THE ROLE OF ENDOTOXIN

AND TUMOR NECROSIS FACTOR

IN THE PATHOGENESIS OF SEPTIC SHOCK

by

Leopoldo Sánchez-Cantú, MD.

Department of Surgery, Division of Surgical Research
McGill University, Montreal.

April, 1990.

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Masters of Science in Experimental Surgery.

I have given you, O Adam, no fixed abode, and no visage of your own, nor any special gift, in order that whatever place or aspect or talents you yourself will have desired, you may have and possess them wholly in accord with your desire and your own decision. Other species are confined to a prescribed nature, under laws of my making. No limits have been imposed upon you, however; you determine your nature by your own free will, in the hands of which I have placed you. I have placed you at the world's very center, that you may the better behold from this point whatever is in the world. And I have made you neither celestial nor terrestrial, neither mortal nor immortal, so that, like a free and able sculptor and painter of yourself, you may mold yourself wholly in the form of your choice.

Oratio de hominis dignitate
Pico della Mirandola

(Quoted in Yourcenar, 1976)

"I don't mind being bone and feathers, mom. I just want to know what I can do in the air and what I can't, that's all. I just want to know."

Jonathan Livingston Seagull

(Bach, 1970)

ABSTRACT

Lethality, weight loss and Tumor Necrosis Factor (TNF) production induced by lypopolysaccharide (LPS) were studied in rats during early and late endotoxin tolerance. Different O-specific types of LPS were used. The TNF serum profile in two models of sepsis and the effect of LPS tolerance in the lethality induced by these models were also investigated. Results show diminished LPS-induced TNF levels during early LPS tolerance either elicited with the same or a different type of LPS. No correlation was found between TNF levels and LPS-induced mortality in naive animals. Low toxicity LPS preparations induced TNF levels similar to levels induced with toxic types of LPS. Late tolerance was associated with low levels of LPS-induced TNF and similar susceptibility to the lethal effect as naive rats. Early endotoxin tolerance conferred high protection from mortality induced with live intra-peritoneal bacteria or caecal ligation and puncture. In these last two animal models of sepsis, high serum levels of TNF were not detected.

RÉSUMÉ

La mortalité, la perte de poids, et la production de TNF ont été étudiées sur des rats rendus tolérants à différents sérotypes O d'endotoxine. Dans deux modèles de sepsis, les taux sériques de TNF et les effets de la tolérance au LPS sur la mortalité ont été investigués. Les résultats montrent une diminution de la réponse de TNF induite par LPS durant la phase précoce de tolérance à LPS, qu'on ait utilisé un type identique ou différent de LPS. Il n'y avait pas de corrélation entre les taux de TNF et la mortalité LPS-induite. Les LPS de faible toxicité et les LPS hautement toxiques ont entraînés des taux de TNF similaires. La tolérance tardive était associée à de faibles taux de TNF LPS-induit, ainsi qu'à un effet léthal identique à celui observé chez des rats intacts. La tolérance précoce a protégé de la mortalité dans les deux modèles utilisés. Dans ces modèles de sepsis, une production de TNF n'a pas pu être démontrée.

PREFACE

The experimental work for this thesis was done while the candidate was under the supervision of Dr. N.V. Christou in the Department of Surgery, McGill University, from May 1988 to March 1990.

All work contained in this thesis is that of the candidate. The L929 assay was adapted by the candidate with guidance from Dr. H.N. Rode. The assays were performed with help from Ms. Marta Bachetti. Ms. Dayle Eshelby assisted the candidate in the initial knowledge related to the animal work. Dr. M.J. McPhee and Dr. H.N. Rode initiated the candidate in experimental design. Ms. Corinne Lacey and Ms. Bisa Mitrovski, helped the candidate to perform the microbiological aspect of the experiment of caecal ligation and puncture (CLP) and Dr. W. Haupt made significant contributions to the surgical part of those same experiments. Bacteria used in this study were grown and prepared by Ms. Betty Giannias who also made the isolation and morphological characterization of all experimental samples cultured. The ELISA technique was adapted by the candidate with the assistance of Dr. Kushi Abikar. Mr. Ted Yun M.R.C.C. gave the candidate significant assistance to perform the experiments and generous help with some of the ELISA and the L929 cytotoxicity assays. The statistical analysis and the graphics were prepared by the candidate using commercial software. This thesis was prepared by the candidate. Luz María Osorio and Rémi Shneider helped with the Résumé.

Portions of the material contained in this thesis have been published elsewhere. A reference for these publications follows:

Sánchez-Cantú L, Rode HN, Christou NV. Lipopolysaccharide and Tumor Necrosis Factor reduce delayed-type hypersensitivity response but not humoral and nonspecific host defense. Surg Forum 1989;40:75-77.

Sánchez-Cantú L, Rode HN, Christou NV. Endotoxin Tolerance is associated with reduced secretion of Tumor Necrosis Factor. Arch Surg 1989;124:1432-1436.

Sánchez-Cantú L, McPhee MJ, Christou NV, Rode HN. Lack of correlation between TNF levels and LPS lethal effect. (Abstract) 7th International Congress of Immunology. Berlin, 1989.

Sánchez-Cantú L, Rode HN, Yun T, Christou NV. Late LPS tolerance is related to low levels of TNF but is not related to the specificity of the inducing LPS. (Abstract). 1st Congress, International Endotoxin Society, San Diego, 1990.

Sánchez-Cantú L, Rode HN, Christou NV. Tumor Necrosis Factor alone does not explain the lethal effect of lipopolysaccharide. (Abstract) 10th Annual Meeting. Surgical Infections Society, Cincinnaty, 1990.

ACKNOWLEDGMENT

I would like to extend my gratitude to Dr. Nicolas V. Christou who made possible the accomplishment of this project. To Dr. Jonathan L. Meakins who opened the doors for me into Surgical Research. To Dr. Harold N. Rode who took me under his tutorial protection and with patience, tolerance and skill showed me beginning with the *abc* one of the most intense intellectual experience of my life. To Dr. Julius Gordon for his restless pursuit of knowledge and open-minded and energetic attitude, an example that will be an inspiration in my life. To Dr. Pnina Brodt and Dr. Deidre Waldon-Edward for their countless contributions to my scientific development. To Dr. Annalise Recklies and Dr. Carl W. Nohr who, as members of my Advisory Committee were vigilant of my progress and ready to help.

To Dr. José I. Santos P. for conceiving this crazy project and for believing in me. To Dr. Luis Torregrosa and the Hospital Infantil de México for their support. To Dr. Pedro Valencia M. Dr. Nicolás Martín del Campo M. and Dr. Luis Eraña G. who from the distance were stimulating friends. To the Surgical Infection Society and the 3M Company for granting to me a very substantial award and to Dr. Fred Nestell for generously giving me the L929 cell line.

To Corinne Lacey and Ted Yun who gave me fraternal friendship and camaraderie on the job. To Werner Haupt and Martin J MacPhee for sharing with me their ways of seeing life and science; a most stimulatory experience. To Jian Wang for reminding me a human soul like her's can be pure and still keep a highest quality as a student and scientist.

To Els Schotman for her warm friendship to me and my family when we most needed it and for making the everyday work enjoyable and productive with the highest quality.

To Brenda Bewick, Ann Gordon and Irene Sydorenko for making the long days at the lab so nice and for their affectionate support. To Maria Ribeiro and Leslie Johnson for their care for my experimental animals and fine touch with me whenever we interacted.

To Betty Giannias for her skillful contributions and kind approach. To Dale Eshelby, Diane Cunningham, Marta Bachetti, Mary de Santis, Louise Chartrand, Francine Lalond, Rose Mary Weidner, Mary Bouldadakis, Rémi Shneider, Kushi Abikar and Bomi Kapadia for all their help.

And to Patricia and Harvey Guyda for showing me the nicest face of Canada, the Canadian noble decency.

DEDICATION

to **Luz María,** my lovely wife;

for believing and sharing and carrying and caring with me and for me with endless solidarity all the way through.
Without her I wouldn't have made it.

to **Rebeca and Alejandra,** my daughters;

for giving light, freshness and meaning to my life.

to **Jaqueline,** my former patient;

a brave little girl who defeated septic shock
in spite of my limitations.

and

To my dear brother **Mauricio X. Zuber,** for his example, advice and support and for being my very much needed **BIG BROTHER** in this adventure.

TABLE OF CONTENTS

ABBREVIATIONS

ABTS ..... 2,2'-Azino-bis(3-ethylbenzoline-6-sulfonic acid)
AGEPC ..... Alkyl-acetyl Glycero Phosphocholine
ARDS ..... Adult Respiratory Distress Syndrome
$BaSO_4$ ..... Barium sulphate
BHI ..... Brain-Heart Infusion culture broth
BSA ..... Bovine Serum Albumin
Cat ..... Catalog number
$CEFD_{50}$ ..... Chick embryo lethal dose 50
CFU ..... Colony forming units
CK ..... Cytokine
CNS ..... Central Nervous System
CoF ..... Cobra venom Factor
CRP ..... C Reactive Protein
CTA_2 ..... Carbocyclic thromboxane A_2
DIC ..... Disseminated Intravascular Coagulation
D/R ..... Dose/Response
DTH ..... Delayed Type Hypersensitivity
ELISA ..... Enzyme Linked Immono Sorbent Assay
F1 ..... First generation subject
FBS ..... Fetal Bovine Serum
FMLP ..... Formyl-Methionyl-Leucyl-Phenylalanine
GI ..... Gastro-intestinal
GM-CSF ..... Granulocyte Monocyte-Colony Stimulating Factor
HBSS ..... Hank's Balanced Salt Solution

HETE..... Hydroxyeicosatetraenoic acid
HSF..... Histamine-induced suppressor factor
IFN..... Interferon
IL-1..... Interleukin 1
IL-2..... Interleukin 2
IL-6..... Interleukin 6
im..... intramuscular
ip..... intraperitoneal
KDO..... Keto deoxy octulosonate
KLH..... Keyhole Lympet Hemocyanin
LD_{50}..... 50% Lethal dose (75%, 90%, 100%)
LPS..... Lipopolysaccharide
LT..... Leukotriene (B, C, D, E)
M..... molar
2-ME..... 2-Mercaptoethanol
MIF..... Migration Inhibition Factor
MK..... Monokine
Mø..... Macrophage
MOF..... Multiple Organ Failure
MPL..... Monophosphoryl lipid A
MPS..... Mononuclear Phagocytic System
MSH..... Melanocyte Stimulating Hormone
MSOF..... Multiple System Organ Failure
MTT..... 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2HPO_4..... Sodium phosphate, dibasic
NK..... Natural Killer Cell
NSAIDs..... Non-steroid anti-inflammatory drugs
OD..... Optical Density
PAF..... Platelet Activating Factors
PBS..... Dulbecco's Phopsphate Buffered Saline
PG..... Prostaglandin (D, E, F, I)
PGI_2..... Prostacyclin
PMN..... Polymorphonuclear leucocyte.
RPMI-1640..... Rockwell Park Memorial Institute media #1640
sc..... subcutaneous
SP..... Substance P

SRS-A ..... Slow-releasing substance of anaphylaxis
rMuTNF-α ..... recombinant Murine Tumor Necrosis Factor-alpha
TNF ..... Tumor Necrosis Factor
TT ..... Tetanus Toxoid
Tx ..... Thromboxane (A_2, B_2)
UTI ..... Urinary Tract Infection
VIP ..... Vasoactive Intestinal Peptide

LIST OF FIGURES

LIST OF TABLES

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. In rats, the early phase of endotoxin tolerance is associated with decreased generation of serum TNF levels after a challenge with the same or a different type O-specific endotoxin compared to naive animals.

2. During early endotoxin tolerance, diminished serum TNF levels produced in response to endotoxin have no correlation with increasing levels of anti-LPS antibodies.

3. In naive rats, there is no correlation between peak serum TNF levels and mortality after an intra-peritoneal injection of LPS.

4. LPS from different types of gram-negative bacteria can induce in rats similar peak serum TNF levels whereas their toxicity varies from highly lethal to mildly toxic.

5. The late phase of LPS tolerance is characterized by the presence of high titers of O-specific anti-LPS antibodies and much lower peak serum TNF levels after a challenge with the same or a different type O-specific LPS compared to naive rats.

6. Rats in the late phase of endotoxin tolerance have similar susceptibility to the lethal effects of LPS as the naive animals but decreased sensitivity to the body wasting effect.

7. Early endotoxin tolerance confers high protection from mortality after the intraperitoneal injection of lethal amounts of live *E. coli* in barium sulfate or caecal ligation and puncture.

8. Animal models of infection such as caecal ligation and puncture or intraperitoneal injection of live bacteria are not associated with the high levels of circulating TNF that are seen in the pure endotoxin administration rat model. This finding questions the role of tumor necrosis factor in the mediation of septic shock.

REVIEW OF THE LITERATURE

BACTERIAL INFECTION AS A PROBLEM IN SURGERY

Since the very early practice of surgery, infection has been and still is one of the unsolved problems in this discipline.

The way infection challenges our skills has varied over the years. In the early times, the cause of death was cardiovascular collapse or acute renal failure as a result of incomplete resuscitative efforts. More recently the Adult Respiratory Distress Syndrome (ARDS) under its many synonyms (Baue 1975) was imposing its toll. Nowadays, what we rather see is a late developing pattern of multiple organ failures (Tilney 1973) linked to intraabdominal and pulmonary infections (Fulton 1975) and extended to the full spectrum of Multiple System Organ Failure (MSOF) (Baue 1975; Polk 1977; Fry 1980) which encompasses ARDS, acute renal failure, disseminated intravascular coagulation (DIC), gastrointestinal tract failure (stress ulcers, hepatic failure or acute acalculous cholecystitis), cardiovascular collapse, metabolic instability and central nervous system dysfunction, all of them sharing a common phathophysiology closely related to microvascular damage seemingly mediated by endogenous products released or produced as response to microbial invasion (Borzotta 1983; Marshall 1988; Kreger 1980).

Sepsis has an incidence of 70,000 to 500,000 cases each year in the United States and some 18,000 of these patients die annually with gram-negative bacteremia (Parrillo 1984). Shock complicates sepsis in about 40% or cases (Parker 1983) and septic shock has a mortality of 30-90% (McCabe 1974; Bone 1989). MSOF on the other hand occurs following 7% to 22% of emergency operations and 30% to 50% of operations for intra-abdominal infection (Bone 1989) and carries a 30% to 100% mortality depending on the number of organs involved (Carrico 1986; Fry 1980; Baue 1980).

Escherichia coli, Pseudomonas aeruginosa, enterococci, and *Staphylococcus aureus* are the most frequently reported pathogens associated with nosocomial infections (Segreti 1989). In Kreger's review of septic patients only in 70% of the patients could the original source of infection be identified and 16% of the positive bacteremia were polymicrobic (Kreger 1980). In another series only 45% of septic

patients were found to be bacteremic (Bone 1989). These patients with overt septic syndrome as well as persistently negative blood cultures are actually very common. The phenomenon was named "nonbacteremic clinical sepsis" by Meakins highlighting the fact that this group of patients had essentially the same clinical course and outcome as that of patients who had demonstrable bacteremia (although Bone reports higher incidence of shock in the bacteremic subgroup, [Bone 1989]), yet no convincing focus of infection could be found (Meakins 1980).

Infection as a microbial phenomenon is the presence of microorganisms invading normally sterile host tissues and requires the demonstration of an infecting organism to be diagnosed, but does not require evidence of a host response. Sepsis on the other hand is a clinical syndrome characterized by fever, tachypnea, tachycardia, leukocytosis, and evidence of inadequate organ function or perfusion; the diagnosis of sepsis does not require the demonstration of a microbial pathogen (Marshall 1990).

The extent of an infection is a complex multifactorial condition determined by the interplay of the host's defense mechanisms and microbial virulence factors. Some failure in local or systemic host defenses must occur for a microbe to cause disease. Once established, bacteria are thought to produce damage in an infected host by two main mechanisms: (1) The production of toxic factors (protein exotoxins and lipopolysaccharide endotoxins). (2) Inducing responses in the host that are harmful to the host tissues and systems (Immunologic injury).

Although there are many examples of immunologic injury resulting from experimental infections in animals and even a few examples in human infections such as rheumatic fever, antigen-antibody complexes causing acute glomerulonephritis and vasculitis, and Delayed-type hypersensitivity reactions. (Howard 1988) a conclusive argument describing the way and weight of the host's response in the pathophysiology of sepsis is not yet available.

The traditional approach to infection treatment is addressed to the agent in the form of antibiotics and passive immunotherapy (Ziegler 1982; Baumgartner 1985), and to the patient with all the life support systems including conventional nutritional support. Enhancement of the immunologic potential of the patient, is only recently

being focused upon as a new and encouraging therapeutical modality (Kotani 1983; Waymack 1984; Bone 1987; Alexander 1988).

Improvement of new therapeutic modalities is of utmost importance considering that the short generation time of microorganisms virtually guarantees that any new antimicrobial chemotherapy will have only a transient period of effectiveness prior to the emergence of microbial resistance. Also modern therapeutic and monitorizing techniques with invasive devices, hyperalimentation, aggressive oncologic chemotherapy, the use of immunosuppressive agents and prolonged survival of severely ill, malnourished or extremely old patients have created new and peculiar conditions for the establishment of sepsis in the immunocompromised host that we are not ready to successfully treat with the traditional therapeutic modalities.

To what extent are bacteria and their toxins responsible for the derangements found in sepsis, and what exactly is the role of endogenous mediators in the pathogenesis of the hemodynamic and metabolic catastrophes that ultimately result in septic shock or the MSOF syndrome is still a matter of debate. Nevertheless, increasing interest and knowledge has recently accumulated in this field and a broader picture is becoming available for us.

Future prospects for improved prevention and treatment of surgical infection hinge upon two pivotal issues: (1) Better definition of the mechanisms involved in the compromised host, and (2) advances in methods of modulation of the immune response for enhancement of the patient's defense against contamination that is inevitable from ubiquitous, potential pathogens. The ability to stimulate non-specific host defense is particularly attractive, since this should minimize the capability of microbial invaders to create a resistant niche that has characterized treatment by antibiotics or specific bacterial vaccines (Polk 1981).

ENDOGENOUS MEDIATORS OF SEPSIS

In pathophysiologic terms the essence of shock is cellular damage secondary to tissue hypoxia, which might be a consequence of hypoxemia or hypoperfusion. Blood flow to the peripheral tissues may be compromised by the fall in driving force

(perfusion pressure) and/or by the baroreceptor-induced vasoconstriction aimed at maintaining the systemic pressure and preferential flow to the more essential cerebral and myocardial tissues. However, the vascular beds that do respond to centrally induced vasoconstriction during systemic hypotension, principally viscera and kidney, do so at the expense of their own well-being. If flow deprivation of this tissues is prolonged, local and systemic accumulation of vasodepressor substances may cause these vascular beds to lose their tone, which allows a local increase of flow but further systemic hypotension (Emerson 1974). This vasodilation called vascular decompensation by some and shock by others will cause an even greater fall in the systemic arterial pressure; it is in fact a breakdown in the normal compensatory response to hypotension (Bond 1983; Green 1990; Parrat 1983).

In the course of an infection, stimuli are generated and perceived by different receptors located peripherally or centrally. Part of these stimuli are sensed locally and induce a local or regional inflammatory reaction (Shires 1989). Others will be detected by specialized neural receptors which transmit afferent signals to the central nervous system (CNS). In the CNS inputs are integrated resulting in the production of efferent signals which amount in the stimulation or inhibition of the release of numerous neuroendocrine products that generate physiologic changes and will ultimately direct an attempt to restore cardiovascular stability, to preserve oxygen delivery, to mobilize energy substrates, to recover fluid and electrolyte homeostasis, and to minimize pain (England 1982, Benedict 1978).

The complete setting for the host's response to sepsis constitutes two global groups: (1) the systemic neuroendocrine environment dedicated preferentially to the homeostasis of the cardiovascular and metabolic systems; and (2) the inflammatory mediators whose duties include the setting up of an inflammatory response aimed to limit bacterial invasion and its eradication, and the mechanisms involved in wound healing, and tissue remodeling and repair (Table 1).

SYSTEMIC NEUROENDOCRINE ENVIRONMENT

The loss of effective circulating volume results in the baroreceptor reflex conserving water and salt and increasing total peripheral resistance. This leads to increased secretion of adrenocorticotropic hormone (ACTH), vasopressin, renin, growth

hormone, β-endorphin and catecholamines (Bereiter 1984 and 1985; England 1986). In turn these neuroendocrine effectors bring about further changes including stimulation of cortisol secretion by the adrenal glands, conversion of angiotensinogen to angiotensin I by renin, stimulation of aldosterone secretion in response to angiotensin II, stimulation of glucagon secretion and inhibition of insulin secretion by the pancreas in response to epinephrine (Claybaugh 1973).

