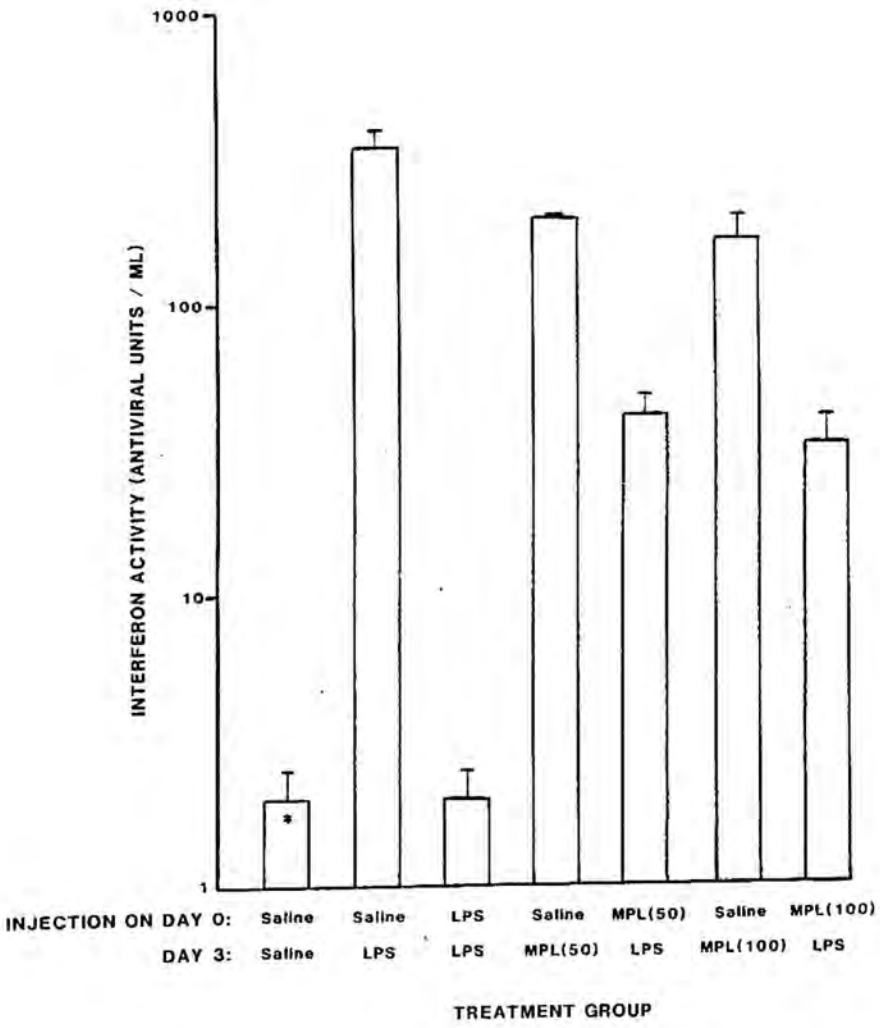

1000
100
10
1
INTERFERON ACTIVITY (ANTIVIRAL UNITS / ML)
*
INJECTION ON DAY 0: Saline Saline LPS Saline MPL(50) Saline MPL(100)
DAY 3: Saline LPS LPS MPL(50) LPS MPL(100) LPS
TREATMENT GROUP

Table XII

Effect of MPL exposure on LPS-induced LD_{50} and symptoms of endotoxicity

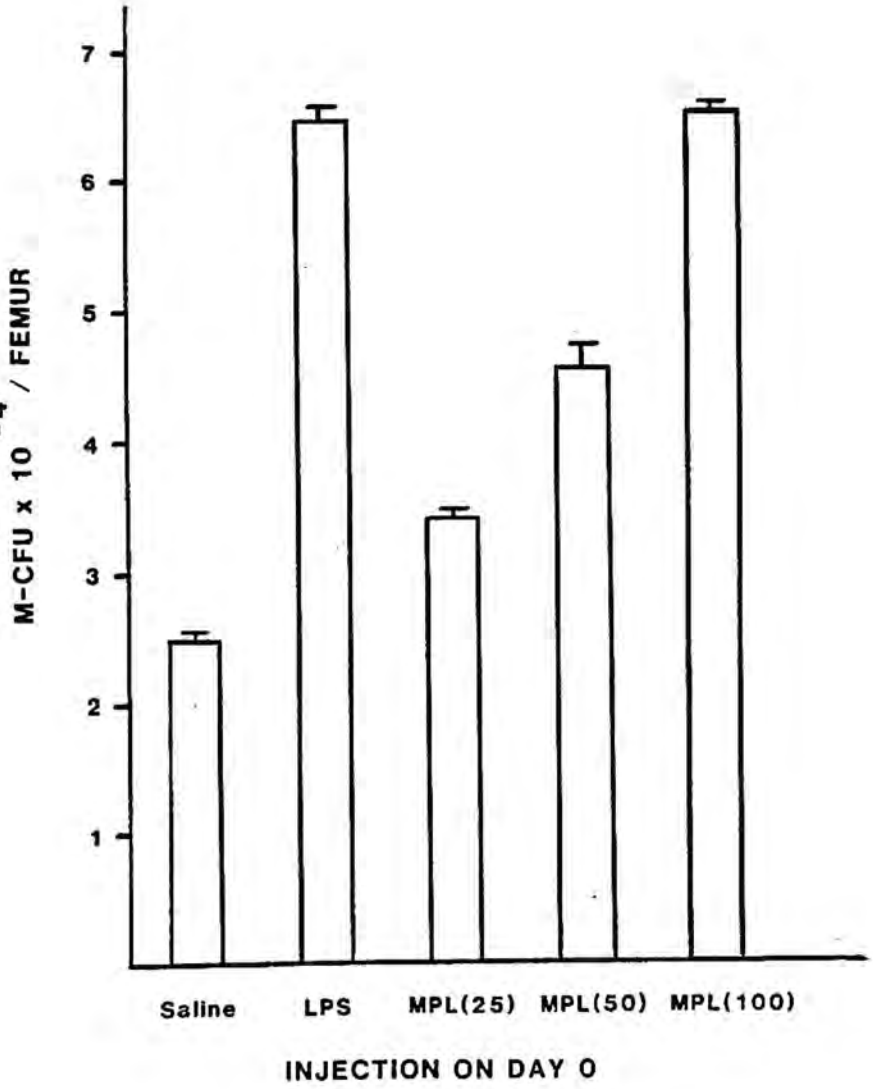
	Experimental Group[a]		
	Non-tolerized (Saline/LPS)	MPL-tolerized (MPL/LPS)	LPS-tolerized (LPS/LPS)
LD_{50}[b]	258 µg	1,156 µg	972 µg
Ruffled fur[c]	10/12	0/15	1/12
Conjunctival discharge[c]	5/12	2/15	0/12
Diarrhea[c]	6/12	1/15	1/12

[a] Mice were injected i.p. on day 0 with saline, MPL (100 µg/mouse), or *E. coli* K235 LPS (25 µg/mouse). Three days later, the mice were challenged with *E. coli* LPS for LD_{50} determination and assessment of symptoms as described in the Materials and Methods section.

[b] Calculated by the method of Reed and Muench (1939). [The LD_{50} for MPL was not determined in this study. The MPL LD_{50} has been reported to be greater than 10 mg for mice (Ribi, 1984)].

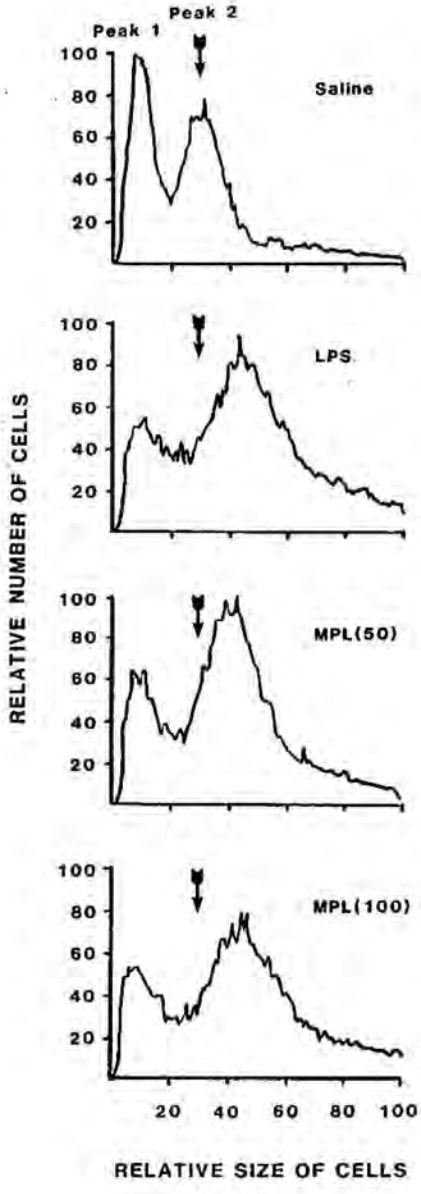
[a] The proportion of mice clearly exhibiting the indicated symptom 6 h after i.p. injection of E. coli K235 LPS (25 µg/mouse) on day 3. Injection of MPL (100 µg/mouse) induced none of the indicated symptoms in any of the 15 mice tested in this experiment.