Table 1. Inflammatory Mediators

Function	Mediators
Increased microvascular permeability	Histamine, bradykinin, C_{3a}, C_{4a}, C_{5a}, LTC_4, LTD_4, PGE_2, prostacyclin, Hageman factor, HMWK, PAF, serotonin, substance P
Vasodilation	PAF, Histamine, Bradikinin, prostacyclin, VIP
Vasoconstriction	TxA_2, leukotrienes C and D, C_{5a}, LTB_4, formyl peptides, $PGF_{2\alpha}$, serotonin
Smooth muscle contraction	C_{3a}, C_{5a}, histamine, LTB_4, leukotrienes C and D, bradikinin, PAF, serotonin
Increased endothelial cell stickiness	IL-1, TNF-α, endotoxin, LTB_4, PAF
Platelet aggregation	PAF, TxA_2, TNFα, serotonin
Phagocytes:	
Recruitment from bone marrow	CSFs, IL-1, C_{3e}
Adherence/aggregation	iC_{3b}, fibronectin, IgG, PAF, IFNα,β1
Chemotaxis	C_{5a}, LTC_4, LTB_4, PAF, IL-1, histamine, β-endorphin, laminin, fibronectin and elastin, formyl peptides, collagen, lymphocyte-derived chemotactic factor, IFN α,β1
Lysosomal granule release	C_{5a}, PAF, formyl peptides
Toxic oxygen product formation	C_{5a}, TNF-α, PAF, most chemoattractants, phagocytic particles; interferon-γ
Phagocytosis	C_{3b}, iC_{3b}, IgG (Fc portion) fibronectin; interferon-γ (increases FcR expression)
Granuloma formation	Interferon-γ, IL-1, TNF-α
Pyrogens	IL-1 α & β, TNF-α, PGE_2, IL-6, IFN-α & γ
Pain	PGE_2, bradikinin, PAF
Acute Phase response	IL-1, TNF-α, IL-6

Changes in the concentration of oxygen, hydrogen ions and carbon dioxide, through the activation of peripheral chemoreceptors located in the carotid and the aortic bodies produce a decrease in cardiac sympathetic nervous system activity and an increase in parasympathetic activity which activates the respiratory center leading to an increase in respiratory rate (Davies 1984).

Pain induces secretion of vasopressin, ACTH, endogenous opiates, catecholamines, cortisol, and aldosterone, and changes in activity of the autonomic nervous system (Vaughan 1982).

Changes in the plasma glucose concentration are sensed by receptors in the hypothalamus and in the pancreas. A decrease in the plasma glucose concentration stimulates the release of catecholamines, growth hormone, cortisol, ACTH, β-endorphin, and vasopressin (Curtis 1980). In addition, the secretion of insulin is inhibited by central pathways and by the pancreas itself (Gann 1989; Lilly 1983).

ENDOGENOUS OPIOIDS

Recently, interest in opioid peptides has resulted from the finding that naloxone improves the hemodynamic response and survival in hemorrhagic, septic and spinal shock (Faden 1978; Curtis 1980; Plotnikoff 1985; Wybran 1985). Several endogenous opioids have been characterized. Two pentapeptides: methionine-enkephalin and leucine-enkephaline; β-endorphin which contains 31 amino acids; α-endorphin with 16, and τ-endorphin and dynorphin both 17 amino-acid peptides; all of these share the amino-terminus Tyr-Gly-Gly-Phe which is responsible for much of biological activity of these neuropeptides.

Opioid peptides are synthesized in the central nervous system, peripheral nerves, neuroendocrine cells, macrophages and in lymphocytes. Thus, these neuropeptides are considered to allow bidirectional interaction between various cells of the immune system as well as between the nervous and immune system (Smith 1985).

Endogenous opiates have analgesic activity, and cardiovascular, metabolic and neuroendocrine modulating properties. Their precise role in the response to infection and other types of injury remains ill defined at present, nevertheless, they have important immunomodulatory capabilities such as enhancing lymphocyte blastogenesis, increasing the size of the thymus or spleen in rodents, stimulating active T cell rosettes and natural killer cell activity, and increasing neutrophil and macrophage chemotaxis and adherence and neutrophil superoxide production (Van Epp 1987, Sharp 1987). It is beleived that in stress, enkephalins modulate the effects of steroid hormones on the immune system (Plotnikoff 1985) and in this

context opioids have been implicated in stress-induced natural killer cell suppression (Shavit 1984). In spite of the high expectations clinically, opiate receptor blockade with naloxone has yielded equivocal results (Tiengo 1980, Peters 1981, Groeger 1983).

INFLAMMATORY MEDIATORS

Many cell types together with soluble serum components and elements of the extracellular matrix are needed to build up a complete local and systemic inflammatory reaction. The leading cell types are the endothelial cells, macrophages and neutrophils; also important are platelets, mast cells, fibroblasts, basophils, eosinophils, T and B lymphocytes and hepatocytes. Nevertheless, macrophages have a privileged role as they can influence almost every aspect of both the immune and inflammatory responses, from acute to delayed hypersensitivity, from the first breach of epithelium to its eventual repair. One of the outstanding capabilities of macrophages is the secretion of some 100 active substances ranging in molecular mass from 32 (superoxide anion) to 440,000 daltons (fibronectin) which allow this cell to play such a central role. Some of the most relevant secretory products for sepsis and shock are listed in Table 2 (adapted from Nathan 1987).

COMPLEMENT SYSTEM

The portion of the complement cascade that affects the hemodynamic status of the host involves the anaphylatoxins C_{3a}, C_{4a} and C_{5a}. Their biologic activities include increased permeability and dilation of the microvasculature; smooth muscle contraction and release of histamine by basophils and mast cells (Grant 1976). Anaphylatoxins act upon neutrophils increasing their chemotaxis, aggregation, secretion of lysosomal enzymes, oxidative metabolism, adherence to surfaces and expression of C_3 receptors (Becker 1974; Tonnesen 1984; Charo 1986; Hoover 1978). They act upon mononuclear phagocytes to increase chemotaxis, secretion of lysosomal enzymes, oxidative metabolism, expression of C_3 and IgG receptors and generation of Interleukin-1 (Goodman 1982). C_{5a} may also prompt release of serotonin from platelets (Meuer 1981). C_{3a} induces release of thromboxane from

macrophages (Hansch 1984), and apparently the effects of human C_{5a} on vascular permeability is mediated by neutrophils and vasodilatory prostaglandins (Issekutz 1980; Björk 1985; Regal 1981).

Complement components are known to be normal or increased in the acute stages of clinical infection but are often decreased once systemic sepsis and septic shock develop. The massive complement activation seen in septic shock could plausibly exhaust the body's synthetic capacity and explain these kinetics. Furthermore, the extreme increase in complement activation that occurs in sepsis has been correlated with an ultimately poor prognosis (Duchateau 1984; Hammerschmidt 1980; Solomkin 1985; Weinberg 1984).

Table 2. Secretory Products of Macrophages

Polypeptides:
IL-1α, IL-1β, IL-6, TNF-α, IFN-α, IFN-β1, IFN-τ, GM-CSF, G-CSF, β-endorphin, Insulin like activity, Epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, Transforming growth factor-β
Complement components:
Classical pathway: C_1, C_4, C_2, C_3, C_5; Alternative pathway: Factor B, Factor D, properdin
Inhibitors. C_{3b} inactivator, β-1H
Active fragments generated by Macrophages: C_{3a}, C_{5a}, C_{3b}, Bb
Coagulation factors:
Intrinsic pathway: IX, X, V, prothrombin. Extrinsic pathway: Factor VII
Surface activities. tissue factor, prothrombinase
Prothrombolitic activity. plasminogen activator
Other enzymes:
Neutral proteases. Plasminogen activator, elastase, collagenases, angiotensin convertase
Lipases. lipoprotein lipase, phospholipase A_2; Glucosaminidase. lysozyme; Deaminase. arginase
Lysosomal acid hydrolases. proteases, lipases, deoxyribonucleases, phosphatases, glycosidases, sulfatases
Binding Proteins:
Fibronectin, Thrombospondin, transferrin, transcobalamin
Bioactive lipids:
PGE_2, PGF_2, prostacyclin, TxA_2, LTB_4, C, D, E, HETE acid, PAF
Reactive oxygen intermediates:
Superoxide, hydrogen peroxide, hydroxyl radicals, hypohalous acids
Reactive nitrogen intermediates:
Nitrites, nitrates
Inhibitors of enzymes and cytokines
Protease inhibitors. α-2-macroglobulin, α-1-antiprotease, plasminogen activator inhibitors, plasmin inhibitors, collagenase inhibitor
Phospholipase inhibitor lipomodulin (macrocortin), IL-1 inhibitors
Low molecular weight inflammatory cytokines:
Monocyte-derived neutrophil chemotactic factor, IP10, β-Thromboglobulin,

The vasodilatory properties of anaphylatoxins can decrease peripheral resistance and subsequently produce hypotension. Severe shock states have been produced in animal models with the injection of the C_{3a} and C_{5a} components. Whether the effects of complement are primary, secondary to interactions with other systems, or both is unclear, but as previously mentioned, complement components induce histamine, thromboxane and serotonin secretion, all which possess significant vasoactive properties and, via complement, could certainly alter the hemodynamic balance during sepsis.

Particularly relevant to show the important role of anaphylatoxins MSOF is the experimental evidence (Till 1982 and 1985) suggesting that systemic complement activation can lead to neutrophil dependent acute pulmonary injury. It now appears that oxygen derived free radicals (superoxide anion, hydrogen peroxide, hydroxyl radicals and others) released from complement-activated sequestered neutrophils may play a major pathogenic role in the microvascular damage that lead to increased permeability and pulmonary edema in the ARDS (Simon 1988; Hammerschmidt 1980; Duchateau 1984; Weinberg 1984; Chenoweth 1981).

Bacterial endotoxins can activate complement both by the classic (through an antibody-dependent as well as an antibody-independent mechanism) and the properdin pathways (Mergenhagen 1969, Loos 1974) and it has been suggested that complement activation may be involved in the lethal effects of endotoxins (Spink 1961 and 1964; Gilbert 1962, Polák 1969, From 1970; Garner 1974, Kitzmiller 1972). On the other side, experiments by several groups have shown contradictory results (Muller-Berghaus 1974). Ulevitch concluded that C_3 and terminal components did not play a significant role in the lethality in rabbits caused by bacterial endotoxins (Ulevitch 1975 and 1978), and experiments by Galanos et al have demonstrated that anticomplementary activity of endotoxin is not fundamental for toxicity (Galanos 1971). Nevertheless, these conclusions are still challenged (Brown 1973; Johnson 1971).

It is clear that the interaction of complement with bacterial endotoxins *in vivo* does, at least in part, contribute to the overall host response to endotoxin, yet the precise role of this interaction in the initiation of pathophysiologic changes leading to tissue injury remains to be defined (McPhaden 1985).

COAGULATION SYSTEM AND PLASMA KININS

Four major plasma protein systems contribute to the host's defense and, at times, participate in the development of inflammatory tissue injury. These are the contact activation (CAS), the coagulation, the fibrinolytic, and the complement systems.

The CAS is composed by Hageman factor, plasma prekallikrein, high-molecular weight kininogen (HMWK), and coagulation Factor XI. Secondary reactions with plasminogen, Factor VII of the extrinsic coagulation system, and complement proteins extend the physiologic effects of the contact system (Kozin 1988).

Two kinins, bradykinin and kallidin are produced through the action of the serine protease kallikrein on high and low molecular weight kininogens in plasma and in tissues respectively. Kallikrein exists in plasma in an inactive form as prekallikrein, whose activation depends upon activation of the clotting system through Hageman factor. All these reactions are tightly regulated by a series of inhibitory plasma proteins; C_1 inhibitor, $\alpha 2$ plasma inhibitor, $\alpha 2$ macroglobulin, antithrombin III, and $\alpha 1$ antitrypsin inhibitor (Miller 1973; Kozin 1988).

The kinins are potent vasodilators that increase capillary permeability, produce edema, evoke pain, increase bronchial resistance, and enhance glucose clearance. As such they are important mediators of the inflammatory response. They participate in the regulation of fluid and electrolytes by the kidney by causing renal vasodilation, reduction in renal blood flow, increase formation of renin and an increase in sodium and water retention (Cochrane 82).

Hageman factor activates the classical complement pathway through C_1 activation or it can be activated by kallikrein which generates a C_{5a}-like chemotactic peptide from C_5 (Wiggins 1981). Bradikinin has been shown to augment arachidonic acid release from cells (McGiff 1976), and on fibroblasts and endothelial cells it induces prostacyclin, PGE_2, and PAF production (McIntyre 1985).

Increased plasma concentrations of kallikrein and bradikinin and decreased plasma concentrations of prekallikrein have been noted during hemorrhagic, endotoxic and septic shock. Furthermore, these changes appeared to correlate with survival and severity of injury, since there is a gradual increase in the concentrations of

prekallikrein in survivors but not in nonsurvivors and a greater activity of kallikrein in patients with septic shock than in patients with sepsis without hypotension (Aasen 1983).

Studies in humans and in non-human primates (Kimball 1972; Nies 1973) have also shown that blood bradykinin levels increase following the intravenous injection of endotoxin, which suggested the generation of activated Hageman factor by endotoxin. Kimball et al, (1973) demonstrated that injection of *S. abortus equi* endotoxin into humans produced a significant elevation in blood bradykinin levels within 30 to 60 minutes after injection. These authors suggested that bradykinin may serve as a trigger vasodilator in endotoxin shock. Other studies have shown that in dogs and in rabbits total blood kininogen also decreases following endotoxin injection.

Mason and Colman observed decreased levels of plasma Hageman factor and prekallikrein in patients with disseminated intravascular coagulation (DIC) in whom endotoxemia was thought to be responsible for initiating the coagulative changes (Mason 1971). Robinson (1975) studied patients undergoing cystoscopy and trans-urethral resection of the prostate and measured blood levels of endotoxin. Positive serum endotoxin or gram-negative bacteremia were associated with decreased levels of plasma prekallikrein and decreased vascular resistance.

Therefore, experimental evidence as well as clinical studies suggest that Hageman factor activation results from exposure of the host to endotoxin. Although direct biochemical measurements of Hageman factor activation or activation of its substrates, are essential to fully evaluate the role of these proteins in endotoxin-induced injury (Shen 1973).

Plasma kallikrein, coagulation factors and fibrinolytic factors can interact with other plasma proteins like the complement system (Ghebrehiwet 1983), prostaglandins (McGiff 1976; Hong 1976), prostacyclin (Moncada 1979; Roscher 1984), platelet activating factors (McIntyre 1985). This implies that the contact activation system elements are principal subjects in the pathophysiology of inflammation and sepsis.

VASOACTIVE INTESTINAL PEPTIDE (VIP) AND SUBSTANCE P

VIP is a recently characterized 28-amino acid peptide with the capacity to affect vascular tone (Said 1970). Originally isolated from the small intestine, it is now known to be widely distributed in both central and peripheral nerves and is also produced by non-beta cells of the endocrine pancreas. Endotoxic animal preparations have shown a significant elevation of VIP, and VIP is a known potent vasodilator. It is postulated that this peptide may also mediate the hemodynamic septic response, although clinical corroboration is lacking at this time (Bjornson 1977). Several modulatory effects of VIP on various aspects of the inflammatory response suggest that VIP is in fact an anti-inflammatory neuropeptide (Aguayo 1989).

Substance P (SP) is an 11 amino acid peptide, localized in the C fibers of sensory nerve endings innervating connective tissue, smooth muscle, and blood vessels of several species, including man (Lundberg 1987). SP causes contraction of smooth muscle, constriction of pulmonary airways leading to bronchoconstriction, and permeability of microvasculature to increase and stimulates epithelial cell secretion in the lungs and the gut. SP also acts indirectly by stimulating the release of mediators such as leukotrienes and histamine. SP stimulates monocyte chemotaxis, induces generation of TxA_2, oxygen radicals and H_2O_2, activates lysosomal enzyme release and stimulates phagocytosis of yeast cells (Shanahan 1985).

HISTAMINE

Histamine, 2-(4-imidazolyl)ethylamine or 5β-aminoethylimidazole is formed by decarboxylation of the amino acid histidine. Most histamine is stored preformed in cytoplasmic granules of mast cells and basophils in close association with proteoglycans comprising the granule matrix (Metcalf 1979).

Activation of human mast cells by antigen bridging of membrane-bound IgE initiates a cascade of membrane lipid metabolic events leading to the opening of calcium channels, cleavage of arachidonic acid from phosphatidylcholine, and release of secretory granules containing preformed granule constituents such as histamine (White 1988). Other substances also cause mast cells or basophils to

release histamine. Among them C_{3a}, C_{5a}, substance P, neurotensin, IL-1 and ATP. Once released histamine diffuses rapidly into the surrounding tissues and appears in draining blood within minutes.

Histamine mediates bronchoconstriction, increased myocardial contractility, intestinal contraction, gastric secretion, increased cardiac rate and pulse pressure reflecting diastolic hypotension, increased vascular permeability and small vessel vasodilation with edema formation (Keahey 1985; Yurt 1986).

Histamine has long been implicated in the pathophysiology of tissue injury and shock and elevated concentrations have been demonstrated after hemorrhagic shock, septic shock, endotoxemia and thermal and nonthermal injury (Hinshaw 1960). The highest levels of histamine seem to occur with sepsis and endotoxemia (Markley 1975). Following the administration of endotoxin to dogs, there is an immediate explosive release of histamine that correlates with the amount of endotoxin administered and with the decline in arterial blood pressure and circulating platelets consequent to the administration of endotoxin.

Serum histamine levels have been inversely correlated with survival in patients with septic shock and after endotoxin administration in rats (Nagy 1986). By contrast, histamine administration could increase the survival rate in endotoxic shock (Fox 1962). Released histamine also failed to worsen or improve vascular and metabolic responses to endotoxin (Jacobson 1964), and long-term infusion of histamine did not elicit pulmonary oedema (Olson 1983). A reliable demonstration of histamine release under *in vivo* septic-endotoxic shock conditions has however not sufficiently been fulfilled mainly because the assays to measure it as well as the design of the studies have not been solid enough (Neugebauer 1987).

Histamine induces the elaboration of several lymphokines that accounts for its immunomodulator role. Among those lymphokines is Histamine-induced Suppressor Factor (HSF) made by suppressor T cells which inhibits lymphocyte proliferation, NK activity, and antibody production (Rocklin 1977 and 1978). Another histamine induced T-cell factor inhibits Migration Inhibition Factor (MIF) production (Center 1983; Reichman 1979).

PLATELET ACTIVATING FACTORS

This family of acetylated phosphoglycerides together with the eicosanoids form a group known as lipid autacoids (substances with autopharmacologic properties and potent local activity). Both PAF and eicosanoids are rapidly generated after cell stimulation or perturbation. PAF is 1-alkyl-2(R)-acetyl-glycero-3-phosphocholine (AGEPC) composed of at least 16 molecular species containing saturated or unsaturated alkyl or acyl chains, or even a polar head group other than choline; each may have distinct although related biological effects.

PAF is a potent mediator of anaphylaxis and hyperreactivity and may be involved in shock, graft rejection, post-ischemic disorders, ovoimplantation and certain central nervous system disorders. It is also a relevant modulator of the immune response (Pinckard 1988). The complete biological role of PAF is unknown because the majority of studies characterizing its biological activities utilize only one or possibly two AGEPC derivates. Also, differences in the rates of synthesis and degradation of the various molecular species of PAF could be anticipated and expected to dramatically influence their biological potentials.

Sensitized basophils and mast cells release PAF in response to antigen or anti-IgE challenge. The release of PAF is the response of monocytes and macrophages to phagocytic stimuli such as bacteria, opsonized zymosan or immune complexes, and to the calcium ionophores A23187, while neutrophils additionally respond to phorbol esters and C_{5a}, C_{5a}-des-Arg and formyl-methionyl-leucyl-phenylalanine (FMLP) releasing PAF. Eosinophils also respond to A23187, C_{5a}, FMLP. Endothelial cells release PAF in response to A23187, thrombin, vasopressin, angiotensin II, anti-Factor VIII and interleukin 1 (Braquet 1987).

PAF is able to mimic the shock state. It produces hypotension, bronchoconstriction, thrombocytopenia granulocytopenia, and monocytopenia, and death in the guinea-pig. (Vargaftig 1980; Bessin 1983; McManus 1980). It is a potent agonist not only for most inflammatory cells, but also for vascular smooth muscle and endothelial cells as well as renal mesangial and epithelial cells. *In vitro* it causes aggregation, chemotaxis and chemokinesis, and effects the release of both specific and azurophil granule contents, including lysozyme, lactoferrin, β-glucuronidase and

myeloperoxidase from isolated rabbit and human PMN through lipoxygenase stimulation of arachidonate metabolism (15-HETE [hydroxyeicosatetraenoic acid] and LTB4). It initiates the respiratory burst in human neutrophils (Smith 1984). The monocyte, eosinophil and vascular endothelial cell are also stimulated by AGEPC increasing vascular permeability through post-capillary venular endothelial cell activation (Yasaka 1982).

In man, the intracutaneous administration of AGEPC results in dramatic and potent inflammatory actions (blanching of the skin, pain and pruritus) followed by the development of erythema and edema (Pinckard 1980). AGEPC induced vasoactivity appears to be platelet-, PMN-, H1 receptor- and cyclooxygense products-independent (Archer 1985). In several species including nonhuman primates, AGEPC induces hemoconcentration and generalized vascular leakage of plasma proteins (Saunders 1987). It has a potent negative inotropic effect in isolated perfused guinea pig and rat hearts; it induces a dose-related decrease in coronary flow and impairs atrioventricular conduction (Kenzora 1984). In dogs, PAF induces hypotension, myocardial contractility impairment, decreased coronary artery flow, systemic and pulmonary vascular changes, renal dysfunction, hemoconcentration and metabolic acidosis (Bessin 1983).

In the rat, shock triggered by ip injections of 2×10^8 CFU of *E. coli* (Inarrea 1985) or *S. enteritidis*.(Braquet 1989) or endotoxin (Dobber 1985) produced a dose-dependent increase in vascular permeability accompanied by the appearance of PAF. And in *S. enteritidis* endotoxin-induced shock, the PAF antagonist BN 52021 confers a significant dose-dependent inhibition of lethality associated with a reduced increase in body temperature suggesting that BN 52021 has an effect on the release of IL-1. (Etienne 1985).

Sun et al (Sun 1988) have demonstrated that TNF induced bowel necrosis is due to PAF release and can be prevented by pretreatment with the PAF antagonist SRI 63-119. High doses of PAF could also induce necrosis and PAF was synergistic with LPS and with TNF.

All this elements have situated PAF among the most important mediators of septic shock and of inflammatory reactions in general.

ARACHIDONIC ACID METABOLITES

Five major eicosanoid (20 carbon, cyclic fatty acids metabolites of arachidonic acid) groups are described. The classic prostaglandins (1) PGD_2, PGE_2, PGF_2; (2) prostacyclin (PGI and its stable metabolite $PGF_{1\alpha}$) and (3) the thromboxanes (TxA_2 and its stable metabolite TxB_2) are generated through the cyclooxygenase pathway. On the other hand, (4) the Hydroxyeicosatetraenoic acids (HETE) and (5) the Leukotrienes are products of the lipooxygenase pathway.

PGE and PGF have direct actions on smooth-muscle cells. PGE_2 causes coronary vasodilation and contraction of the uterus. $PGF_{2\alpha}$ is a universal vasoconstrictor that exerts potent effects in the coronary, the mesenteric and the renal circulation important in the pathogenesis of circulatory shock (Fink 1985). PGE_2 has also important immunologic functions. It inhibits clonal proliferation of the committed granulocyte-macrophage stem cell, augments phagocytosis, increases the numbers of Con A and Fc receptors, and induces T suppressor cell activity (Stenson 1980; Chouaib 1984).

Thromboxane and Prostacyclin (PGI_2) play a major role in the control of blood flow. On the one side, thromboxane is a potent vasoconstrictor and platelet aggregator (Ogletree 1987) and is responsible for repair of vascular injury. Thromboxane A_2 (TxA_2) and Carbocyclic thromboxane A_2 ([CTA_2] a stable analog of TxA_2) render lysosomal membranes leaky. TxA_2 has also been implicated in tumor cell growth and metastasis (Honn 1983), and in the pathophysiology of preeclampsia (Walsh 1985). PGI_2 on the other hand, is a potent inhibitor of platelet aggregation (Bertele 1984), and has vasodilator, bronchodilator and membrane-stabilizing effects which evidence a relevant anti-shock role for prostacyclin. Its primary source appears to be vascular endothelium.

In sepsis, endotoxin, thrombin, bradykinin, angiotensin II, C_3, C_5 all augment the biosynthesis of PGI_2 and epinephrine, histamine, and serotonin may also stimulate PGI_2 biosynthesis.

Leukotrienes (LT) are potent mediators of inflammation. They are produced by a host of cell types including the pulmonary parenchymal cells, macrophage, mast cells, leukocytes, and several types of connective tissue and smooth muscle cells,

particularly vascular smooth muscle (Ford-Hutchinson 1985). LTB_4 causes chemotaxis of eosinophils and macrophages; it promotes the release of lysosomal hydrolases from various cell lines and induces edema, capillary dilation, and transudation of fluid across vessel walls (Staub et al 1985). In contrast to LTB_4, the peptide leukotrienes (LTC_4, LTD_4, LTE_4) are more active as stimulators of smooth muscle contraction (Feuerstein 1987).

Plasma concentration of eicosanoids are increased during septic and endotoxic shock and following thermal and nonthermal injury (Fink 1985). They have been implicated in pathologic conditions such as ARDS (Brockmann 1986, Deby-Dupont 1987), myocardial ischemia (Smith 1980) vascular occlusion (Bridenbaugh 1976, Flynn 1976), and hemorrhagic (Collier 1973), endotoxic (Aderem 1986, Anderson 1972, Carmona 1984, Casey 1985), septic (Ball 1986, Fink 1985, Halushka 1985) and cardiopulmonary bypass shock.

Intravenous injection of LPS results in elevated plasma levels of PGE_2 (Cook 1980), $PGF_{2\alpha}$, TxB_2, and 6-keto $PGF_{1\alpha}$ (Anderson 1972). Levels of TxB_2 typically rise dramatically within minutes after the injection of endotoxin, whereas levels of 6-keto $PGF_{1\alpha}$ tend to increase more gradually. This observation has led to the hypothesis that thromboxane mediates the acute pulmonary hypertension characteristic of early endotoxic shock (Winn 1983), whereas the vasodilator effect of PGI_2 may contribute to peripheral vascular collapse later in the shock syndrome.

The Slow-releasing substance of anaphylaxis (SRS-A) is a member of the leukotriene family, specifically LTC_4 and LTD_4. This substance is produced by stimulated neutrophils, eosinophils and some macrophages in different tissues. In animals it can cause bronchospasm and increased vascular permeability (Taylor 1983; Dahlen 1981; Samuelsson 1983) and increase adherence of neutrophils to surfaces (Goetzl 1983).

The finding that massive doses of aspirin enhanced the survival rate of endotoxin-treated dogs has been confirmed using other non-steroid anti-inflammatory drugs (NSAIDs), lower doses, other species and models of bacteremia using live *E. coli* organisms (Jaffe 1988; Revhaug 1988). The effect of these NSAIDs on survival, hemodynamic values, systemic coagulopathy, and ischemic sequel have been assessed, generally with positive results. Their major mode of action is inhibition of

cyclooxygenase with reduced synthesis of prostaglandins and thromboxanes. Also, fatty acid-deficient rats which release limited amounts of endogenous prostanoids are less susceptible to endotoxic shock and have a markedly reduced mortality (Cook 1980). Acute arachidonic acid supplementation given to this rats restores lethal sensitivity to the endotoxin, which can be reversed with the thromboxane synthetase inhibitor, 7-imidazole-heptanoic acid (Ball 1986). Imidazol therapy diminished the permeability edema of acute respiratory failure resulting from complement activation (Krausz 1982) and prevents formation of the toxic myocardial depressant factor. These results lead to the conclusion that TxA_2 is probably involved in the pathophysiologic processes resulting in ischemic myocardial damage (Lefer 1985).

INTERLEUKIN-1.

Microbial invasion, tissue injury, immunologic reactions, and inflammatory processes induce a constellation of host responses collectively referred to as the acute-phase response characterized by changes in metabolic, endocrinologic, neurologic, and immunologic functions. Most of these changes are observed within hours to days after the onset of infection or inflammation. The acute-phase response has the outstanding characteristic of being a generalized host reaction irrespective of the localized or systemic nature of the inciting disease. Interleukin-1, IL-6 and TNF are the main integrators of the acute-phase response (Ramadori 1988).

IL-1 previously known as Lymphocyte Activating Factor (LAF) is a 12,000 to 15,000-dalton polypeptide that augments the mitogenic response of thymocytes and promotes thymocyte helper functions and B cell antibody production. IL-1 induces fever (Atkins 1960), activates and stimulates the production of IL-2 by T cells (Mizel 1982), induces fibroblast growth (Krane 1982) and stimulates hepatocytes to produce acute phase proteins (Oppenheim 1982).

IL-1 is primarily produced by mononuclear phagocytic cells: blood monocytes, phagocytic lining cells of the liver and spleen and other tissue macrophages, keratinocytes, gingival and corneal epithelial cells, renal mesangial cells, and brain astrocytes (Dinarello 1984). Nearly all infections, immunologic reactions, and inflammatory processes stimulate mononuclear-cell phagocytes to synthesize and

release IL-1. Some of the IL-1 that is synthesized remains associated with the plasma membrane and induces changes in local tissues without producing systemic responses (Dinarello 1988).

IL-1 affects nonleukocytic targets, such as the liver, pancreas, bone, muscle fibroblasts, and brain (Dinarello 1984) and is thought to have a role in tissue remodeling by inducing mitogenic activity on fibroblasts and smooth muscle (Rainess 1989), bone resorption (Gowen 1983), and stimulation of synovial cell prostaglandin and collagenase synthesis in tissue culture (Mizel 1981). IL-1 initiates fever by inducing an abrupt increase in the synthesis of PGE_2 in the anterior hypothalamus (Dinarello 1982). It promotes an increase in the number and immaturity of circulating neutrophils, apparently by direct action on the bone marrow. IL-1 is chemotactic for neutrophils and T cells, and local concentrations of IL-1 in inflammatory sites probably contribute to cellular infiltration (Lugler 1983), increase in binding of human B and T lymphocytes to endothelial cells (Cavender 1986) and synthesis and cell surface expression of procoagulant activity in human vascular endothelium (Bevilacqua 1984).

Administration of human IL-1 or a homogeneous preparation of monocyte-derived IL-1 to mice and rats increased blood levels of ACTH and glucocorticoids. TNF, IL-2 and τ-interferon had no such effects at equivalent doses (Basedovsky 1986).

Like TNF, IL-1 can also suppress (although not completely as TNF does) lipoprotein lipase activity apparently through a different mechanism (Beutler 1985), and it is cytocidal for several tumor cell lines (Onozaki 1985). IL-1 alone or in combination with other cytokines (TNF, IFN-τ) increases antibacterial resistance in different animal models (Kurtz 1989; Minami 1988; Cross 1989).

Rabbits given a single intravenous injection of recombinant human IL-1β rapidly develop decreased systemic arterial pressure with the lowest levels after 50-60 minutes, systemic vascular resistance and central venous pressure fall while cardiac output and heart rate increase. Ibuprofen prevents these responses, and the combination of IL-1 with TNF at low doses results in a profound shock-like state. Ibuprofen prevents the hemodynamic, leukocyte and platelet changes induced by the low-dose cytokine combinations (Dinarello 1989).

In rabbits, IL-1 has been detected in the circulation as an endogenous pyrogen following the injection of submicrogram amounts of endotoxin, other pyrogenic toxins and synthetic adjuvants (Dinarello 1988).

In brief, the multiple biological properties of IL-1 seem to fall into two categories: (a) abrupt changes such as fever, hemodynamic shock, increased slow-wave sleep, sodium excretion, ACTH and insulin release, and degranulation of eosinophils and basophils; and (b) slow-onset changes such as hepatic protein synthesis, production of growth factors, cell proliferation, cytotoxicity, and induction of synthesis of a variety of enzymes and structural proteins. The lymphokines IL-2, interferons, IL-4 and IFN-β2 (IL-6) are in the latter category. Finally, the proinflammatory action of IL-1 is closely linked with the pathophysiology of septic shock.

INTERLEUKIN-6

Interleukin-6 was first identified by Gauldie and co-workers (Gauldie 1987). The mature form is a 184 amino acid peptide with glycosylation at both N- and O-linked sites (Hirano 1986) and has about 23 to 32 kilodalton molecular weight.

IL-6 also known as interferon-β2 (IFN-β2), hybridoma plasmacytoma-growth factor (HGF), hepatocyte-stimulting factor (HSF), B cell stimulating factor 2 (BSF-2) and 26K protein has antiviral activities, stimulates hybridoma and plasmacytoma growth, stimulates hepatocytes and B cells. IL-6 is also believed to stimulate macrophage differentiation, to stimulate multipotential colony formation in hematopoietic stem cells and to induce neural differentiation (Kishimoto 1989).

T cells, macrophages, fibroblasts, myeloma cells and other transformed cells can produce IL-6 in response to different type of stimuli including endotoxin (Heflgot 1988), live bacteria (de Man 1989), RNA and DNA viruses (Tamm 1989), IL-1 (Sironi 1989), TNF (McIntosh 1989), IL-2 (Jablons 1989), IL-4 (Kupper 1989), lymphotoxin, and IFN-β1. As would therefore be expected, IL-6 levels increase rapidly when the body is invaded by an infectious agent or injured. It has actually been detected in blood from patients with gram-positive and gram-negative bacteremia, meningitis and acute trauma, and also in synovial fluids from inflammatory arthropaties (Tamm 1989, Waage 1989a).

Some of IL-6 activities overlap those of IL-1 and TNF, and similarly, IL-6 is considered a major immune and inflammatory mediator. It is released coordinately with IL-1 and TNF from macrophages. Also, IL-1 or TNF can induce IL-6, TNF can induce IL-1, and IL-1 can induce IL-1.

Fever and the acute phase proteins α_1-acid glycoprotein, C_3, factor B, serum amyloid A, serum amyloid P, C reactive protein and hemopexin and haptoglobin in the rat are produced by the three cytokines, but only IL-6 decreases albumin and transferrin production and increases production of α_1-antichymotrypsin. α_2-macroglobulin, α1-proteinase inhibitor, ceruloplasmin, C_1 esterase inhibitor, contrapsin, cystein proteinase inhibitor, fibrinogen, and haptoglobin and hemopexin in humans (Durum 1989, Gauldie 1989). Interestingly, IL-6 response is controlled by the lipopolysaccharide gene, *Lps* and its production is as expected, markedly decreased in the HeJ mice (de Man 1989).

The physiologic and physiopathologic role of IL-6 in inflammation and sepsis is yet to be ascertained. So far it is believed that the pattern of the acute phase proteins synthesized by liver cells may be accurately tuned by the action of various inflammatory cytokines with IL-6 as the main signal and IL-1 , TNF and IFN-τ as accessory signals or modulators. In such a way, the organism may achieve the most effective homeostatic response to infection and inflammation due to the broad spectrum of biological activities of the acute phase proteins (Koj 1989).

INTERFERON GAMMA

IFN-τ was the first secretory product of T cells to be discovered and cloned on the basis of its ability to interfere with viral replication in fibroblasts. It is a polypeptide (143 amino acids) with no homology with IFN-α or IFN-β which are not T cell products as IFN-τ is. It exists as a glycosylated molecule with a molecular weight by gel filtration ranging from 40 to 70 kD, although, on reducing SDS polyacrylamide gel electrophoresis 20 and 25 kD forms are observed (Yip 1982).

IFN-τ is produced by conditions known to cause the activation of T lymphocytes: mitogens, specific antigens, anti-lymphocyte sera and alloantigens in the Mixed Lymphocyte Culture [MLC] reaction (Kirchner 1984).

Its effects on macrophages seem to be more central to the chief physiologic functions of these cells than for any other cell type (Nathan 1988). Upon binding its receptor, IFN-τ induces synthesis of several proteins (2'5'oligoadenylate synthetase, protein kinases, nucleases) that are important for the development of the antimicrobial state (Lengyel 1982). IFN-τ activates macrophages as shown by increased metabolic functions; enhanced phagocytosis; acquisition of cytotoxicity against tumor cells; expression of the class I and II histocompatiblility antigens (Basham 1983; Pace 1983); increased secretion of reactive oxygen intermediates, and increases resistance to a host of gram-positive, gram-negative, protozoa, chlamidia, and fungi in different cell populations (Nathan 1988). IFN-τ also increases macrophage production of IL-1 (Arenzana-Seisdedos 1985), TNF (Nedwin 1985), C_2 and factor B (Strunk 1985), plasminogen activator (Collart 1986), eicosanoids (Boraschi), and other mediators. Other complex functions of macrophages induced with IFN-τ include inhibition of migration (Thurman 1985) and the formation of giant cells (Weinberg 1985). The enormous spectrum of actions attributed to some of these mediators, especially interleukin-1 and TNF disclose the potential impact of the secretion of IFN-τ (Nathan 1987).

Alteration of the IFN-τ paracrine pathway of macrophage activation are associated with some of the most prevalent of the serious, chronic infectious diseases, such as acquired immune deficiency syndrome (Murray 1986), lepromatous leprosy (Nathan 1986), visceral leishmaniasis (Carvalho 1985), and possibly tuberculosis (Vilcek 1986), and in experimental animals rickettsia and schistosomes (Hamilton 1986). Interferons in general and τ-inteferon in particular may also be immunosuppressive (Merigan 1983).

INTERFERON ALPHA AND BETA-1

Molecular cloning of macrophage derived interferons (α and β1) has revealed at least 20 subtypes of IFN-α grouped into class I (18 Kd) and II (20 Kd) based on their structure (Weissman 1986) and two of IFN-β (20 kD).

In vitro activated macrophages produce predominantly IFN-α but they also secrete low levels of IFN-β1. Viruses and many nonviral agents such as polynucleotides and bacterial, fungal, parasitic, mycobacterial agents or their products can induce IFN-

$\alpha,\beta 1$ to be produced by a wide variety of cell types including fibroblasts, macrophages, T and B lymphocytes and endothelial and epithelial cells (Fiedman 1988).

Macrophages from endotoxin-resistant HeJ mice do not produce interferon after endotoxin exposure and are highly susceptible to viral infection. This suggests that "natural resistance" to virus infection is mediated by endogenous interferon production which occurs in response to stimuli, such as endotoxin (Bocci 1987).

IFN-$\alpha,\beta 1$ have antiviral activities; they suppress the proliferation and often concomitantly promote the differentiation of normal and tumor cell types (Chen 1987) and also can counteract the mitogenic activity of a number of other growth factors (Moore 1984). Their effects on macrophages include increasing accessory cell, bactericidal and tumoricidal functions (Chen 1987). They promote differentiation of macrophage, cell enlargement, increased adherence, spreading, pseudopod formation and vacuolization. They also increase the synthesis of lysosomal enzymes and expression of receptors for the Fc portion of immunoglobulin. These in turn promotes increased phagocytosis of immune complexes and increases capacity to lyse antibody-coated bacteria, parasites, and tumor cells by antibody-dependent cell-mediated cytotoxicity reactions (Vilcek 1985). IFN-$\alpha,\beta 1$ also inhibits the migration of macrophages and is therefore said to exhibit macrophage migration inhibitory activity. Finally, IFN-$\beta 1$ enhances macrophage production of IL-1 and TNF that augment lymphocyte activation (Rhodes 1986).

TUMOR NECROSIS FACTOR ALPHA

TNF was discovered based on its ability to induce hemorrhagic necrosis in certain tumors in vivo (Carswell 1975) which raised great hope for its use in cancer therapy. It was independently discovered as cachectin, when it was detected during parasitic disease in rabbits infected with *Trypanosoma brucei* (Rouzer 1980) which became cachectic. The capacity of TNF to inhibit lipoprotein lipase (Kawakami 1981) lead to the assumption that the wasting effect seen in the rabbit model could be secondary to the presence of TNF. Once isolated, purified and the gene cloned, it was demonstrated that cachectin and TNF were identical proteins (Beutler 1986a).

The demonstration that TNF/cachectin is capable of inducing many of the deleterious effects of endotoxin shock (Beutler 1985e, Tracey 1986) finally helped to ascend TNF to the level of the cytokine of the 80's.