Figure 19. Effect of a tolerizing injection of MPL on the number of macrophage precursors in the bone marrow -- ICR mice (5 mice/group) were injected on day 0 with either saline, E. coli LPS (25 µg/mouse), or MPL (at the indicated doses). Three days later, the bone marrow cells were collected and pooled (5 mice per treatment group). 5×10^4 cells from each cell suspension were then plated in duplicate in an excess of CSF-1 and the number of M-CFU determined as described in the Materials and Methods section. The results represent the arithmetic mean ± one standard deviation of duplicate determinations.


M-CFU x 10 -4 / FEMUR
7
6
5
4
3
2
1
Saline
LPS
MPL(25)
MPL(50)
MPL(100)
INJECTION ON DAY 0

bone marrow cell sizing profiles obtained using a Coulter Channelyzer. Bone marrow cell suspensions from groups of 5 mice injected 3 days earlier with saline, E. coli LPS, or MPL were compared. As can be seen in Figure 20, like LPS, injection of MPL induced a marked reduction in the relative number of bone marrow cells in Peak 1 (20% of total) and a compensatory increase in the number of cells within Peak 2 (80%). This was accompanied by an increase in cell size in Peak 2. As was previously observed with E. coli LPS, this change in cell sizing profile induced by MPL coincided temporally with the acquisition of optimal tolerance.

Figure 20. Effect of tolerizing doses of MPL on bone marrow cell sizing profiles — ICR mice (5 mice/group) were injected i.p. on day 0 with either saline, E. coli LPS (25 µg/mouse), or MPL (at the indicated doses) and sacrificed three days later. Cell sizing profiles were obtained from suspensions of bone marrow cells using a Coulter Channelyzer. The arrow indicates the initial position of Peak 2 in mice injected with saline on day 0.


Peak 1
Peak 2
Saline
LPS
MPL(50)
MPL(100)
100
80
60
40
20
RELATIVE NUMBER OF CELLS
20 40 60 80 100
RELATIVE SIZE OF CELLS

DISCUSSION

For some time, investigators have attempted to clarify the extremely complex cellular mechanisms which underlie responsiveness to Gram negative bacterial endotoxin (LPS). By far, one of the most extensively used animal models has been the C3H/HeJ mouse strain, which, due to the presence of a single autosomal mutation (Watson et al., 1978), is markedly hyporesponsive to LPS (reviewed by Morrison and Ryan, 1979). This genetic defect (Lps^d) is reflected in a number of cell types, e.g., decreased mitogenic response of B cells (Sultzer and Nilsson, 1972), reduced ability of C3H/HeJ pre-T cells to express Thy-1 antigen (Koenig et al., 1977) or to proliferate (Vogel et al., 1983) in response to LPS, reduced ability of C3H/HeJ macrophages to respond to LPS (reviewed in Vogel et al., 1981), and the failure of C3H/HeJ fibroblasts to be altered metabolically by LPS (Ryan and McAdam, 1977). Using this model in conjunction with syngeneic LPS-responsive mice (Lps^n), much information has been obtained that points to the macrophage as the cell which figures centrally in mediating endotoxicity. Michalek et al. (1980) demonstrated that the cells principally responsible for endotoxin responsiveness are radiosensitive and bone marrow-derived. Further, it was shown that many soluble, macrophage-derived factors which are induced by LPS in vitro [e.g., endogenous pyrogen (Atkins et al., 1967), colony stimulating factor (Apte et al., 1976; Apte and Pluznik, 1976; Metcalf, 1971; Quesenberry et al., 1972),

interferons (Ho, 1980), and prostaglandins of the E series (Skarnes and Harper, 1972)] are associated with *in vivo* endotoxicity. In addition, an association between macrophage differentiation and macrophage sensitivity to LPS has been demonstrated (Suter *et al.*, 1958; Benacerraf *et al.*, 1959; Rosenstreich and Vogel, 1980; Moore *et al.*, 1980).