TNF is produced mainly by activated macrophages but other cells such as T cells (Steffen 1988) and T cell lines (Sung 1988a), B cells (Sung 1988b) and B cell lines (Williamson 1983), mast cells and natural killer cells can also produce it (Cuturi 1987). Its mature peptide is 157 amino acids (MW 17,356 dalton). No N-glycosylation sites are present but TNF binding to lentil lectin has been reported (Rubin 1985).

Priming macrophages *in vivo* with agents such as *Bacillus Calmette-Guerin* (Old 1985), *C. parvum* (Green 1977, Satomi 1981) *C granulosum*, or zymosan (Carswell 1975) or *in vitro* with interferon-τ (Gifford 1987) increase the capacity of LPS to induce TNF *in vitro*. Interferon-τ, low concentrations of PGE_2 (Renz 1988), GM-CSF, CSF-1, and TNF itself are also reported to promote TNF synthesis and/or release from the activated macrophage. In contrast, compounds toxic for the macrophages (carragenan), or lysosomal inhibitors such as trypan blue (Satomi 1981), and other compounds like PGE_1, theophylline, isoproterenol, high concentration of PGE_2 (Renz 1988) and hydrocortisone or dexamethasone (Satomi 1981, Beutler 1986b, Waage 1987) decrease TNF production induced by LPS.

TNF is one of the major endotoxin-inducible secretory proteins. It constitutes 1-5% of the total protein secreted by stimulated RAW 264.7 cells (Beutler 1985c). According to Mathison, more than 5 μg of TNF per mL of serum is found, and an estimated total of 130 μg TNF are released into the plasma compartment 30-200 minutes after injection of 10 μg of LPS into rabbits (Mathison 1988). Although endotoxin is the premier inducer of TNF, other inducers have been reported including muramyl dipeptide (Kidahl 1985), other bacterial products (Männel 1987; Fast 1989), protozoan products (Rouzer 1980), Poly inosinate-Poly cytidylate (Bloksma 1983), mitogens and viruses (Aderka 1986; Nedwin 1985).

TNF can be detected in peripheral blood within 15 minutes after LPS injection into animals (Green 1977) or humans (Michie 1988). Serum levels reach a maximum between one and two hours and decline thereafter (Gifford 1987). *C. parvum* primed mice produce 200 times as much TNF than non-primed ones after

equivalent doses of LPS (25 μg) (Flick 1986a). Once produced, TNF is cleared from the plasma with a half-life of 6.5 to 10.5 minutes, and has a volume of distribution consistent with the extracellular space (Beutler 1985d, Flick 1986b, Mathison 1988). TNF activity can also be detected in peritoneal exudate after 2 hours of LPS injection but in Satomi's report it was lower than that of sera (Satomi 1981).

Receptors with high affinity ($K_a = 10^{10}$) for TNF are present at up to 10,000 copies per cell in normal tissues (Beutler 1985). The action of TNF is powerfully augmented by interferon τ on several target cell types, at least partially through increasing TNF receptor expression (Aggarwal 1985).

Lymphotoxin (LT or TNF-β), is the lymphokine counterpart for TNF-α/Cachectin. It is a T cell product related to the DTH reaction with capacity to kill rat embryo fibroblasts (Ruddle 1967). LT induction is antigen specific but not it's effect which is mediated through binding to the same receptor as TNF-α. Both cytokines (TNF-α & LT) have a 35% amino acid homology in the mouse and 28% in the human. LT is believed to share most of the biological properties of TNF-α and its main function is considered to be mediation of cytolytic T cell killing of antigen bearing cells (Paul 1988).

The biological effects of TNF relevant to the shock state, include: on adipocytes, diminished lipoprotein lipase activity (Rouzer 1980); on endothelial cells, increased leukocyte adhesiveness, procoagulant activity and increased IL-1 and GM-CSF synthesis (Cavender 1987, Bevilaqua 1986, Gamble 1985, Nawroth 1986); on hepatocytes, secretion of acute phase reactants such as C-reactive protein, and transferrin (Andus 1988). On the central nervous system, it induces fever and slow-wave sleep (Kawasaki 1989; Beutler 1989; Dinarello 1986). On macrophages, it increases cytotoxicity, hydrogen peroxide production, Fc receptor expression and production of PG-E_2 and IL-1 (Tamladge 1988; Philip 1986; Hoffman 1987). On PMNs it induces chemotaxis, adhesiveness, phagocytosis, degranulation and oxygen radicals production (Clark 1987; Seow 1987) and according to some reports it provokes hypotension (Weinberg 1988) and suppresses bone marrow activity (Talmadge 1988).

Other effects of TNF include stimulation of proliferation or stimulation of mediator release from some types of normal cells such as fibroblasts (Sugarman 1985; Elias

1987) bone marrow hemopoietic cells (Slordal 1989; Vogel 1987) and thymocytes (Hurme 1988) as well as neoplastic cells (Schutze 1988). It also induces collagenase production (Dayer 1985), bone resorption (Bertolini 1986), angiogenesis (Leibovich 1987) and removal of advanced glycosylated end products (Vlassara 1988). All of these suggest an important role in tissue remodeling, wound repair, and potential pathologic effects seen in fibrosis induced by chronic inflammation (Sugarman 1985).

In addition TNF has several immunomodulatory effects such as enhancement of B cell proliferation and differentiation (Jelinek 1987) induction of cytostatic properties of NK cells and cytotoxic T-cells (Talmadge 1988), stimulation of lymphocyte migration (Issekutz 1989), cytotoxicity for a series of human tumor cell lines (Onozaki 1985, Old 1985) and as mediator of IL-2 toxicity when administered for cancer therapy in clinical trials (Fraker 1989). It is important to mention that although their might be a direct cytotoxic effects on the tumor, the *in vivo* antitumor effects of TNF are mainly related to disruption of the tumor's blood supply (Palladino 1987a).

As a generalization we can say that cachectin exerts a catabolic influence over most tissues. The benefits to be derived from cachectin effects are not fully understood at present. Possibly, the actions of the monokine are chiefly beneficial at a local level, and are deleterious when the hormone is expressed systemically in large quantity, in combination with other cytokines or with bacterial products, or in certain specific pathophysiologic conditions not related with its physiologic role.

Considerable information has shown that at a minimum, TNF has a prominent role in mediating LPS toxicity. Passive immunization with polyclonal (Beutler 1985) or monoclonal (Tracey 1987) anti-TNF antibody given before the administration of either TNF or LPS (Tracey 1987, Fraker 1989, Mathison 1988) has protected animals from lethality.

One of the main arguments that supports the hypothesis of TNF as THE mediator of sepsis is the protection of baboons with monoclonal anti-TNF F(ab') fragments from a lethal challenge with *E. coli* (Tracey 1987). It showld be considered however, that baboons like vervets and C3H/HeJ mice are poorly sensitive to endotoxin (according to Westphal 10-25 kg baboons can stand iv injections of 4-5 mg/kg LPS)

and can survive up to 100 mg of *S. abortus equi* endotoxin without systemic reactions as those seen in sensitive species (Westphal 1975, Sultzer 1968). The difference in sensitivity between primates and humans is in the order of 1:>1,000,000 .

In the same report it is suggested that 1 hour might have failed should uniform distribution and tissue penetration of the antibody not have occurred. Considering that probably not until 3 hours after the -1 hour administration scheme of antibody high levels of TNF occur, by that time enough intravascular distribution of the antibody fragments is certain. The argument of tissue distribution depends upon a mechanism of action of TNF out of the intravascular space. However, even in the endotoxin model, the only mechanism of action that has been proposed, at least under current time knowledge, is the endothelial injury that the TNF might mediate upon endothelial cells as well as upon other targets. Interestingly, in an experiment in rabbits, polyclonal anti-TNF administered before the injection of LPS did not prevent LPS induced-leukopenia, development of LPS tolerance or fever although 30 minutes were enough to prevent both TNF-like cytotoxicity in the serum and decrease in the mean arterial pressure and 45 minutes were enough to prevent mortality and all but the minimal histopathologic changes (Mathison 1988).

Administration of recombinant cachectin to rabbits caused fever or hypothermia, diarrhea, cyanosis, hemoconcentration, lactic acidosis, a transient phase of hyperglycemia followed by hypoglycemia, hypotension, and death. At necropsy, animals treated with cachectin showed widespread visceral necrosis, particularly involving the caecum and large bowel. Acute renal tubular necrosis was also evident, as was severe interstitial pneumonitis, increased number of Kupffer's cells and intracellular hepatocyte edema and increased liver and lung weight (Kettelhut 1987, Gaskill 1988; Tracey 1986). These effects were blocked by pretreatment with a monoclonal antibody capable of neutralizing recombinant human cachectin.. These and other metabolic and hormonal changes characteristic of endotoxic shock were also induced in dogs by recombinant TNF administration (Tracey 1987b). The acute LD_{50} for recombinant human TNF is 700 μg/kg in the rat, 50 μg/kg in the dog and in the baboon 30-50 μg/kg.

Both endotoxin and TNF cause the release and production of many other potent mediators with the capacity of inducing shock like syndrome. Among them PGE_2 and thromboxanes (Dayer 1985, Kettelhut 1987), leukotrienes (Huber 1988) IL-1

(Dinarello 1986, Okusawa 1988), PAF (Sun 1988) and IL-6 (Jablons 1989) are prominent candidates.

Table 3. Effects of Tumor Necrosis Factor α

T lymphocytes	IL-2 receptor expression, cytotoxicity, differentiation and proliferation
B lymphocytes	proliferation and differentiation.
Neutrophils	Activation, endothelial binding, increased respiratory burst, degranulation, inhibition of precursors, expression of $C_{3b}R$, aggregation
Monocyte/Mø	Activation, IL-1 and PGE_2 production, tissue factor production
Endothelial cells	Increase stickiness (ICAM-1), TNF, IL-1, GM-CSF and PGE_2 production, Procoagulant activity (tissue factor), increases MHC class I antigen expression, decrease exp. of thrombomodulin, decrease fibronectin.
Adipocytes	Suppresses lipoprotein lipase activity, inhibition of lypogenic enzyme gene transcription, Mobilization of stored lipids with return to morphology of fibroblasts.
CNS	Fever (synthesis of PGE_2), slow-wave sleep
Some tumor cells	Cytotoxicity
Fibroblasts	PGE_2 and collagenase production, proliferation, increases MHC class I antigen expression, interferon $\beta 1$ and $\beta 2$ production
Synoviocytes	Proliferation, IL-1, IFN-$\beta 1$ and IFN-$\beta 2$, PGE_2 and collagenase synthesis
Bone/Cartilage	Resorption with Ca^{++} release
Bone marrow	Increases production of granulocyte-monocyte lineage, radioprotection, differentiation of some myelogenous leukemia cell lines
Skeletal muscle	Reduction of resting transmembrane potential, Acceleration of glycogenolysis with lactate release, glucose uptake and expression of hexose transporters.
Hepatocytes	Acute phase genes transcription
Others	Anti *Plasmodium*, *Candida* and schistosome larvae activity, Angiogenesis

Although this evidence points to TNF playing a central role in endotoxic shock, there is also evidence to the contrary. It has been recently reported that TNF itself can't induce hemorrhagic necrosis (Rosthstein 1988a), or lethal shock (Rothstein 1988b, Neilson 1989) unless it is combined with endotoxin or other bacterial products, IL-1, or maybe some yet non-defined endogenous products. High levels of TNF may be induced with non-toxic forms of lipid A (Kiener 1988; Desiderio 1987), and in Mathison experience, the hypotension and leukopenia induced in rabbits by LPS were not induced by the infusion of comparable amounts of human recombinant TNF as those endogenously generated by LPS.

This has lead to the idea that TNF is a primary mediator of the toxic effect of LPS administration though not sufficient to provoke full development of injury, and may

act by sensitizing the host to the action of other mediators induced by LPS with which great synergism has been reported (Mathison 1988).

In humans, TNF has been detected after LPS administration with the same kinetics as in other animals (Michie 1988a). The effects of TNF administration itself show similar metabolic and toxic responses as the administration of LPS with fever, pituitary and stress hormone release and acute phase changes (Michie 1988b, Warren 1987). The presence of TNF in natural human diseases has been inconsistent. The first report associated it to parasitic infections with an incidence of 66% in patients with kala-azar and 70% in patients with malaria (Scuderi 1986). In contrast, this same study reported a less than 12% incidence in healthy subjects, 7.9% in cancer patients and none in umbilical cord blood. Simultaneously, a report found TNF in 3 cases (out of 23) of severe infections, in none of 23 cancer patients and in none of 25 normal controls (Waage 1986). Subsequent reports by Waage more consistently related TNF levels with the presence, severity and lethal outcome from meningococcal septicemia (Waage 1987 and 1989a) and have related the clinical presence of meningitis with detectable TNF levels in the cerebrospinal fluid (Waage 1989b).

Balkwill et al (1987) could relate TNF serum levels with clinically evident neoplasic activity in 50% of 226 patients (specially in patients with ovarian or oat-cell carcinoma), and although they do not mention number of cases or any other methodological data, Debets et al (1989) claim that TNF detection can be related to unfavorable outcome from sepsis. It is of interest however that cancer patients under clinical trials with TNF are remarkably resistant to levels of TNF exceeding concentrations found to be related to lethal outcome in patients suffering from meningococcal sepsis (Chapman 1987, Blick 1987).

The mechanisms of tissue damage that results from high TNF levels still remains unresolved. Direct cytotoxic effect has clearly been ruled out (Palladino 1987b). A number of studies have provided evidence that interactions between TNF and endothelial cells and PMN could produce changes that are relevant to many LPS induced pathophysiologic changes (Heflin 1981). C_{3a}, C_{5a} IL-1 and TNF-induced increased adherence of neutrophils to endothelial cells combined with the stimulation of respiratory burst, degranulation, and increased production of reactive oxygen radicals, associated with expression of tissue factor-like procoagulant

activity, diminished expression of thrombomodulin activity, and the cooperation of thromboxanes, PAF, leukotrienes, lysosomal enzymes, histamine, serotonin and bradikinin, altogether have an enormous potential for producing endothelial damage, increasing microvascular permeability and resulting in the pathologic characteristics of septic shock or MSOF.

A gap of intriguing facts still remains between the experimentally induced toxic effects of LPS/endotoxin and the damage and response inflicted by live bacteria in the infected host; not absolute evidence has yet linked human bacterial infection with LPS toxicity. It is clear that in the endotoxin model, macrophages, platelets and their products acting upon, neutrophils and endothelial cells have the leading roles, and that the direct toxic effect of LPS is at best the spark that starts a seemingly kaotic situation. At this point it seems that the initial production of TNF is necessary, though not sufficient to lead to full development of injury, and may act through potential autocrine effects of TNF itself.

BACTERIAL ENDOTOXINS

It is known that bacterial products can exert profound effects on humans and other animals. One such class of products exists exclusively in association with the bacterial wall of gram-negative bacteria and is released mainly upon bacterial lysis. These toxic materials have been designated "endotoxins" to distinguish them from toxic substances of protein nature synthesized and excreted by intact bactera, known as "exotoxins". Endotoxins consist of aggregates of lipopolysaccharides and protein and to a lesser extent, loosely bound lipids released into the surrounding medium upon bacteriolysis.

STRUCTURE OF LPS

The bacterial surface consists of an inner cytoplasmic membrane and a trilayer outer cell wall structure composed of: 1) Mucopolysaccharide-peptidoglycan layer; 2) phospholipid protein layer; 3) the outermost Lipopolysaccharide layer.

Some strains of gram-negative bacteria contain, in addition, a capsular polysaccharide layer.

Several extraction methods have been devised to obtain different preparations of LPS from the bacterial wall. Two of the most popular ones are the procedures described by Boivin (1935) and Westphal (1952). Boivin's procedure involves extraction with ice-cold trichloroacetic acid (TCA) and results in endotoxin preparations which primarily consist of lipopolysaccharides but contain, in addition, protein and lipid. Westphal's procedure utilizes a hot aqueous phenol extraction of bacterial suspensions and yields a pure protein-free lipopolysaccharide, which also manifests potent endotoxin activities (Morrison 1978).

Virtually all of the biologic activities normally attributable to bacterial endotoxins could also be elicited with isolated chemically pure lipopolysaccharides and in most instances the lipid portion of the LPS molecule. It should however be recognized, that endotoxin and LPS or even fractions of LPS may be significantly different, both with respect to chemical composition and biological activity. Endotoxins are actually LPS-protein complexes contained in cell walls of gram-negative bacteria and thus the presence or absence of the protein component serves to distinguish endotoxins from purified lipopolysaccharides.

The LPS molecule (Figure 1) which has the function of a selective permeability barrier in controlling the transport of molecules into the cell (Leive 1965) consists of a polysaccharide region covalently bound to a lipid region, the lipid-A (which is different from the noncovalent loosely bound lipids associated with the endotoxin complex). The polysaccharide portion consists of two distinct regions: a core polysaccharide and an O-antigen polysaccharide. The core polysaccharide region which is often identical for large groups of bacteria, contains a unique deoxysugar 2-keto,3-deoxy-octulosonate (KDO) as well as heptose, phosphoryl-ethanolamine, and several hexoses which together define the core structure. The O-antigens normally exist as repeating oligosaccharide units containing three to four different hexose units each. The number of repeating oligosaccharide units can be as few as two (present in so called semi-rough strains) or as many as 10 (in smooth strains) even within the same bacterium. A number of mutant (rough) strains of bacteria which synthesize deficient LPS molecules have been isolated. The LPS isolated from the Re 595 mutant of *Salmonella minnesota* (Kim 1967) which lacks the ability to

synthesize heptose, consists only of a Lipid A, the trisaccharide of KDO, and phosphoryl-ethanolamine (Figure 1).

The three regions of the LPS molecule, the O polysaccharide, the core, and the Lipid A, are immunogenic. While the O polysaccharide exhibits a high diversity in its structure and composition and thereby in its serologic specificity among gram-negative bacteria, the core polysaccharide is less variable, being similar in larger groups of bacteria. For this reason, antibodies to the various core structures, usually prepared by immunizing with defective R-mutant bacteria, are expected to interact with a large number of gram-negative bacteria, provided the various core antigens are exposed in the intact LPS (Galanos 1984).

LIPID A, LIPID X AND RELATED COMPOUNDS

As mentioned, lipid A represents the covalently bound lipid component of lipopolysaccharides. Lipid A of endotoxically active LPS consists of a β1-6 linked D-glucosamine disaccharide that carries phosphoryl groups in positions 1 and 4' (Figure 1). Depending on the origin of lipid A, nonacylated, nitrogen-containing residues may be bound to these phosphoryl groups. Lipid A also contains amide-linked fatty acids, that appear to be uniformly β-OH substituted. The basic structure of the Lipid A region of LPS isolated from a broad spectrum of gram-negative bacteria is remarkably similar (Hase 1976, Rietschel 1984).

Lipid X is thought to represent a very early precursor involved in the biosynthesis of lipid A. It is a diacylglucosamine 1-phosphate fraction with two β-hydroxymyristoyl groups at positions 2 and 3 (Kusumoto 1984). Another form of mutant lipid A called lipid Y has the same structure as lipid X , except for the additional presence of a palmitoyl moiety on the N-linked β-hydroxymyristate (Nishijima 1985). Figure 1 shows these molecules and Figure 2 shows a schematical representation of the basic structure of LPS from smooth and rough strains.

The demonstration that endotoxins prepared from polysaccharide-deficient mutants still manifest virtually all of the endotoxic properties of polysaccharide-containing endotoxins firmly established that lipid A plays a dominant role in endotoxicity (Takayama 1984, Homma 1985). Further evidence of the importance of lipid A is

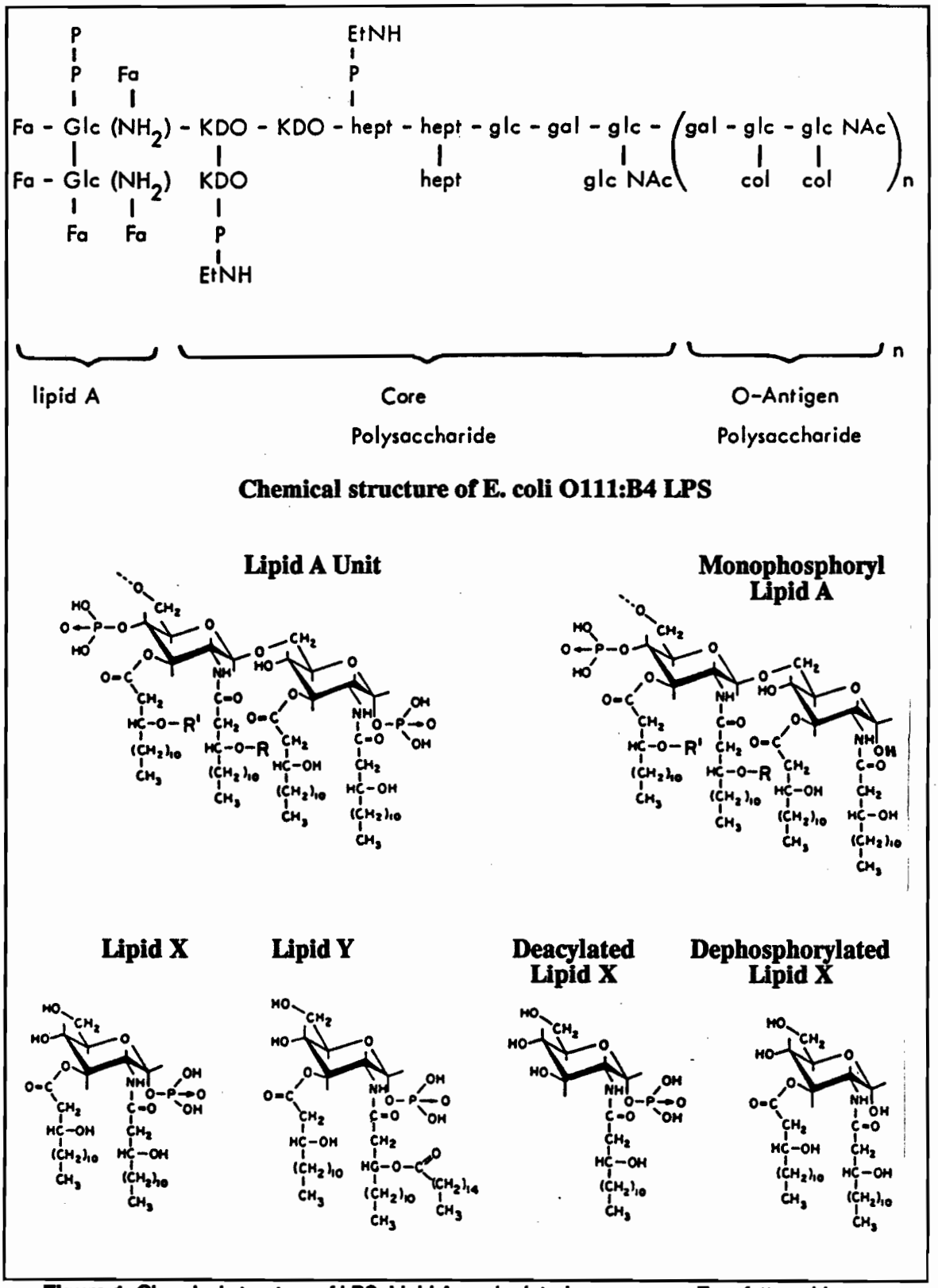

Figure 1. Chemical structure of LPS, Lipid A, and related precursors. **Fa** = fatty acid; **Glc(NH$_2$)** = Glucosamine; **P** = Phosphoryl; **KDO** = Keto-deoxy-octulosonate; **EtNH** = Ethanolamine; **hept** = heptose; **glc** = glucose; **gal** = galactose; **col** = collateral chains; **NAc** = N-acetylglucosamine.

that its biological effect can be neutralized with Polymyxin B which binds stoichiometrically to the lipid A region of LPS (Morrison 1976).

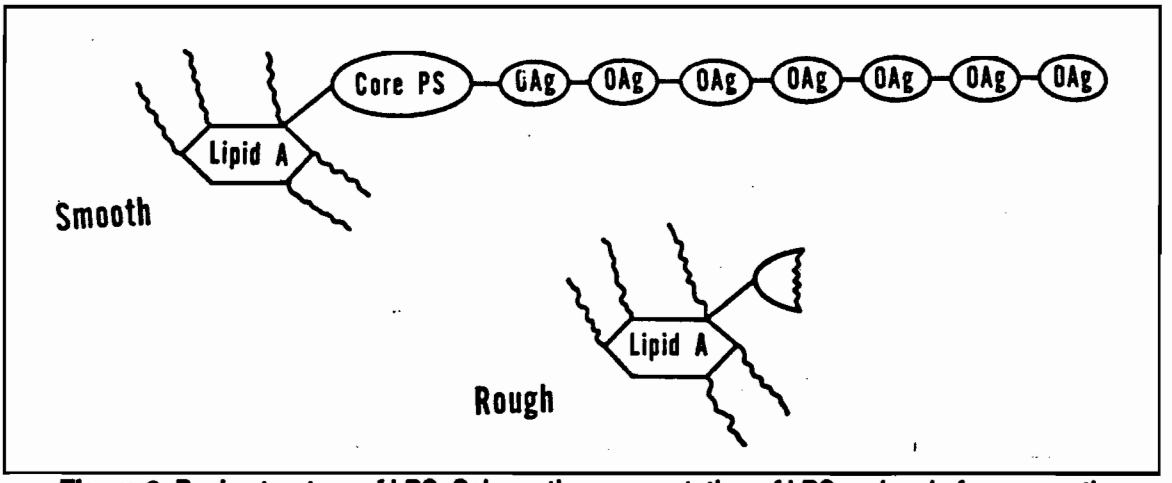

Figure 2. Basic structure of LPS. Schematic representation of LPS molecule from smooth or rough strains of gram negative bacteria.

It has been proposed that in order to induce toxicity and pyrogenicity, lipid A must contain: (1) a glucosamine disaccharide, (2) a sugar-1-phosphate, and (3) normal fatty acids. Most reports agree with these conditions in terms of toxicity (Takayama 1981 and 1984, Galanos 1984, Homma 1985, Golenbock 1987) although there is disagreement in terms of lethality and the capacity of inducing a Shwartzman reaction. Some groups report high lethal toxicity for both natural and synthetic lipid A and for monophosphoryl lipid A (Takayama, Galanos and Homma) while others deny such a degree of toxicity (Burhop 1983, Kiener 1988). All reports agree in the concept that dephosphoryl lipid A, lipid X or smaller fractions are non-lethal, non- or poorly toxic and unable to induce fever or even mitogenicity on B cells.

Nontoxic and still biologically active LPS derivates have been searched for their potential role as an immunogen and biologic response modulators. Progressively smaller fractions or modified preparations have been tested for the various effects: gelation of the *Limulus amebocyte* lysate; toxicity for chick embryo, sheep, guinea pigs or mice; pyrogenicity; induction of TNF production *in vivo* or *in vitro*; or induction of tolerance to more toxic preparations.

In 1981 Takayama et al reported that it was possible to prepare detoxified fractions of lipid A (probably monophosphoryl lipid A) with a Chick embryo lethal dose 50

($CELD_{50}$) > 10 μg, non-pyrogenic to rabbits, and non-toxic to guinea pigs and still effective inducing tumor regression (Takayama 1981). Although some subsequent reports support this concept, others seem to be in conflict with it.

There is agreement in relation to the capacity that differnt compounds have in provoking LAL gelation. Natural and synthetic lipid A, monophosphoryl lipid A and lipid X gave a positive reaction while deacylated and dephosphorylated lipid X were negative (Elin 1976, Takayama 1984, Kanegasaki 1984, Homma 1985, Proctor 1986). This concept demonstrates that LAL is not a valid measure of all parameters of toxicity of lipid A or lipid A-like compounds and can yield false positive results while no toxic condition are actually produced. Desphosphorylated lipid A, and desphosphorylated or deacylated lipid X do not gelate the LAL (Takayama 1984, Homma 1985).

In terms of the capacity of the different compounds to induce TNF production or LPS tolerance *in vivo* or *in vitro* reports are again divergent. According to some publications natural or synthetic lipid A (Galanos 1984, Männel 1989), monophosphoryl lipid A (Madonna 1986) and natural lipid X (Proctor 1986, Golenbock 1987) are capable of inducing LPS tolerance. Nevertheless, Sayers et al (1987) using a synthetic lipid X *in vitro* could not induce in bone marrow-derived macrophages unresponsiveness to lipid A for TNF production.

Great differences in opinion prevail in relation to TNF production. Some authors suggest that monophosphoryl lipid A (Chen 1985) and lipid X (Feist 1989) are unable to induce TNF production, while others sustain that monophosphoryl lipid A (Feist 1989), natural (Amano 1986) or synthetic lipid X (Sayers 1987), or even desphosphorylated lipid A (Homma 1985) are capable of inducing TNF production.

Ribi reported that like endotoxin or diphosphoryl lipid A, the non-toxic monophosphoryl lipid A retains the ability to synergistically enhance the antitumor activity of mycobacterial cell wall skeleton adjuvant (Ribi 1984).

Finally, Nishijima et al (1985) has reported that lipid X and lipid Y can activate macrophages, and according to Homma et al (1985) desphosphorylated lipid A can also do so. Desphosphorylated and deacylated lipid X seem to have lost the

capacity to provoke any of the biological effects of endotoxin (Takayama 1984, Amano 1986).

Antibodies to Lipid A occur naturally in the serum of normal humans and many animal species. They have been found in 10%-34%, and even up to 73% of individuals tested as detected by indirect hemolysis and ELISA. Antibodies to lipid A interact with free lipid A but not with lipid A as present in the intact lipopolysaccharide molecule. Because of similarities between the lipid A of many gram-negative bacteria, antibodies to lipid A showed wide cross-reaction. With regard to biologic activity, antibodies to lipid A have been variously shown to be protective, damaging, or without effect (Galanos 1984).

Natural or exogenously administered anti-LPS antibodies in have been extensively studied but there is still disagreement both in the mechanism of action of the antibodies and in the particular benefits they confer. Some models have used anti-oligosaccharide antibodies and others have precluded the superiority of anti-core raised antibodies.

The use of passive or active immunization with anti-core LPS relies on the following principles: (1) LPS plays a central role in gram-negative pathogenicity. (2) Antisera raised aginst rough mutants contains antibodies directed anaginst core LPS. (3) These antibodies are able to interact with LPS from smooth strains of multiple species of enterobacteria sharing common antigens. The protective effect is thought to be acting as both antitoxin and opsonin.

In an animal model Abernathy first reported that late endotoxin tolerance conferred protection against the lethal effect of homologous endotoxin that persisted uniformly for two months wherein heterologous protection was not impressive (Abernathy 1957).

McCabe observed that the highest levels of passive protection against endotoxin lethality were provided with O-specific rabbit antisera and that no protection was conferred using antisera to lipid A in lead-sensitized rats (McCabe 1977), but this same author found that naturally occuring high titers of O-specific antibodies did not prevent shock and death, whereas both complications were 1/3 as frequent among patients with high titers (1:80 or above) of Re antibody (McCabe 1972b).

From animals work it has been concluded that passive immunization with rough mutants may prevent death after lethal challenge with various smooth heterologous gram-negative bacteria or endotoxins (Tate 1966; Chedid 1968; McCabe 1972 and 1972a; Ziegler 1973a and 1975; Marks 1982, Dunn 1982). The antibodies have also precluded localized and generalized Shwartzman reactions after injection of LPS (Braude 1972 and 1973; Ziegler 1973b; Davis 1978) and have suppressed hypotension after injection of endotoxins (Young 1975).

There are however, conflicting reports. One group supported that anti-native LPS antibodies did not protect granulocytopenic animals from a bacterial challenge but anti rough type of LPS did (Ziegler 1973); while in another study monoclonal antibodies directed against the oligosaccharide side chain determinant of LPS provided protection against challenge with the specific bacteria (Kirkland 1984).

Using monoclonal antibodies against *E. coli* J5 a high degree of protection has also been reported in the dermal Shwartzman reaction and against lethal bacteremia with different bacteria (Teng 1985; Dunn 1985). Aldridge proposed that J5 antiserum exerts its beneficial effect by nonspecific enhancement of rethiculoendothelial function rather than by a specific effect on clearance of LPS (Aldridge 1987).

In clinical trials the story is even more confusing. Based in an uncontrolled study of 20 patients with severe pseudomonas infections it was concluded that human anti-*Pseudomonas* IgG fraction improved opsonic activity and bacterial killing (Jones 1987). In a now classical report septic patients were treated with human antiserum to *E. coli* J5 endotoxin and compared it with the effect of pre-immune serum. From improved survival rate both in a bacteremic group (22% vs. 39% in the controls) and in a group in profound shock (44% vs 77% in the controls) the authors concluded that their treatment could substantially reduce death from gram-negative bacteremia (Ziegler 1982). However, using the same preparation for prophylaxis in neutropenic patients, no benefit could be demonstrated as assessed by the number of febrile days, the number of gram-negative bacteremic episodes, or death from these infections (McCutchan 1983). On the contrary, in Baumgartner experience, prophylactic administration of J5-immune plasma prevented the development of gram-negative septic shock and death after abdominal surgery in patients at high risk of infection (Baumgartner 1985).

More recently, an intriguing study using human IgG antibody to *E. coli* J5 compared with a standard IgG preparation failed to show any advantage of the hyperimmune IgG in survival, systemic complications or time of death due to septic shock (Calandra 1988).

That antibodies are not essential for late endotoxin tolerance to be manifested is perhaps best illustrated by the studies of Zaldivar and Scher wherein B-lymphocyte-defective (CBA/N) mice exhibited high levels of late tolerance to endotoxin lethality tested 8 days after a single priming injection of endotoxin, despite the inability of this murine strain to produce any detectable levels of anti-endotoxin antibodies (Zaldivar 1979).

Table 4. Biological Effects of Endotoxin

Leukopenia	Nonspecific resistance to infection
Leukocytosis	Induction of endotoxin tolerance
Pyrogenicity	Adjuvant activity in promoting IgG synthesis
Depression of blood pressure	Polyclonal B lymphocyte mitogen
Enhanced dermal activity to epinephrine	Decrease expression of Fc receptor on Mø
Tumor necrotic activity	Mø secretion of tissue factor of coagulation
Bone marrow necrosis	Mø and endothelium production of Colony stimulating factors and fibroblast proliferation factors
Embryonic bone reabsorption	
Limulus lysate gelation	
Shwartzman phenomenon	Increase expression of Ia molecules on macrophages
Lethal toxicity	
Toxicity increased by adrenalectomy	Macrophage activation
Toxicity increased priming with BCG, *C. parvum*, *C granulosum* and Zymosan	Direct cytotoxic effect on endothelium
	Pro-coagulant activity on endothelium
Production of eicosanoids PGE_2, $PGF_{2\alpha}$, TxB_2, 6-keto $PGF_{1\alpha}$, LTB_4 and C_4	Increased adherence of endothelium to PMN and lymphocytes
	PMN and Mø Production of PAF
Production of cytokines TNF α, IFN-α and β1,IL-1 α and β and IL-6	Increased PMN and Mø production of active oxygen metabolites and phagocytosis
Production of lymphokines LT and IFN-τ	Chemotactic for PMN and Mø
Complement, Hageman factor, Plasminogen and Kinin activation	Degranulation of PMN

Because of the power of LPS to promote inflammation, LPS must be rapidly neutralized. Considering the vast amount of LPS in the gut, the mechanism for neutralizing LPS must be powerful enough to keep subthreshold levels in the circulation and prevent monocyte activation (Pabst 1989).

Ulevitch and Johnston demonstrated that combination of LPS with high density lipoproteins (HDL) through *in vitro* or *in vivo* exposure to normal rabbit serum, greatly reduced LPS-induced pyrogenicity, toxicity, neutropenia, and anticomplementary activity suggesting that this may account for a major intravascular pathway of LPS detoxification (Ulevitch 1982). This same group described that there is a rapid phase of LPS clearance where one third of the administered LPS is cleared from blood in 30 minutes. The rest of the LPS complexes with HDL and is then slowly eliminated with a half life of 12 hours and no evidence of alteration in the chemical structure of LPS. The slow phase is longer for the S-form than for R-form of LPS (Freudenberg 1980). In the liver, LPS is first detected in Kupffer's cells and granulocytes; from there it is later redistributed (after two days) into hepatocytes. Chemically altered LPS excretion continued via the feces for several weeks (Freudenberg 1984). Supporting this same line of thinking, it has been demonstrated that pre-incubation of LPS with serum reduced its potency for *in vitro* LPS-induced production of TNF by rabbit peritoneal macrophages (Mathison 1988) or the production of TNF, IL-1 or IL-6 by human monocytes (Flegel 1989) by a factor of 10 to 100 which was true for different types of LPS and even for lipid A. Umbilical cord derived sera and five batches of FBS and pooled rat sera were effective. On the other side natural anti-LPS IgG antibodies present in human sera and complement components did not seem to affect LPS-induced IL-1 production (Flegel 1989).

It has been uncertain that chemically extracted LPS actually mimic the properties of native LPS generated in the host of an infectious process *in vivo*. Addressing this problem phenol extracted and native forms of LPS from *S. typhimurium* G-30 were compared for various biological properties (*in vivo* fate, pyrogenicity, LAL assay activity) and were found quite similar (Munford 1982). Phenol extraction did not alter the biological properties of LPS but as the authors alert, the disaggregation of purified LPS (a pre-requisite for combination with HDL) and the removal of LPS from membrane fragments before HDL binding may be quite different *in vivo*, and so could be the biological behavior of bacterial endotoxins different to the one tested.

LPS is concentrated in the liver, spleen, and lung while the HDL-LPS complex primarily concentrates in the adrenal glands and ovaries, which as speculated, might compromise the ability of the host to survive LPS-induced shock (Mathison 1979).

Even more, treatment of experimental animals with dexamethasone abolishes specific binding of both HDL and HDL-LPS complexes to the adrenal gland while administration of ACTH increases the specific adrenal binding of the two preparations (Munford 1981)

ENDOTOXIN TOLERANCE

Acquired endotoxin tolerance was encountered almost a century ago by physicians employing bacterial vaccines for fever therapy who noticed that increasing resistance developed to the pyrogenic and subjective toxic effects of the bacterial products when they were repeatedly injected (Beeson 1947).

Endotoxin tolerance is defined as the unresponsiveness to bacterial endotoxins. It may occur as a inborn attribute genetically determined where the tolerant species (or strain) exhibit minimal response to an initial intravenous injection of relatively massive quantities of endotoxin. Examples are baboons, vervets, and C3H/HeJ mice (Sultzer 1968; Westphal 1975). In contrast to such natural resistance, other species respond to an initial intravenous injection of endotoxin with striking physiologic and biochemical alterations, man being one of the most highly responsive (Greisman 1973). Most of these responses decrease progressively in two temporally distinct phases; early and late endotoxin tolerance.

EARLY LPS TOLERANCE

The early phase of endotoxin tolerance develops in as little as one hour after the first exposure to LPS (Galanos 1979) it is transient, and requires closely spaced or continuous endotoxin infusion for maximal maintenance (Greisman 1965). It is not O-antigen specific and is not associated with increments in circulating anti-endotoxin antibodies but is specific for endotoxin as a class, and no tolerance occurs to other pyrogens such as staphylococcal enterotoxin, influenza virus, or tuberculin in sensitized animals (Greisman 1966). The level of tolerance is directly proportional to the magnitude of the initial pyrogenic response but it is still relative, and can be overcome by increasing the rate of endotoxin infusion (Greisman 1965). In rabbits, nonpyrogenic doses of LPS do not induce tolerance (Greisman 1965). It

cannot be transferred with plasma and cannot be overcome with normal plasma or whole blood. Refractoriness results from inability of the host to continue to mobilize endogenous pyrogen during sustained endotoxemia (Greisman 1966), and responsiveness persists to preformed endogenous pyrogen (Greisman 1983). These same authors and others demonstrated that tolerance is a macrophage-mediated phenomenon (Dinarello 1968; Galanos 1979).

Recently, it has been demonstrated that LPS-unresponsive C3H/HeJ mice are sensitive to the toxic effect of recombinant TNF administration, and what is more interesting, such animals develop a state of unresponsiveness comparable to the early phase of endotoxin tolerance which can be induced both with native murine TNF (Cerami 1985) as with recombinant human TNF (Socher 1988). IL-1 is also capable of inducing refractoriness to its own effects *in vivo* as the IL-1-induced anorexia disappear under continuous IL-1 administration (Otterness 1989).

Repeated expossure to IL-1 may induce down-regulation of specific surface receptors as shown *in vitro* on a murine T cell lymphoma, EL4, a murine fibroblast cell line, Swiss 3T3 (Mizel 1987), or a human large granular lymphocyte cell line, YT (Matsushima 1986). Even more, Fraker et al demonstrated in rats that in a dose dependent manner, TNF can induce tolerance to its own toxic effects as well as to the toxic effects of endotoxin and *vice versa* (Fraker 1988), and Holtmann et al demonstrated that cells exhibited a lesser vulnerability to the cytolytic effect of TNF associated with a reversible reduction in TNF receptors initiated within minutes of application of IL-1 or phorbol-myristate-acetate [PMA]. Interestingly, TNF receptor level was fully recovered within a few hours of removal of the agents and yet at that time resistance to the cytotoxicity of TNF was still prominent (Holtmann 1987).