A second, less frequently employed model of LPS-hyporesponsiveness is an inducible state of "endotoxin tolerance", as it was designated originally by Favorite and Morgan (1942). This condition is characterized by a progressive decrease in LPS responsiveness when endotoxin is re-administered at appropriate intervals (reviewed by Greisman, 1983). An "early phase" of endotoxin tolerance occurs within the first few days after exposure to LPS, is transient (subsides within a week), and is not O-antigen-specific (Greisman and Hornick, 1976). Previous studies which employed this model also suggested macrophage participation in the mediation of LPS-induced toxicity. Specifically, it was shown that the levels of endogenous pyrogen and prostaglandins produced *in vitro* by macrophages derived from tolerized mice were decreased (Dinarello *et al.*, 1968; Rietschel *et al.*, 1980). The data presented herein demonstrated that the sterile inflammatory agent, thioglycollate, elicited markedly reduced numbers of peritoneal exudate macrophages from endotoxin-tolerized mice (see Table IV). This reduction in the relative numbers of peritoneal macrophages in mice pre-treated with LPS suggested that, in addition to LPS-induced macrophage

refractoriness to LPS, fewer macrophages might be available to respond to LPS in the periphery. Taken together, these findings prompted us to test the hypothesis that early endotoxin tolerance is related to the presence of immature monocytic cells which accumulate in the bone marrow and could, in turn, limit the potential number of LPS-responsive cell types in the periphery.

Using colony stimulating factor (CSF) as an indicator of *in vivo* LPS-responsiveness, we confirmed the findings of Williams *et al.* (1983), that early endotoxin tolerance was optimal at 3 and 4 days after the initial exposure to LPS and that this state of hyporesponsiveness waned within a week. We found that the acquisition of early tolerance followed a normal response to LPS. Optimal levels of serum CSF were demonstrated 6 h after the first exposure to LPS, as had been reported originally by Metcalf (1971). On days 1 and 2 post-LPS injection, the number of nucleated cell types in the bone marrow was markedly reduced. This latter observation was consistent with the findings of Sultzer (1969) and Moeller *et al.* (1978) who have shown an efflux of granulocytes and monocytes from the bone marrow into the blood and peritoneum following intraperitoneal injection of LPS. Furthermore, we have shown that the acquisition, maintenance, and loss of tolerance coincides temporally with an increase (followed by a decrease) in the number of macrophage precursors (M-CFU and HPP) in the bone marrow. The increase in macrophage precursor frequency correlated well with: (1) an alteration in bone

marrow cell sizing profiles, (2) an enrichment in a denser cell population which contained all of the precursor activity, and (3) an increase in immature macrophage forms, as shown by cytology. Taken collectively, these results indicated that the development of early endotoxin tolerance is associated with an abrupt depopulation of the nucleated cells in the bone marrow which is replaced by a less mature population of cells enriched for macrophage precursors.

The increase in the level of macrophage precursors was sustained in the bone marrow for at least 24 h after a second (challenge) injection, but returned to normal after a series of repeated injections. Thus, it is possible that in the initial phase of endotoxin tolerance, those macrophages which are available to respond to the second challenge dose of endotoxin are immature and less responsive to the LPS challenge. The hypothesis that immature macrophages are less LPS-responsive has been supported by the independent findings of a number of investigators. Specifically, Neumann and Sorg (1980) showed that bone marrow-derived macrophage precursors grown in the presence of CSF-1, acquire LPS-sensitivity late in culture relative to the acquisition of other macrophage differentiative functions. Similarly, Moore et al. (1980) have shown that CSF-1-propagated bone marrow macrophages (from endotoxin responsive mice) coordinately exhibited enhanced differentiation and LPS-sensitivity in vitro. In addition, it has been shown that lymphokine-rich culture supernatants induced increased differentiation of C3H/HeJ macrophages in

vitro, and rendered them partially responsive to LPS (Vogel and Mergenhagen, 1982). It is conceivable that the sequential expression of certain functions during macrophage development and differentiation may be a prerequisite for full expression of endotoxin sensitivity. A blockade in development which results in the accumulation of macrophage progenitors in the bone marrow may limit the availability or, if released, dilute the numbers of mature LPS-responsive effector cells in the periphery. This latter possibility was supported by the finding that the number of M-CFU in the spleens of mice injected 3 days previously with *E. coli* LPS was increased fourteen-fold. One approach which could be taken to test this hypothesis would be to reproduce the hematopoietic alterations seen in response to LPS with a non-endotoxic agent, then subsequently challenge with endotoxin. If the mice were found to be endotoxin hyporesponsive, it would strengthen the notion that the induction of early endotoxin tolerance depends upon a repopulation of the bone marrow with immature macrophages.

The studies of van Waarde *et al.* (1978) provide possible mechanisms by which increased numbers of macrophage precursors could accumulate in the bone marrow. These investigators have shown that the injection of certain inflammatory agents results in the induction of two soluble factors (i.e., factor increasing monocytopoiesis (FIM) and monocytopoiesis inhibitor (MPI)). FIM was shown to decrease cell cycle time for promonocytes and results in increased numbers of monocytes, while MPI, which is produced after the

inflammatory stimulus has been removed, curtails monoblast proliferation and acts to curtail the action of FIM. Although endotoxin was not utilized as an inflammatory stimulus in these studies, it is possible that the increase in macrophage precursor pools which we observed in tolerized mice resulted from an overproduction of FIM or a failure of LPS-tolerized mice to produce the inhibitor, MPI.

The finding that tolerance is sustained with repeated LPS injections (homologous or heterologous), even though the number of precursors returns to normal levels, suggests that additional mechanisms for sustaining the tolerant state come into play. One possibility is that repeated LPS injections result in the appearance of anti-LPS antibodies which act to clear the endotoxin from the circulation. However, since this was seen for both homologous and heterologous LPS injection sequences, one would need to invoke the presence of either cross-reactive anti-Lipid A or anti-core antibodies which could act to clear both E. coli and Ps. aeruginosa LPS from the circulation or a polyclonal activation event, in which B cell clones with antibody specificities for an assortment of LPS species are expanded. Although the LPS and Lipid A of Ps. aeruginosa share some structural similarities with those produced by E. coli (Wilkinson, 1977), neither the Lipid A nor the core moieties are extremely antigenic in the mouse (Galanos et al., 1971). For this reason, the first possibility is less likely. Nevertheless, since it is well known that LPS acts as a polyclonal B-cell activator, such that a spectrum of antibody

specificities is induced (Andersson et al., 1972), repeated injections of either homologous or heterologous LPS could possibly expand B-cell clones with specificity for a heterologous LPS preparation.

A second possible mechanism to account for the finding that tolerance is sustained even after M-CFU numbers return to normal comes primarily from the work of Ulevitch et al. (1979, 1981, 1984). These authors reported that the binding of LPS to high density lipoprotein (HDL) markedly affected the kinetics and specificity of LPS-cell interactions and the biological activity of LPS. Specifically, pyrogenic activity and the ability of LPS to induce neutropenia in rabbits were inhibited. Further studies were performed by Ulevitch and co-workers to compare the binding of LPS to HDL in normal rabbit serum and serum derived from LPS-pretreated rabbits. Their results suggested that a new protein, which they termed gp60, was produced as an "acute phase reactant" (as defined by Sipe and Rosenstreich, 1981) in response to LPS, and that gp60 increased the binding of LPS to HDL. Perhaps increased LPS-HDL binding, facilitated by an LPS-induced acute phase reactant, occurs following repeated injections with homologous or heterologous LPS preparations. This, in turn, could limit the availability of LPS to induce toxic effects, even after the number of immature macrophages had returned to normal. Such a mechanism could be operative, even in heterologous injection sequences, without invoking the presence of antibodies to clear the LPS. The effect of acute phase proteins on LPS activity and their

relationship to the tolerant state remains to be explored.

It is also possible that the alterations in macrophage progenitor numbers is an epiphenomenon; the actual mechanism which underlies acquired unresponsiveness to LPS is that exposure of macrophages to LPS renders them refractory to subsequent stimulation. This could be due to receptor blockade and/or down-modulation of the putative "LPS receptor" from the surface of the cell. The findings of Sullivan et al. (1983) are consistent with such an hypothesis. These investigators found that in response to an initial exposure to endotoxin in vitro, human monocytes produced CSF; however, the cultures rapidly became refractory to further stimulation by LPS, such that by the fourth day in culture, CSF secretion was not detectable. The diminished response was shown not to be due to degradation or "detoxification" of endotoxin, accumulation of a soluble inhibitor of monocyte synthesis, or negative feedback inhibition by newly-synthesized CSF. To date, theirs is the only report which suggests that human monocytes can be rendered tolerant to LPS by in vitro exposure to LPS. Precedent for receptor down-regulation following ligand-receptor interaction has been demonstrated in a number of systems. For example, after epidermal growth factor (EGF) binds to specific receptors on target cells, aggregation of the hormone-receptor complexes occurs and occupied EGF receptors in coated pits are endocytosed. The rapid degradation of receptor molecules by lysosomal enzymes results in their down-regulation because the receptor molecules cannot be recycled to the membrane

(Schlessinger et al., 1984). Other studies have shown that down-regulation of insulin receptors occurs following binding of insulin to its receptor (Marshall and Olefsky, 1979; Conti et al. 1976; Marshall and Olefsky, 1980). This receptor-ligand interaction induces clustering of insulin receptors (Schlessinger et al., 1978), and this clustering leads to internalization of the ligand-receptor complex and, like EGF, a failure to recycle the receptor to the cell membrane (Anderson et al., 1976).