Nevertheless, opposite results have been obtained by others. Vogel et al could not induce LPS tolerance with TNF or with IL-1 alone, but combined administration of both cytokines which were not toxic when administered individually led to synergistic toxicity (as assessed by death or weight loss). However, within nontoxic range, the two cytokines synergized to induce a significant reduction in the capacity to produce colony-stimulating factor and bone marrow changes characteristic with LPS-induced tolerance (Vogel 1988).

The role and capacity of TNF in inducing LPS tolerance is controversial. Mathison reported lack of induction of LPS tolerance by *in vitro* (peritoneal macrophages) or *in vivo* exposure (rabbits) to recombinant TNF, and interestingly, the administration of anti-TNF antibody previous to the *in vivo* LPS challenge prevented detectable levels of TNF in serum, prevented hypotension, prevented all but the minimal histopathologic changes and lethality but did not prevent induction of LPS tolerance (Mathison 1988).

Several mechanisms have been invoked to explain LPS tolerance. LPS could induce inhibitors that would: (a) block directly the activity of TNF and other mediators of LPS effect, (b) block further LPS-induced release of endogenous mediators. Alternatively (c) the cell population responsible for the release of mediators becomes desensitized and refractory to subsequent exposure to LPS, or (d) the target cells for the effects of TNF and the other mediators become refractory through various possible mechanisms such as down-regulation of receptors, inactivation of intracellular enzyme-activating targets or secondary mediators, or repression of genes responsive to the various LPS-induced mediators including TNF.

Although several concepts are available to support some hypothesis, no definitive explanation has yet been formulated. A brief comment on those concepts follows.

In vitro models using alveolar (Fisch 1983) or peritoneal macrophages (Männel 1980) obtained from animals during endotoxin tolerance have shown a lack of TNF production. LPS induced endotoxin tolerance like state with lack of generation of TNF after a challenge with LPS has also been induced *in vitro* in the RAW 264.7 cell line (Beutler 1985). Preliminary experiments in our lab have shown similar low *in vitro* LPS-elicited TNF levels using spleen, peritoneal cavity or liver (Kupffer's cells) macrophages obtained from endotoxin tolerant rats (in preparation, Yun 1990).

These *in vitro* experiments suggest that endotoxin tolerance is neither due to inhibitors of TNF activity present in plasma nor to the presence of serum factors that inhibited LPS induction of TNF production by monocytes (Mathison 1988).

Using a TNF sensitive cell line, it has been shown that although exposure to TNF decreases the number of TNF receptors on the cell surface and supressed the

susceptibility to the cytotoxic effect of TNF, the kinetics of recovery of TNF receptors and susceptibility to the cytotoxic effect is not similar. Within a few hours of removal of TNF, IL 1 or phorbol diesters from pre-treated cells previously decreased TNF receptors are fully regained, yet, the cells still maintain their resistance, indicating that some induced changes other than decrease in TNF receptors participates in the protection from cytolysis (Israel 1986), and a similar argument might be applicable to refractoriness to LPS- and other TNF-dependent effects.

Additional evidence for the mechanism of endotoxin tolerance was presented by Madonna and Vogel who associated tolerance to the presence of increased numbers of bone marrow-derived macrophage progenitor cells and suggest that the lack of responsiveness is related to failure of these immature cell types to respond to LPS (Madonna 1985). Pilaro and Laskin reported increased numbers of activated mononuclear phagocytes in the liver following LPS treatment of rats (Pilaro 1986). Similar non-published experience has been obtained in our lab (Yun 1990).

An important difference between aquired endotoxin tolerance and the natural tolerance of the C3H/HeJ mouse is that the HeJ mouse is insensitive to LPS but sensitive to TNF itself while LPS-induced tolerance is effected not only at the endotoxin-TNF link but also beyond this level because LPS tolerance can not be overcome by exogenous administration of TNF (Fraker 1988; Wallach 1987) . The C3H/HeJ mouse lacks the gene controlling responsiveness to LPS and such mice fail to produce TNF, however such mice also do not produce IL-1 (Rosenstreich 1978), interferon (Apte 1977; Vogel 1980; Watson 1978) or prostaglandins E_2 and $F_{2\alpha}$ (Rietschel 1982) in response to endotoxin.

The complexity of endotoxin tolerance where not only endotoxin fails to induce a response but animals *in vivo* or cells *in vitro* are also refractory to the effect of LPS-induced mediators has motivated other ways to study the mechanism of tolerance. Holtmann et al working with TNF sensitive cell lines studied the mechanism of sensitivity or resistance to the cytolytic effects of TNF. The working hypothesis were two: (1) That resistant cells may be incapable of responding to TNF because they do not express those activities which lead to their destruction, or alternatively, (2) they may express those cytolytic mechanisms just as effectively as sensitive cells, and yet be also endowed with additional mechanisms which counteract the cytolytic

ones. Their results support the last hypothesis: Applying TNF in the absence of inhibitors of protein synthesis, upon cells whose killing by the protein depends on treatment with such inhibitors, resulted in induction of resistance to the cytolitic activity of TNF when they were tested in the presence of the inhibitors, reflecting that an active process occurred while expossed to TNF in the inhibitor-free condition. Such resistance is also inducible with IL-1 and with tumor promoting phorbol diesters (Holtman 1987 and 1988). This information suggests that tolerance to the effects of TNF might be mediated not through a literally speaking unresponsiveness or insensitivity but rather through the development of a TNF neutralizing process. At the moment is not clear if such a mechanism is also present in endotoxin tolerance at the cellular or the molecular level.

LATE LPS TOLERANCE.

The characteristics of the late phase of endotoxin tolerance have been described in relation to the pyrogenic and the Schwartzman reaction eliciting properties of endotoxin. Such characteristics are: that it is delayed, requiring 72 hours or more after the first exposure to LPS in order to appear. It is enduring, persisting for weeks to months. It is highly, although not completely, specific for the O endotoxin employed for the initial injection. It bears no direct relationship to the intensity of the initial pyrogenic response, it is associated with increments in anti-endotoxin antibodies, and is retarded in hosts with impaired antibody synthesizing capabilities. It can be transferred with serum, and with IgG and IgM fractions. It is accelerated in onset if the host has previously been injected with the homologous endotoxin, i.e. O-specific anamnesic tolerant responses can be elicited.

These findings, considered collectively, indicate that the late phase of endotoxin tolerance is mediated by the production of anti-endotoxin antibodies. If endotoxins from rough gram-negative bacterial mutants are used to evoke late tolerance, the tolerance transferable with serum is no longer primarily O-specific, but now extends to endotoxins from other bacterial species (Greisman 1973). Such findings indicate that, in the absence of the O-specific terminal antigenic side chains, common core antigens are unmasked in the endotoxin molecule and that antibodies to these common antigens can provide broad spectrum protection against endotoxins from diverse gram-negative bacteria (Greisman 1969).

It has been demonstrated that *in vitro* anti-endotoxin serum inhibits endotoxin from evoking endogenous pyrogen release from isolated hepatic Kuppfer cells and spleen cells, but exerts no such protective effect against the endogenous pyrogen evoking activity of endotoxin from peripheral blood leukocyte (Dinarello 1968).

ENDOTOXIN AND ENDOTOXIN TOLERANCE IN HUMANS

The human is one of the species most reactive to LPS. Gross inflamatory responses are readily elicited with 10^{-3} to 10^{-4} μg/0.1 mL (10^{-11} M according to Colditz 1984b) and leukocytosis can occur with subpyrogenic doses (Mechanic 1962). An elevation of temperature can be evoked with less than 0.001 μg/kg of purified endotoxin (Greisman 1973). On a per/kg bases, rabbit and man are almonst equally reactive to threshold pyrogenic quantities of endotoxin and this is why the rabbit model has so profusely been used. However, as the quantity of endotoxin is increased, the dose response relationship becomes considerably steeper for man, and the intensity of the subjective human toxic responses increases in parallel with the pyrogenic response (Greisman 1969). Interestingly, most laboratory rabbits have no coliform bacteria in ther bowel in clear distiction from humans so the comparison between rabbits and humans in terms of LPS effects should be carefully evaluated (Braude 1969).

When endotoxin is administered to humans in single, daily, iv injections, tolerance to both febrile and subjective toxic reactions of endotoxin becomes evident within 48 hours, and with certain endotoxins, the febrile and subjective toxic responses are actually increased 24 hours after the initial iv administration. Such enhanced febrile response at 24 hours are never seen in the rabbit (Greisman 1964).

According to Greisman, no tolerance develop to LPS induced dermal inflammatory reactivity when humans are rendered tolerant by daily iv injections of LPS or when endotoxin is administered daily into the same site for one week (Greisman 1973). Other authors have found in rabbits inhibition of the neutrophil inflammatory response at 6 hours when restimulated with the same agent. This has been corroborated for PAF, LTB_4 and LPS but not for α-casein which repeatedly induced neutrophil infiltration without desensitization (Colditz 1984a). Local development of tachyphylaxis to histamine-, bradikinin- and FMLP-dependent capillary leakage has also been demonstrated which develops in 30 minutes to one hour and

progressively decreases over 4 days (Colditz 1985). And tachyphylaxis to the local inflammatory effect of LPS and IL-1 has been observed with a cross-tachyphylaxis between endotoxin and IL-1 and vice versa (Cybulsky et al 1986).

In the naive rabbit, the injection of endotoxin induces a biphasic febrile curve with a rapidly appearing first-fever peak with a latent period of approximately 15 minutes and a summit at about 90 minutes. This early reponse is absent in man. A second phase of fever develops after that first peak which closely parallel the time course of the human response. After induction of tolerance, the rabbit manifests a monophasic fever curve equivalent to the first phase of the usual response, whilst in tolerant humans the febrile response may completely disappear (Greisman 1973). Tolerance also lasts a much shorter period of time in humans than the rabbit, but this might be a reflection of the very low doses that can be used in man due to the extraordinary high sensitivity of the species to LPS (Wolf 1973).

Volunteers infected with *Salmonella typhosa* develop a remarkable hyperreactivity to the pyrogenic and subjective toxic activities of homologous (*S. typhosa*) and heterologous (*Pseudomonas*) endotoxins. Significant tolerance to these endotoxins can be readily induced within the framework of the hyperreactive state. In Greisman experience tolerance induced before illness by repeated daily intravenous injections of the endotoxins remained demonstrable during overt illnes. Daily intravenous injections of the endotoxins begun during overt illness evoked progressively increaseing tolerance and continuous intravenous infusion of *S. typhosa* endotoxin during illness rapidly induced a pyrogenic refractory state. Similar results have been found in volunteers with *Pasteurella tularensis* and patients with brucellosis (Greisman 1969).

Using the *Limulus* lysate technique , Caridis et al (1972) identified endotoxemia in a group of patients with septic and non-septic disorders. Unfortunately, there were no controls and it is only a small collection of 13 cases where endotoxemia was detected of whom 8 died; one had no evidence of infectious process. Positive *Limulus* test in plasma from apparently healthy humans or from portal blood in non infected patients are also common (Jacob 1977; DuBose 1978).

Substances other than LPS can produce false positive result for endotoxin on the *Limulus* assay (Brunson 1976; Susuki 1977; Wildfeuer 1974; Elin 1973, 1976 and

1978), and many patients with infections due to gram-negtive rods do not yield positive reactions on the test. Positive reactions are also not strong indicators of bacteremia or fatal outcome (Kass 1973).

Hesse et al administered endotoxin (20 U/kg) to 4 adults. Peak amounts of serum TNF were detected at 1.5 hours and returned to basal levels at 2.5 hours. Peak levels of IL-1 were reached at 2 hours but were not statistically different from basal levels. IFN-τ and lymphotoxin were not detected. The subjects presented a temperature increase of 0.8 to 2.6 ^{o}C within 4 hours preceded by myalgia, headache and rigors. All of the symptoms appeared as early as 90 minutes and resolved within six hours (Hesse 1988).

Revhaug compared the effect of *E. coli* endotoxin iv in normal human volunteers to Ibuprofen treated subjects. Endotoxin administration produced a response similar to an acute illness, with flu-like symptoms, fever, tachycardia, increased metabolic rate, and stimulation of stress hormone release (corticotropin, cortisol, growth hormone, norepinephrine and epinephrine). These changes, including the subjects' discomfort were markedly attenuated by cyclooxygenase inhibition. The leukocytosis, lymphopenia, hypoferremia, and elevation of the C-reactive protein level induced by endotoxin were unaffected by Ibuprofen treatment (Revhaug 1988).

SOME INTRIGUING ELEMENTS.

1) Endotoxin, has been proposed as a major factor contributing to the pathogenicity of bacterial infection in humans. Unfortunately, direct measurement of circulating endotoxin during clinical gram-negative infections has been inconsistent. Blood levels of endotoxin have correlated poorly with clinical manifestations and outcomes (Stumacher 1973; Elin 1975; Almdahl 1987; Ramsay 1988; Schoeffel 1989). One exception is meningococcal septicemia with shock where good correlation between endotoxin levels and outcome has been reported (Brandtzaeg 1988, Waage 1989).

2) Responses elicited by endotoxin administration in animal models are non-specific and can be mimicked by other substances (gram-positive bacteria, fungi,

toxic compounds, etc.) and the same problem has actually been documented in clinical sepsis (Parker 1975).

3) Endotoxin tolerance does not develop in clinical conditions of sepsis (i.e. typhoid fever), whilst it is a particularly relevant phenomenon in all species of mammals tested. Even more, volunteers render tolerant to endotoxin exhibited no discernible reduction in pyrexia or toxemia during infection with *S. typhosa* or *P. tularensis* (Greisman 1968) suggesting that endotoxemia can play no major role in the sustained fever and toxemia of typhoid fever and tularemia (Hornick 1978; Butler 1978).

4) The C3H/HeJ strain of mice which is naturally resistant to LPS and fails to produce TNF and other mediators that are considered to be the reason for damage after a challenge with LPS is more sensitive to *Salmonella* (McCabe 1981) or *E. coli* (Cross 1989) infection than related LPS sensitive strains, contrary to what would be predicted from the classical belief. A paradox exists where those animals virtually unresponsive to the lethal activity of LPS are more highly susceptible to mortality from gram-negative bacterial infection (von Jerney 1977). Furthermore, pre-treatment of the HeJ mice with a combination of TNF & IL-1 protected these mice from infection with an inoculum of *E. coli* of greater than 20 LD_{50} (Cross 1989).

5) BCG, zymosan, *C. parvum* and *C. granulosum* primed mice are more sensitive to LPS (and produce higher levels of TNF after an LPS injection) than non-primed controls but primed animals are not more sensitive to *Salmonella typhimurium* or other infections than the non-primed counterparts, and actually in most cases, the same priming treatment that induces reticuloendothelial hyperplasia and hypersensitivity to endotoxin, induces higher production of TNF after LPS injection (Carswell 1975; Green 1977) and confers resistance from infections (Senterfitt 1968; Joyce 1978).

In experimental models, exogenous TNF confers resistance to infections with *E. coli* (Cross 1989), cutaneous leishmaniasis (Titus 1989), *L. monocytogenes* (Havell 1987 and 1989; Desiderio 1989; Nakane 1988), *Plasmodium chabaudi* (Clark 1987) and Bacillus Calmette Guérin (Kindler 1989). TNF also enhances cytotoxicity for schistosomes of both eosinophils (Silberstein 1986) and platelets (Damonneville

1988) and is able to activate neutrophils to kill *Candida albicans* (Djeu 1986) and macrophages to kill *Trypanosoma cruzi* (Wirth 1988); administered TNF is also able to increase survival after caecal ligation and puncture in mice (Urbaschek 1987).

6) In humans, high serum TNF levels have been beneficial and not necessarily lethal in large proportion of patients with parasitic infections (Scuderi 1986), and cancer (Balkwill 1987); TNF levels have very rarely been identified in clinical sepsis or bacteremia other than meningococcemia or other forms of meningitis (Waage 1986), while high serum TNF levels are readily detectable after LPS injection even when very low doses are administered (Michie 1988a).

7) Once TNF was cloned, it was realized that with one exception, recombinant TNF behaved exactly as one would have predicted from work with the natural product, that is, it caused hemorrhagic necrosis of mouse tumors, showed the same pattern of cytotoxic reactivity for specific murine and human cell lines, and had synergistic cytotoxic activity with interferon. What Old an colleagues had not seen in a large experience using natural TNF was lethality, and even though mice injected with nonrecombinant sources of TNF showed some ruffling of fur and weight loss; they never saw death attributable to TNF alone. Old speculates that TNF dose, impurities contaminating the cytokine or different structure in the recombinant preparations could be the cause of this discrepancy (Old 1985). In agreement with this speculation, several groups have recently shown that ultra-pure preparations of rTNF are non-lethal or incapable of inducing necrosis of the skin (Neilson 1989; Rothstein 1988). In Mathison's experience with a rabbit model, during the iv infusion of TNF fever was registered but no changes were detected on the mean arterial pressure of white blood cells (Mathison 1988).

8) Inter-species differences in TNF potency and effects are also important. We (not shown) as well as others (Fiers, Aggarwal and Baglioni in: Palladino 1987) have detected that using the L929 assay, natural murine TNF which is glycosylated is several-fold more toxic than natural or recombinant human TNF both which are non-glycosylated; also according to Smith (1986) human TNF competes 30-fold less effectively than murine TNF for binding to murine TNF receptors. Another example of inter-species variation was presented by Zuckerman and Bendele who showed that dexamethasone was unable to reduce LPS induced TNF levels in serum

and to affect survival in guinea pigs challenged with a lethal dose of endotoxin while being able to do so in the mouse (Zuckerman 1989).
These facts again alerted us not to over-interpret results obtained by different preparations of TNF nor to neglect important inter-species differences in susceptibility to the mediators but rather to consider that in different species a highly conserved gene such as TNF might have qualitatively or quantitatively different roles and regulatory mechanisms.

Although the above observations do not entirely exclude the participation of endotoxin in the pathogenesis of the clinical manifestations of gram-negative bacillary infections, they do cast considerable doubt on the widely held concept that circulating endotoxin released during the course of gram-negative infections is primarily and almost totally responsible for the pathophysiologic changes observed during such infections.

In a recent publication, Simpson et al stated that "...at the very least, the evidence for TNF as a major, or even "the" ("the" in quotation marks in the original) major mediator of septic and endotoxin shock is quite compelling" (Simpson 1989). Whereas TNF may be able to induce all the signs of septic shock, a host of other mediators many of them with lethal potentialities are induced by LPS and to disregard their existance and participation in this complex process is quite fatuous. This overestimation of a single mediator as a causal factor for the irreversibility of the shock syndrome is not new. Parratt put it this way: "... one might legitimately conclude from much of the published literature that catecholamines, or histamine, of prostaglandins or angiotensin or endorphins etc. are each ultimately responsible for the irreversibility in shock. Such conclusions have in fact been drawn" (Parant 1983). It is possible that Simpson is neglecting that there is a gap that separating gram-negative bacterial infection from the cascade of events triggered by lipopolysaccharide administration.

The body's infection-fighting capabilities were designed to remain localized with the ultimate goal of containment and subsequent eradication of the infecting insult. The mediators discussed in this section for the most part appear to perform most appropriately on this localized level. If the infectious process escapes the localizing efforts and becomes systemic, the organism's infection-fighting process also becomes systemic, and on this level it may be more toxic than therapeutic (Meakins 1988).

CONCEPTION AND SCOPE OF THIS THESIS

One of the most unique features of endotoxin is its capacity to activate soluble factors, immune and inflammatory cell populations and to elicit the release of a wide spectrum of host effector molecules that in one way or another have been linked to the pathogenesis of septic shock.

While attempting to develop an animal model of anergy (lack of response to a Delayed Type Hypersensitivity Reaction [DTH] eliciting stimulus) by the systemic administration of LPS or TNF (Sánchez-Cantú 1989), several observations lead us to the study of endotoxin tolerance.

As proposed by Lefer (Lefer 1973) and expanded by Meakins and Marshall (Corrico 1986), Multiple System Organ Failure (MOSF) is associated with changes in intestinal flora, coupled with impairment of the normal barrier function of the GI tract which allow the bowel to serve as a reservoir of endotoxin and pathogens that can enter the portal and systemic circulation and fuel the ongoing septic process. Along similar lines, in the model we used, it was assumed that exogenous continuous administration of endotoxin or TNF could alter the immune system provoking among other consequences, anergy.