In the second section of this study, we sought to characterize further the cell types which participated in the induction of early endotoxin tolerance. To this end, other murine models with deficiencies or altered responses in specific cellular compartments were subjected to the same tolerizing regimen which had been established for outbred ICR mice. Early endotoxin tolerance was induced in inbred, LPS-responsive (Lps^n) C3H/OuJ mice, but not in syngeneic, LPS-hyporesponsive C3H/HeJ (Lps^d) mice, as assessed by increased serum CSF, bone marrow M-CFU numbers, and a marked shift in bone marrow cell sizing profile. These results indicated that expression of the Lps^d gene defect resulted in: (1) an inability to respond to an initial exposure of LPS to produce serum CSF [as had been reported previously (Apte et al. 1976)]; and (2) no alteration in either bone marrow M-CFU numbers or in the bone marrow cell sizing profile. These findings are consistent with the hypothesis that C3H/HeJ mice fail to respond to LPS due to an Lps^d-encoded membrane defect

(Coutinho et al. 1977; Jakobovitz et al. 1982). If the mechanism(s) by which C3H/HeJ and endotoxin-tolerized mice fail to respond to LPS were the same, one would expect to observe both a shifted bone marrow cell sizing profile and increased M-CFU numbers in untreated C3H/HeJ mice. Neither were observed in this study. However, one report in the literature has suggested that M-CFU numbers are increased in C3H/HeJ mice when compared with C3HeB/FeJ (Lps^n) mice (MacVittie and Weinberg, 1980). Further studies will be required to confirm these observations in light of recent findings that C3HeB/FeJ mice express other gene defects (distinct from Lps^d) which may compromise the functioning of their macrophages (O'Brien and Rosenstreich, 1983).

Whereas normal responsiveness to endotoxin was not seen in C3H/HeJ (Lps^d) mice, BALB/c euthymic (nu/+) and athymic (nu/nu) mice responded comparably to LPS to produce CSF and to become tolerized. The nude mice used in this study were found to be extremely T-cell deficient by FACS analysis of their splenocytes (Table VIII). These findings suggested that T cells do not contribute significantly to the generation of LPS-induced serum CSF or to the induction of early endotoxin tolerance. Previous studies indicated that the presence of the nude gene on either an Lps^n or Lps^d background failed to alter LPS-responsiveness or hyporesponsiveness, respectively (Vogel et al., 1979).

CBA/N mice express an X-linked genetic lesion (xid) which results in a failure to respond to certain B cell

mitogens, including LPS (Amsbaugh et al., 1974; Scher, 1982). This genetic defect manifests itself as an absence of the Lyb 5 positive B cell subset in these mice (Ahmed et al., 1977). In the present study, a third murine model, the C3.CBA/N mouse strain was utilized because it carries this X-linked genetic defect (Mond et al., 1983). This strain was developed by introducing the xid gene into the C3H/HeN inbred mouse strain by repeated cycles of backcrossing. The initial cross consisted of mating a female CBA/N (homozygous for the xid gene) with a male C3H/HeN. One of the male progeny (hemizygous for the xid gene) was then backcrossed to his mother. Lastly, females from this cross, now termed C3.CBA/N and homozygous for the xid gene, were crossed with male C3H/HeN mice. C3.CBA/N mice used in this study were at the 13th cycle of this backcrossing procedure. The probability of homozygosity at a specific locus after 12 generations of backcrossing to an inbred strain has been calculated to be 99.9% (Klein, 1975).