Rats previously sensitized with Keyhole Lympet Hemocyanin (KLH) were immunized with tetanus toxoid (TT) and implanted with osmotic intraperitoneal pumps filled with 0.9 mg of LPS (*E. coli* O127:B8) or 50 μg of rMu-TNF-α. Appropriate controls were included. DTH reaction to KLH, anti-TT antibody production, and a nonspecific immune response (containment of an intradermal inoculum of *S aureus*) were measured at appropriate times.

A modest reduction in the size of DTH skin reaction in the LPS treated group was shown. No difference in the resulting *S. aureus* skin lesion in any of the groups was disclosed and equally positive anti-TT antibody production in all groups were measured demonstrating no effect in antibody dependent immunity or in non-specific immune mechanisms (data not shown).

Weight loss accompanied by piloerection, poor ingestion, apathy, diarrhea, purulent urine and conjunctival secretion were seen for the first 2 days in the experimental groups, more pronounced in the LPS group. In spite of continued LPS or TNF infusion, a state of refractoriness seemed to develop as improvement in the general physical conditions of the animals, and increased food and water ingestion and body weight were evident starting on day three (Figure 3).

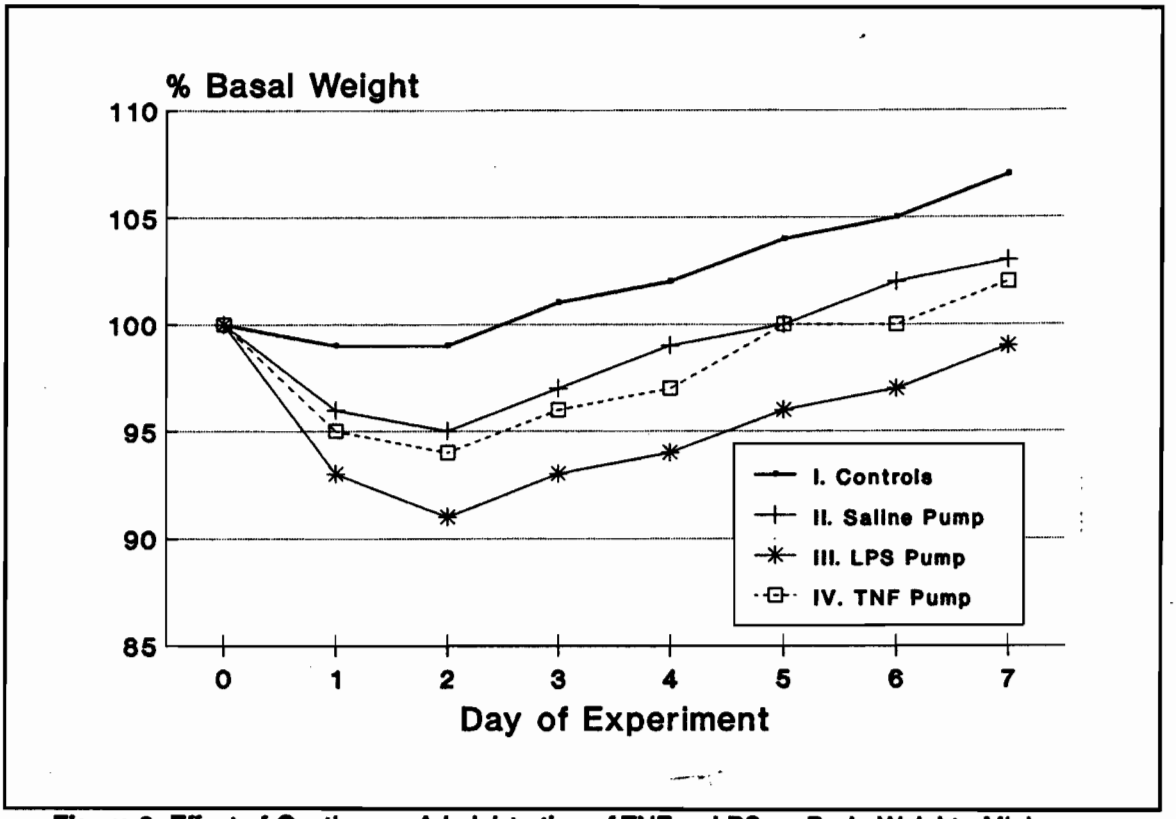

Figure 3. Effect of Continuous Administration of TNF or LPS on Body Weight. Minipumps containing *E. coli* LPS (900 μg), or rMuTNF-α (50 μg) in a volume of 0.2 mL to be delivered in 7 days were implanted on Day 0 in the peritoneal cavity of a group of rats. One group was implanted with pumps containing saline solution and another control group was exposed only to anesthesia (Controls). Daily body weight measurment is reported.

The model showed that cell-mediated immunity decreased somewhat by the systemic administration of LPS or TNF while apparently having little effect upon the humoral and nonspecific inflammatory responses. More striking however was that eventhough there was continuous administration of LPS or TNF, a refractoriness to their effects was taking place.

We tried to compare what was present in our model of LPS or TNF administration with endotoxin tolerance as described in the literature under three general premises: (1) the remarkable similarity between the effects of endotoxin in experimental models and the symptoms observed in patients with gram-negative sepsis have generated a broadly accepted belief in their cause and effect relation; (2) it is the host's response to LPS rather than a direct toxic effect seemingly the most important factor in the pathophysiology of sepsis in gram-negative clinical infections; (3) if tolerance to the toxic effects of endotoxin could be induced in patients with high risk of developing sepsis, this could be a promising model for immunomodulation protecting infected patients from septic shock or MSOF syndrome.

Moreover, as the evidence that links endotoxin with septic shock is largely circumstantial and more research in the immune and inflammatory response to endotoxin are unquestionably needed, this line of research promised to be rewarding in every way.

With these facts in mind we set up our objectives and studied the following problems.

The role of TNF in mediating the toxic effects of LPS.

The role of TNF in endotoxin tolerance.

The role of TNF in models of sepsis that use live gram-negative organisms instead of pure endotoxin.

Therapeutic value of endotoxin tolerance in the prevention of septic shock in a relevant model of sepsis.

The achievement of these objectives was guided by the following working hypothesis.

> Tumor Necrosis Factor is not a *sine qua non* condition for lethality in endotoxin shock. Bacterial endotoxin and TNF are not mandatory elements in the pathophysiology of models of sepsis other than administration of pure endotoxin. Thus in the clinical forms of gram-negative sepsis endotoxin and/or tumor necrosis factor might not play a major role.

MATERIALS AND METHODS

ANIMALS

F1 Lou/Lewis (inbred) male or female rats (150-400g weight) were bred in our animal colony.

Wistar (outbred) male rats Crl:(WI)BR were purchased from Charles River Canada Inc. St-Constant, Quebec.

All animals were fed with regular rat chow and tap water. They were housed at 2 or 3 per cage and maintained in a 12 hr dark/light cycle at a temperature of 70 to 73 °F.

All procedures were approved by the Animal Care Committee of McGill University.

LIPOPOLYSACCHARIDES

E. coli O127:B8, Cat. 3880-25 Bacto, Difco Laboratories, Detroit, Mich (TCA acid extract).

E. coli O26:B6, Cat. L-3254 Sigma Chemical Co., St Louis, Mo (Phenol extract).

E. coli O111:B4, Cat. L 2630 Sigma (Phenol extract).

Pseudomonas aeruginosa, Cat. L 9143 Sigma (Phenol extract).

Salmonella typhosa, Cat. L 6386 Sigma (Phenol extract).

All LPS preparations were diluted in pyrogen free 0.9% NaCl solution for administration in a volume of 1 mL/100 g body weight unless stated otherwise.

DEVICES

Alzet miniosmotic pumps model 2001 Cat. 4708-0, Alza Corporation, Palo Alto, Calif.

Filters. 0.22 μm Stock No. 140666 Nuclepore, Toronto, Ontario.

96 flat bottom well tissue culture plates, Cat. 3072 Falcon, Becton-Dickinson Canada, Mississauga, Ontario.

96 flat bottom well EIA microtitration plates, Cat.76-381-04, Linbro/Titertek, Flow Laboratories Inc. McLean, Va.

ELISA reader, Minireader II Dynatech, Alexandria, Va.

Capillary tubes. (Plain, soda lime glass) Cat. 2502, I.D. 1.1-1.2 mm, Chase instruments Co. Glen Falls, NY.

Autoclips. (9 mm stainless steel), No.B2355-100 wound clips, Clay-Adams Inc., Parsippany NJ.

Spectronic 710 spectrophotometer, Cat 34-4002, Bausch & Lomb, Rochester NY.

DRUGS

Atropine sulfate, 0.6 mg/mL, DIN 705500, Prod. No 454, Astra Pharma Inc. Mississauga, Ontario.

Ketalar (ketamine hydrochloride) Cat. 26980, Parke-Davis Canada Inc. Scarborough, Ontario.

Rompun (xylazine) Cat. 6894, Miles Pharmaceuticals, Rexdale, Ontario.

Ethyl Ether for Anesthesia, E136-1. Fisher Scientific, Fair Lawn, NJ.

TISSUE CULTURE CONSTITUENTS

RPMI-1640, Cat. 430-1800 Gibco, Burlington, Ontario.

Fetal Bovine Serum [FBS] Cat. 29-161-54 Flow Laboratories Inc.

L-Glutamine, Cat. 320-5039, Gibco.

Gentamycin sulfate Cat. G-3632, SIGMA.

2-Mercaptoethanol, Cat. MX309 CQ6377 Matheson Coleman & Bell, Norwood, Ontario.

Dulbecco's Phosphate Buffered Saline [PBS] Cat. 450-1300 Gibco.

OTHER REAGENTS

Saline Solution, 0.9% Sodium Chloride Injection USP, JB1302, DIN 309400, Travenol Canada Inc., Mississauga, Ontario.

10% Dextrose Injection USP, JB0163, DIN 308811, Travenol.

Actinomycin D, Cat. A-1410, SIGMA.

rMuTNF-α, a gift of Genentech, Inc. South San Francisco, Calif.

MTT, ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Cat. M-2128, SIGMA.

Isopropyl Alcohol, Cat. A-419, Fisher Scientific.

Hydrochloric Acid, Cat. A-144-500, Fisher Scientific.

$BaSO_4$. U.S.P. XIX, Cat. K90266, BDH Chemicals, Montreal, Quebec.

Peroxidase Conjugated, AffiniPure Rabbit anti-rat IgM, μ chain specific, Cat. 312-035-020, Jackson Immunoresearch Labs. West Grove, Pa.

Peroxidase Conjugated, AffiniPure Rabbit Anti-Rat IgG, $F(ab')_2$ fragment specific, Cat. 312-035-006, Jackson Immunoresearch Labs.

BSA. Bovine Serum Albumin. Fraction V Cat. 735 108. (Lyophilised), Boehringer Mannheim GmbH. West Germany.

Sodium Azide. Cat. S-227^I, Fischer Scientific.

ABTS. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt. Crystalline. Cat. A 1888, SIGMA.

Citric Acid (anhydrous). Cat. BP339-500, Fisher Biotech.

Na_2HPO_4, Sodium Phosphate, dibasic (anhydrous). Cat. S-374, Fisher Scientific

H_2O_2 Hydrogen Peroxide (0.003%) Cat. H324-500, Fisher Scientific.

$NaHCO_3$. Sodium bicarbonate, Cat. S-233 Fisher Scientific.

Blood/Agar Culture Dish. Quelab #1440 Columbia Sang, Montreal, Quebec.

Brain-Heart Infusion Broth (BHI) McGill University, Microbiology Department.

LPS TREATMENT

Lipopolysaccharide (*E. coli* 0127:B8) was either injected intra-peritoneally (ip) in 1 mL/100 g body weight volume of pyrogen free saline solution or delivered by continuous infusion through an ip implanted osmotic pump with a capacity for 0.2 mL delivered over 7 days.

BLOOD SAMPLES

Blood was obtained by retro-orbital plexus puncture (Waynforth 1980), with a capillary tube, under light ethyl ether anesthesia and centrifuged (3000 rpm x 20 min. at 6°C). Serum was stored at -70 °C until processed. For TNF determination, samples were thawed at room temperature immediately before processing, diluted 1:10 in complete RPMI media, and filter-sterilized (0.22 μm filters). For quantitative blood cultures, samples were obtained inside a laminar flow-hood by retro-orbital plexus puncture with a sterile capillary tube. Five drops of freely running blood (considered as 0.2 mL) were collected into 1.8 mL

of ice-cold 0.9% NaCl sterile solution (1:10 dilution). After vigorous vortexing, three further tenfold dilutions were produced in saline and 3 spots of 20 μL from each dilution were plated on blood/agar plates. After 24 hours of incubation at 37 °C, the number of colonies were counted for each dilution and were morphologically identified. Peritoneal cavity exudate cultures were done essentially in the same way from a 0.2 mL sample obtained under direct view after reopening the abdominal incision.

INTRAPERITONEAL PUMP PLACEMENT

The Alzet osmotic infusion pump consists of a rigid case made of a semipermeable polymer containing hyperosmotic fluid. The case also contains a flexible reservoir which holds the experimental solution (LPS or TNF in these experiments). As free water moves through the semipermeable case from the peritoneal cavity into the hyperosmotic fluid the experimental solution is displaced from the pump reservoir through an exit port-hole into the abdominal cavity at a constant rate. The capacity of the reservoir in this model is 0.2 mL and it delivers the content over 7 days (Alzet 1986). The pumps were surgically implanted as follows. Atropine sulfate 0.5 mg/kg was given intramuscularly (im) 15 minutes before anesthesia with Ketamine (20 mg/kg) and Xylazine (4mg/kg) im. The abdomen was clipped and sterilized with 70% ethyl alcohol and through a 1 cm midline incision, the pre-loaded pump (0.2 mL volume) was introduced into the peritoneal cavity. Thirty mL/kg of 0.45% NaCl solution in 5% dextrose were administered ip at the same time. The incision was closed in two layers, 4-0 silk running suture for peritoneum and muscle, and skin with wound clips. While still under the effect of anesthesia which lasted about 15 minutes, animals are kept under a radiant heat lamp. The animals were allowed free access to food and water in the post-operative period.

These osmotic pumps have a latent period of four hours after implantation to start the infusion of their content so for the analysis of the kinetics of TNF production under continuous LPS administration, the pumps were "primed" before being installed incubating them 4 hours at 37 °C immersed in isotonic saline solution; under these conditions the latent period required was obviated so the content started to flow immediately upon implantation of the pump into the abdominal cavity of the animals (Alzet 1986).

CAECAL LIGATION AND PUNCTURE

Rats were fasted overnight, water allowed *ad lib*. At 8:00 (Time 0) atropine (0.5 mg/kg) and 20 minutes later ketamine (20 mg/kg) and Xylazine (4 mg/kg) were injected im (Canadian Council on Animal Care, 1984). Once unconscious the abdomen was clipped, sterilized with 70% ethyl alcohol and a 2 cm midline celiotomy practiced. The caecum was identified, exposed and filled with feces (if not all ready full) by milking stool back from the ascending colon. The avascular aspect of the caecal mesentery was severed to free the organ as much as possible. The caecum was then ligated on its base distal to the ileocaecal valve with a 3-0 silk suture (bowel continuity preserved), and the antimesenteric surface punctured twice with a 18 gauge needle. It was ensured that holes in the punctures were properly created by squeezing some stool out of them. The bowel was replaced into the peritoneal cavity, and the abdomen closed in two layers (Wichterman 1980). Rats received subcutaneously 5 mL/100 g body weight of saline solution.

In the postoperative period animals were allowed free access to water and food. Twelve hours post-operative, survivors received an other sub-cutaneous saline injection of 5 mL/100 g body weight.

TNF BIOASSAY

The standard cytotoxicity assay was used on the TNF-sensitive L929 cell line (Mosmann 1983; Green 1984). The cell line was kindly provided by Dr. Fred Nestell, Department of Physiology, McGill University. One hundred μL of 2.5 x 10^5 cells/mL of L929 cells suspended in complete RPMI-1640 supplemented with 10% FBS (heat inactivated at 56 °C for 30 min.), L-Glutamine (300 μg/mL), Gentamycin sulfate (10 μg/mL) and 2-Mercaptoethanol (5 x 10^{-5} M) were seeded on 96-well flat bottom plates and cultured overnight at 37 °C in a humidified atmosphere of 5% CO_2 95% air to establish a subconfluent monolayer (L929 cells are more sensitive to TNF when subconfluent, that is, touching each other but before they become round and packed together at maximum). The media was removed and 50 μL of 4 μg/mL actinomycin D in complete RPMI media was added to each well followed by 50 μL of serial dilutions (begining with 1:10) of serum samples in complete media obtaining a final concentration of 2 μg/mL actinomycin D. Serial dilutions of rMuTNF-alfa

(specific activity 2 x 10^7 Units/mg) were included to construct a standard curve and to detect major variations in sensitivity (Figure 4). After 18 to 20 hours of further incubation 20 μL/well of freshly prepared MTT (4 mg/mL in PBS) were added and the plates incubated for an additional 4 hours at 37 °C. Thereafter, the supernatant was removed, 100 μL of acid isopropyl alcohol (0.04 N HCl) added and 5 minutes later 100 μL of distilled water. Plates were read within 15 minutes with an ELISA reader using a 550 nm filter.

Each sample was assayed in triplicate and all samples from each experiment were tested in a single assay. The results are expressed as Units/0.1 mL instead of weight of TNF in order to correct for minor differences in sensitivity from assay to assay. One Unit of activity was defined as the amount of TNF required to produce a 50 % decrease in absorbance relative to control cells exposed to actinomycin D alone. After several assays with the standard rMu-TNF-α, 1 unit was found to be equivalent to 35 ± 17 pg TNF and when the sensitivity was found to be outside this value, the assay was repeated. The dilution chosen to do the calculations for each sample was that which more closely approached the 1 Unit reading on the standard curve. The quantity of TNF in the experimental samples is equivalent to the activity (in units) read on the standard curve (Figure 4) for the optical density of the sample dilution, times the reciprocal of the dilution (U/0.1 mL= units in the curve for the OD in the sample x 1/dilution).

MEASUREMENT OF ANTIBODIES

ELISA Technique (Voller et al, 1979). Flat bottom wells (EIA microtitration plate) were loaded with the antigen (50 μg/mL solution of *E. coli* O127:B8 or *S. typhi* LPS in coating buffer: Na_2CO_3 PBS with 0.01 M $NaHCO_3$ pH 9.6 and 0.02% NaN_3, 200 μL/well) and left overnight at room temperature in a humid environment.

On the following morning, wells were emptied and washed twice with 250 μL/well 0.1% BSA in PBS (with 0.02% azide). Wells were then loaded with 200 μL of 1% BSA in PBS and incubated for 1 hour at room temperature (to block the non-specific receptor sites on the well's surface). Wells were then washed twice with 150 μL/well 0.1% BSA in PBS (with Azide). Serial dilutions of the samples were loaded (100 μL/well) (1:100 - 1:12800) and incubated 1 hr at room

temperature. After 3 more washes with 0.1% BSA in PBS (azide free), 100 μL of peroxidase conjugated Rabbit anti-rat IgG or IgM (1:1000 dilution of stock solution) was added, plates were incubated for 1 hour at room temperature and then again washed 3 times with 0.1% BSA in PBS (azide free). The substrate (0.5g/L ABTS, 0.1M citric acid, 0.05M Na_2HPO_4, 0.003% H_2O_2, pH adjusted to 4.3) was added (100 μL/well), and incubated in the dark for 15 min. 100 μL of 0.1% azide in PBS was added to each well to stop the reaction and the plate read with a 410 nm filter on the ELISA reader.