Mond et al. (1983) verified that, like CBA/N mice, C3.CBA/N spleen cells have a greatly reduced response to LPS in vitro and the B cell deficit in this mouse strain could be demonstrated by FACS analysis of cells from the spleen, lymph nodes, and Peyer's patches. It was shown that there are 21.9%, 2.7% and 18.0% sIgM positive cells, respectively, in these organs from the C3.CBA/N mouse, whereas cells from C3H/HeN spleen, lymph nodes, and Peyer's patches yielded 51.9%, 20.9% and 30.4% sIgM positive cells, respectively. In the current study, these mice responded normally to an initial injection of

LPS with a characteristic increase in the level of serum CSF. This increase was shown to be suppressed after a second (challenge) injection of LPS on day 3. C3.CBA/N mice also displayed the tolerized bone marrow profile and an increase in bone marrow M-CFU numbers 3 days post-LPS injection. Mice which express the xid defect have been shown to respond normally to the toxic effects of LPS in vivo, and their macrophages produce normal levels of IL 1 in response to LPS in vitro (Rosenstreich et al., 1978). Thus, the population of LPS-responsive B cells which is deficient in this strain does not appear to play an essential role in either LPS responsiveness or the induction of endotoxin tolerance.

The recent findings of Williams et al. (1983) indicated that injection of normal spleen cells into LPS-tolerized ICR mice restored the capacity to respond to LPS, as assessed by serum CSF induction. These investigators, however, made no attempt to tolerize normal ICR mice by transfer of spleen cells from tolerized mice to normal mice because the number of cells necessary to override the normal level of responsiveness was deemed by the investigators to be experimentally prohibitive (D. Pluznik, personal communication). In order to evaluate the contribution of the spleen in CSF production and in the induction of tolerance, splenectomized ICR mice were subjected to the standard tolerizing protocol which was established for normal mice. The data indicate that the presence or absence of a spleen had no influence on the ability of mice to respond to LPS to produce serum CSF and to be tolerized. Thus, spleen

cells cannot be considered as a major source of LPS-induced CSF, as suggested by Williams et al. (1983). This supports the alternative suggestion by Williams et al. (1983) that other organs, such as lungs or resident peritoneal macrophages, may contribute significantly to the production of soluble serum factors after administration of LPS. Changes in M-CFU numbers and alterations in bone marrow cell sizing profiles in tolerized, splenectomized mice were also consistent with the notion that the spleen plays little role, if any, in these manifestations of LPS-induced tolerance.

The next phase of this project sought to establish whether acquired resistance to LPS could extend to protection against bacterial infection. The responses observed in the early phase of endotoxin tolerance and endotoxin-induced stimulation of resistance to infections are antibody independent (Milner, 1971). Studies by Landy (1956) showed that various bacterial lipopolysaccharides evoked in mice a rapidly developing, non-specific increase in resistance to different Gram negative bacterial infections. The development of increased resistance to infection by E. coli, Proteus vulgaris, Ps. aeruginosa and Salmonella typhosa following LPS injection was shown by Landy and Pillemer (1956) to be associated with elevated properdin levels, which, in control animals was found to fall progressively as they became infected. These investigators also showed that resistance to Ps. aeruginosa infection was increased optimally 24 h after injection of 1 µg Salmonella typhosa LPS i.p. (65% survivors),

but was reduced only slightly (50% survivors) if mice were injected with Ps. aeruginosa 72 h or 120 h after LPS administration.

Increased resistance to infection stimulated by LPS may also be related to the production in vivo of a number of biologically active materials which have been found to stimulate macrophages to become activated. Production of CSF during infections is associated with enhanced functional activities of macrophages. CSF has been shown to stimulate macrophages to produce prostaglandins (Kurland et al., 1979), plasminogen activator (Lin and Gordon, 1979), and Interleukin 1 (Moore et al., 1980). In one report, Handman and Burgess (1979) demonstrated that partially purified granulocyte-macrophage colony stimulating factor caused a dramatic increase in the uptake and killing of the intracellular parasite, Leishmania tropica by macrophages. Trudgett et al. (1973) studied the effect of CSF on the in vitro phagocytic and microbicidal activity of mouse peritoneal macrophages incubated with Salmonella typhimurium. They found that CSF induced a significant enhancement of S. typhimurium killing by macrophages, although there was no effect on the number of bacteria phagocytosed. Another macrophage product that is produced in response to LPS is interferon (Youngner and Stinebring, 1965). Infection of experimental animals with E. coli (Weinstein et al., 1970) or Listeria monocytogenes (Remington and Merrigan, 1970) has been shown to be suppressed by prior injection of interferon or interferon inducers.

Lastly, the survival rate of mice injected with Klebsiella pneumonia one day after injection with endotoxin or other known interferon inducers was shown to be increased (Pindak, 1970).

In order to determine the temporal relationship of early phase endotoxin tolerance to LPS-induced non-specific resistance to infection, control and tolerized ICR mice were challenged with Pseudomonas aeruginosa using the burn model of Pavlovskis et al. (1977). The rapid progression of fatal Gram negative infection associated with this model was necessary if resistance to infection were to be detected during the transient early phase tolerance period (refer back to Figure 1). The two bacterial products most likely implicated in the systemic infection caused by Ps. aeruginosa are lipopolysaccharide and exotoxin A. Evidence associating Ps. aeruginosa endotoxin with sepsis is extensive (reviewed by Young, 1979). Exotoxin A (Liu, 1966) in its purified form is highly lethal for animals and produces shock in dogs (Atik et al., 1968) and rhesus monkeys (Pavlovskis et al., 1975). Tolerized mice initially displayed a high degree of resistance when compared to similarly infected control mice, but such resistance was shown to wane rapidly (2 days post-bacterial challenge; 5 days post-LPS administration), at a time when early endotoxin tolerance would normally be less than optimal (see Figure 1). It is interesting to note that a statistically significant delay in death was observed only at the highest two doses of bacteria. This may reflect a contribution of LPS from the bacterial challenge, which acts to serve as an additional

"tolerizing" injection. Morrison and Curry (1979) have estimated that approximately 40 µg of LPS could be expected from 10^{10} Gram negative organisms. In consideration of these results, repeated tolerizing injections prior to bacterial infection would have, perhaps, proven to be more efficacious in the protection of mice against Pseudomonas infection. A reduced time interval between LPS administration and bacterial challenge also could have yielded a greater degree of resistance to infection.

The major obstacle which has limited the clinical application of endotoxin for its beneficial effects has been its toxic side effects. Chemical modification of the endotoxin molecule has resulted in a non-toxic, Lipid A derivative, monophosphoryl Lipid A (MPL) (Qureshi et al., 1982) which is greatly reduced in its toxic activity. In the final section of this study, the efficacy of MPL to replace intact LPS in the early endotoxin tolerance protocol was examined. Unlike initial exposure to wild type LPS, MPL did not induce symptoms of endotoxicity, i.e., ruffled fur, conjunctival discharge, or diarrhea, even at a dose of 100 µg per mouse. Despite the absence of these overt manifestations of endotoxicity, MPL was shown to induce both serum colony stimulating factor and interferon to levels induced by wild type LPS. It was also found that injection of MPL on day 0 reduced the LPS-induced serum CSF upon challenge with wild-type E. coli LPS 3 days later. MPL was also shown to increase greatly the LD_{50} to challenge injection with E. coli LPS and effectively reduce

overt symptoms of endotoxicity upon challenge with intact LPS. As seen in the LPS-induced tolerance system, which was established in the first phase of this study, MPL administration greatly increased the number of macrophage progenitor cells in the bone marrow and this increase in M-CFU coincided with an increase in cell size and cell number in Coulter Channelyzer profile "Peak 2". Taken collectively, these results indicate that MPL possesses the necessary structural component(s) required to induce a state of early endotoxin tolerance. Most importantly, MPL failed to induce the initial toxic reactions typically observed with wild-type endotoxin or diphosphoryl Lipid A preparations. One intriguing aspect of this study was that tolerance, as assessed by IFN production, was much less affected by MPL than were the other manifestations of LPS responsiveness. One possible implication is that IFN may be less involved in the induction of the toxic effects of LPS than other serum factors, such as CSF. In this regard, previous work by Moore et al. (1980) supports a role for CSF as an immunoregulatory agent capable of rendering macrophages more sensitive to LPS in vitro, as assessed by increased production of IFN and Interleukin 1.

In summary, an early phase endotoxin tolerance model was established in mice based on a system developed by Williams et al. (1983). The results presented herein indicate that the state of early tolerance is associated with marked alterations in bone marrow progenitor pools. The cellular mechanisms which underlie this state of induced hyporesponsiveness were probed

further using various murine models to assess the relative contribution of certain lymphoid cell subsets. The temporal association of early phase tolerance and non-specific resistance to Gram negative infection was examined using Pseudomonas aeruginosa as the infecting agent. Lastly, a non-toxic Lipid A derivative, monophosphoryl Lipid A, was shown to be efficacious as an inducer of early phase tolerance. These findings, taken collectively, extend our present understanding of the cellular mechanisms which contribute to the acquisition of hyporesponsiveness to the toxic manifestations of endotoxin. An understanding of acquired LPS-hyporesponsiveness in experimental animals should further our understanding of LPS responsiveness. Moreover, the use of non-toxic derivatives of LPS to induce a hyporesponsive state to the toxic effects of endotoxin holds great potential for their use in therapeutic intervention.

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