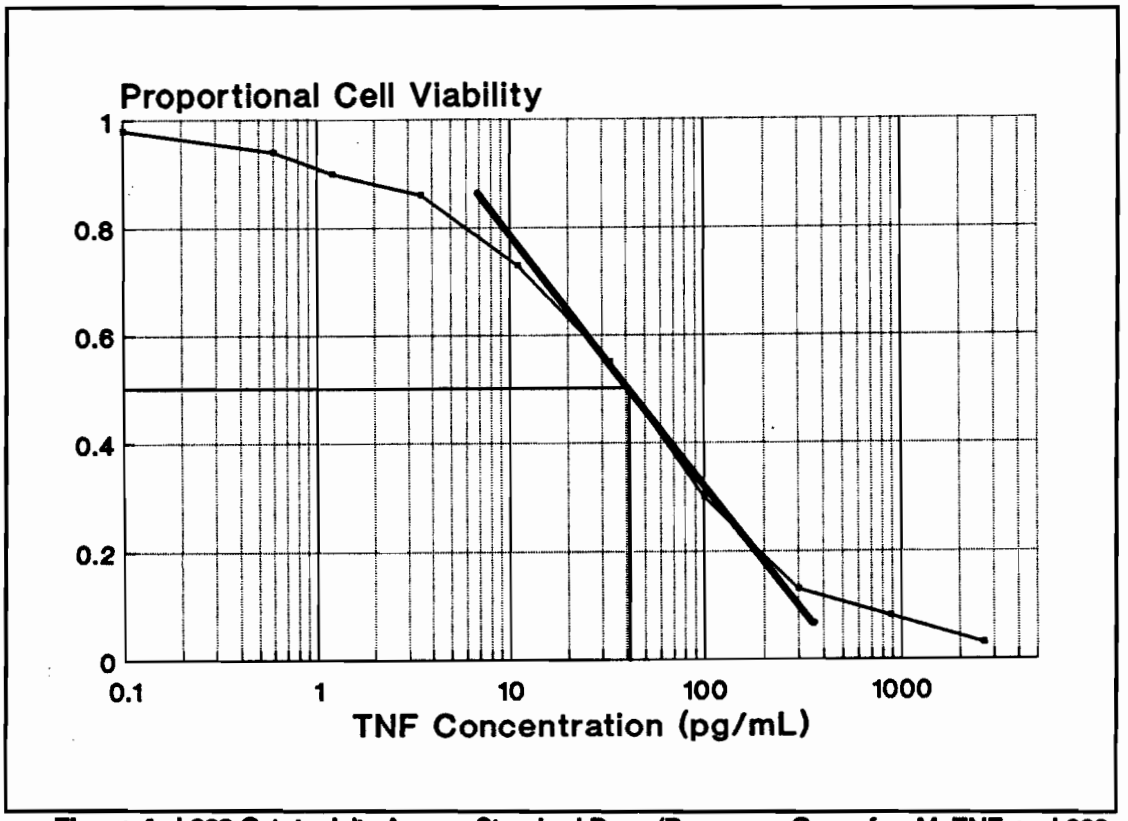

Figure 4. L929 Cytotoxicity Assay. Standard Dose/Response Curve for rMuTNF-α. L929 cells were incubated with serial dilutions of rMuTNF-α. Cytotoxicity was measured in a colorimetric assay and expressed as proportional cell viability. The OD was plotted to obtain a standard curve in each assay. Proportional cell viability of 1 was equivalent to the OD registered from control wells not exposed to TNF. One unit of activity per 0.1 mL was defined as the amount of TNF required to produce a 50% decrease in absorbance relative to control cells. In the curve shown, 1 U/0.1 mL is equivalent to 42 pg/mL. Serial dilutions of the test samples were incubated in the same way and OD readings were performed on each dilution. The straight segment of the standard curve was used to read out from the OD readings TNF activity present in the samples. This was expressed in Units/0.1 mL and the number of units was then multiplied by the reciprocal of the corresponding dilution factor. The dilution chosen to do the calculations reported as results was the one that more closely approached the 1 unit reading on the standard curve.

To calculate the amount of antibody in the samples, a standard curve was generated with a pool of serum samples selected from the group of rats where we expected high antibody titers. A value of one unit/mL of activity was arbitrarily assigned to the activity present in the dilution that produced an 50% absorbance compared to the highest optical density reading in the undiluted sample pool. The OD in the sample was transformed into an activity value (units/mL) using the standard curve. The reported amount of antibody was calculated from the dilution where the OD more closely approximated the 1 unit/mL absorbance in the standard curve, times the reciprocal of the dilution.

LIVE E. COLI PREPARATION

An *E. coli* (NTC-9001)-containing agar slope obtained from the Department of Microbiology and Immunology, McGill University, was subcultured overnight on a blood-agar plate at 37°C. Next morning a sample was inoculated into 10 mL of BHI broth and incubated in a water bath at 37°C for 90 minutes. One milliliter was then inoculated into 100 mL of BHI and incubated again in the water bath for 90 minutes. A standard curve was prepared from the spectrophotometric readings. Fifty μL aliquots were obtained every 30 minutes for 6 hours. Serial 10-fold dilutions (10^{-2} to 10^{-7}) were done at each time point and OD readings (660 nm filter) as well as further seeding on blood-Agar plates were performed for each dilution. OD readings were plotted against CFU.

To prepare the bacterial suspension, the same steps were followed using up to the 100 mL culture in BHI. This was incubated in accordance to the approximate time needed as estimated from the curve. At that time an OD was taken, the bacterial concentration was adjusted to the one desired and used within 30 minutes while kept on ice. A sample was also plated for further verification of the CFU generated.

EUTHANASIA TECHNIQUE

For terminating an experiment or for ethical reasons, euthanasia was performed by placing the animal for a minimum of 5 minutes in a chamber filled with CO_2 (Canadian Council on Animal Care, 1984).

EXPERIMENTAL METHODOLOGY

Endotoxin tolerance was used as a tool for the analysis of the pathophysiology of septic shock. The experimental methodology can be divided in five parts:

I. Response of the naive animal to endotoxin administration.

Some of the effects provoked by the administration of endotoxin to naive animals, namely lethality, body weight loss and serum TNF levels were described. A naive animal is considered one that has not been previously injected or artifitially exposed to LPS.

II. Modifications of the response to LPS during the early phase of endotoxin tolerance.

Responsiveness to a secondary injection with LPS, referred to as "challenge" dose, was modified by previous administration of sublethal doses of LPS referred to as "priming" doses. A systematic study of this modified response in the so called "early tolerant" or simply "tolerant" animal during the early few days (less than 15 days) after the "priming" dose was studied.

III. Modification of the response to an unrelated type LPS during early endtoxin tolerance.

The response of naive rats to different types of LPS and the modified response of the "early tolerant" rat when the type of LPS used to "challenge" was unrelated to the LPS used to "prime" the animal were studied.

IV. Response to LPS during the late phase of endotoxin tolerance.

A modified response to LPS effects known as late LPS tolerance follows the early phase of tolerance once this fades away. This type of response was studied considering 21 days as a landmark defining a complete cycle since the administration of a "priming" dose of LPS until recovery from early endotoxin tolerance and establishment of late phase tolerance was attained. The response to a challenge with an unrelated LPS (an LPS from a different bacteria than the

one used to "prime") during late endotoxin tolerance was evaluated. IgG and IgM anti-LPS antibodies (for both types of LPS used) were also measured.

V. Serum TNF levels and endotoxin tolerance in models of sepsis other than LPS administration.

Serum TNF levels generated after the intraperitoneal injection of pure or mixed live bacteria (Browne 1976; Bartlett 1978) or following caecal ligation and puncture were determined. Protection conferred by LPS tolerance using CLP or intraperitoneal *E. coli* injection as lethal challenge was also studied.

STATISTICAL ANALYSIS

Unpaired Student t-test was used for comparison of two means. X^2 was used for comparison of proportions. Linear regression analysis through the 'least squares' estimation was used to calculate the lethal potency of the different LPS preparations and other values obtained through regression analysis (Colton 1970). One-way ANOVA was used to make comparissons of one variable between several groups (Armitage 1987). A p value < 0.05 was taken to indicate statistical significance.

In every graphic representation of data, error bars for arithmetic means are always shown as ± one Standard Deviation.

RESULTS

RESPONSE OF THE NAIVE ANIMAL TO ENDOTOXIN ADMINISTRATION

A series of control experiments were initially performed to determine the effects of endotoxin in naive animals.

I. LETHALITY

To define the Dose/Response curve for LPS (*E. coli* O127:B8) lethality, single doses of 1, 5, 10, 25, 40 and 60 mg/kg of LPS were injected ip into F1 Lou/Lewis female rats (165-180 g body weight, 10 animals per dose). Figure 5 shows survival rate at 72 hours. None of the animals died in the two lowest dose groups (1 and 5 mg/kg) and all rats in the 60 mg/kg group died. The LD_{50} was estimated as 24.2 mg/kg and the LD_{100} as 47.1 mg/kg by regression analysis.

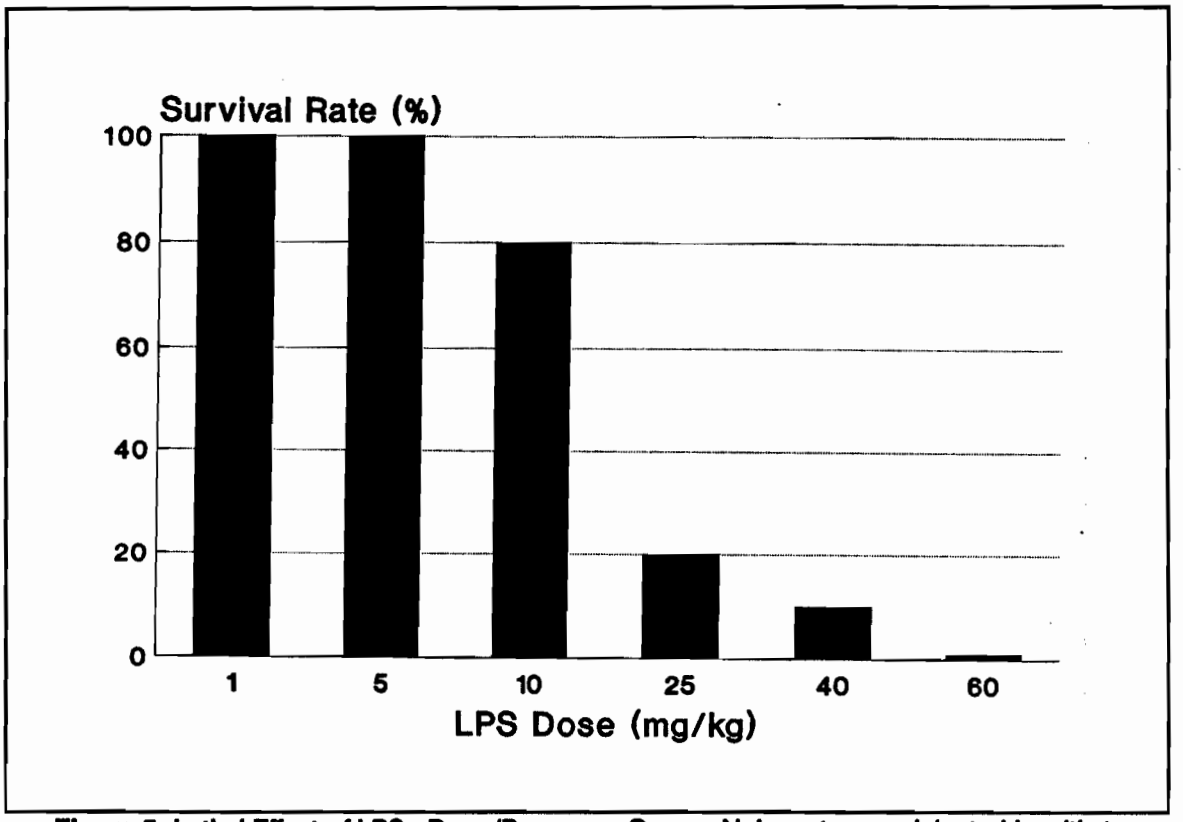

Figure 5. Lethal Effect of LPS. Dose/Response Curve. Naive rats were injected ip with 1, 5, 10, 25, 40 or 60 mg/kg of LPS (10 rats/dose). Survival at 72 hours is shown.

II. WEIGHT LOSS

The effect of single intraperitoneal injections, repeated daily doses and constant intraperitoneal infusion of LPS on weight loss was determined.

A) Single Injections of LPS.

Doses of 6.25, 10, or 12.5 mg/kg of LPS were injected into F1 (Lou/Lewis) male rats (6 rats/group). A control group received saline (10 mL/kg). Body weight was monitored for 7 days. One rat died in the 6.25 mg/kg group and 2 in the 12.5 mg/kg group and are excluded from the curves. The animals exhibited a steady weight loss for 2 to 3 days, which was most pronounced in the highest dose group, followed by recovery from day 3 onwards as represented in Figure 6.

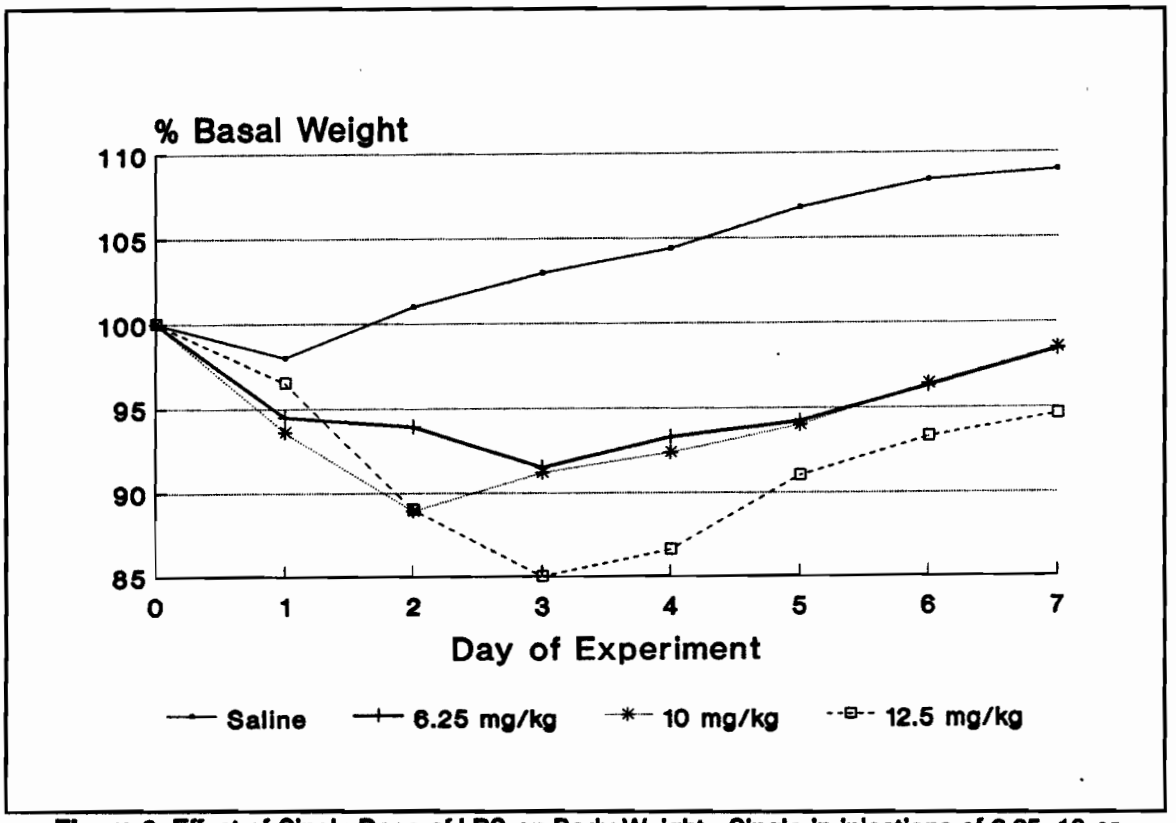

Figure 6. Effect of Single Dose of LPS on Body Weight. Single ip injections of 6.25, 10 or 12.5 mg/kg of LPS were given on Day 0 to 3 groups of naive rats. A control group was injected with saline (6 rats/group). Body weight was measured for 7 days.

B) Repeated Injections of LPS.

Daily doses of 1.25, 2.5, 5, or 10 mg/kg/dose of LPS were given on four consecutive days. Controls received 10 mL/kg saline solution daily for four days. Body weight and overall clinical effect were registered. Male F1 Lou/Lewis rats

(345 ± 30 g) were used (5 rats/group). One rat died in the 10 mg/kg/dose group and is excluded from the analysis.

Weight loss was again evident for three days (Figure 7) except in the highest dose group which still had a mild average loss until day 4. During the first 2 days, rats were lethargic most of the time, had liquid stools, purulent urine, conjunctival bloody secretion and poor appetite. In spite of being injected once more on day 3, most of the rats started to regain weight from day 3 onward and had improved general conditions starting on day 3 which substantiates the fact that they were no longer sensitive to the effect of LPS.

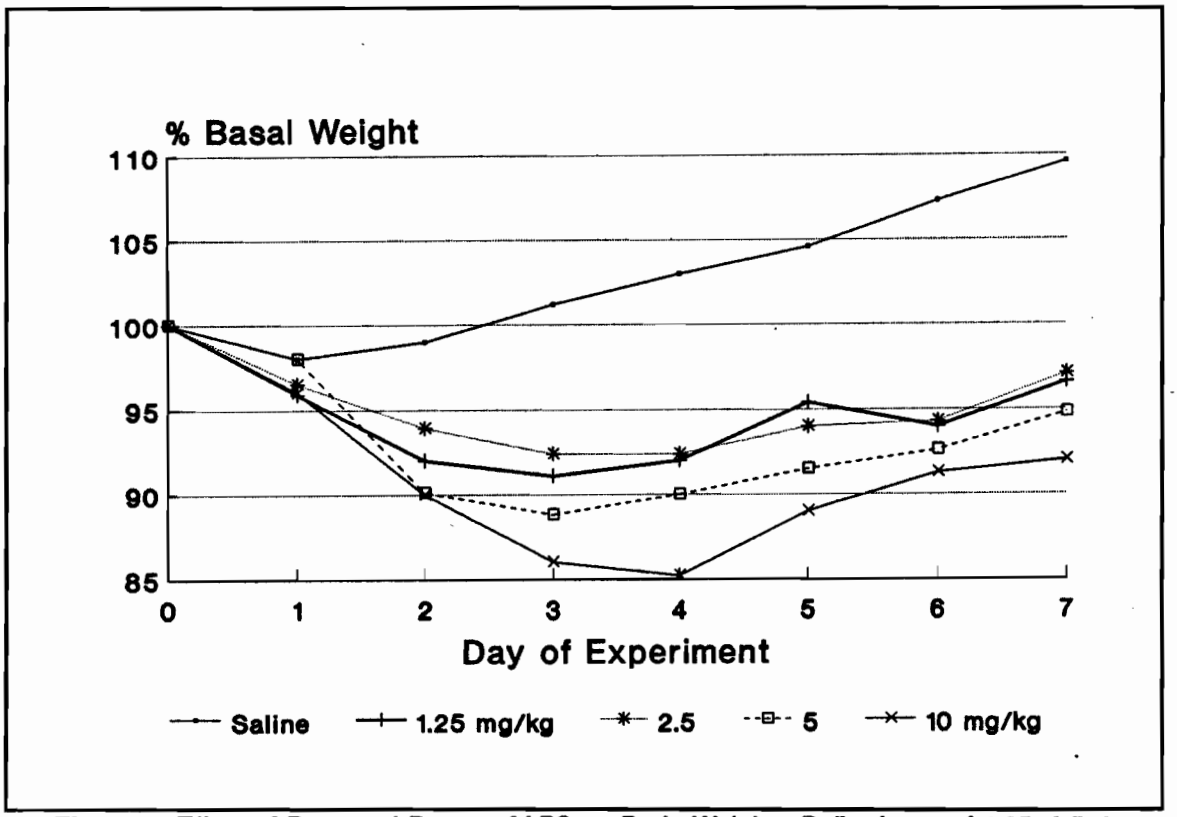

Figure 7. Effect of Repeated Doses of LPS on Body Weight. Daily doses of 1.25, 2.5, 5 and 10 mg/kg per dose of LPS were given to 4 groups of rats on four consecutive days starting on Day 0. A control group received four injections of saline solution. Body weight was measured for 7 days.

C) Continuous Intraperitoneal Infusion of LPS.

Pumps containing different concentrations of LPS to be delivered over 7 days were implanted in the abdominal cavity and body weight was monitored.

Symptomatology was also registered. F1 male Lou/Lewis rats (weights 250-277 g) were used (4 rats/group). Pumps were loaded with saline (controls) or with the following amounts of LPS: 4.5 mg/mL (0.9 mg/pump), 16 mg/mL (3.2 mg/pump), 32 mg/mL (6.4 mg/pump) or 64 mg/mL (12.8 mg/pump). At the end of the experiment rats were killed, the peritoneal cavity was examined and the pumps inspected.

As in the previous 2 experiments, weight loss proportional in magnitude to the concentration of LPS was observed for 2 days after the infusion began and was followed by recovery thereafter. Figure 8 shows weight curves. This was duplicated in the general conditions of the rats. No deaths were observed in any group. Examination of the pumps showed that in every case their content was fully voided. There was little peritoneal reaction manifested in the form of loose omental adhesions to the pump. No gross inflammatory exudate or macroscopic evidence of chronic inflammation were detected.

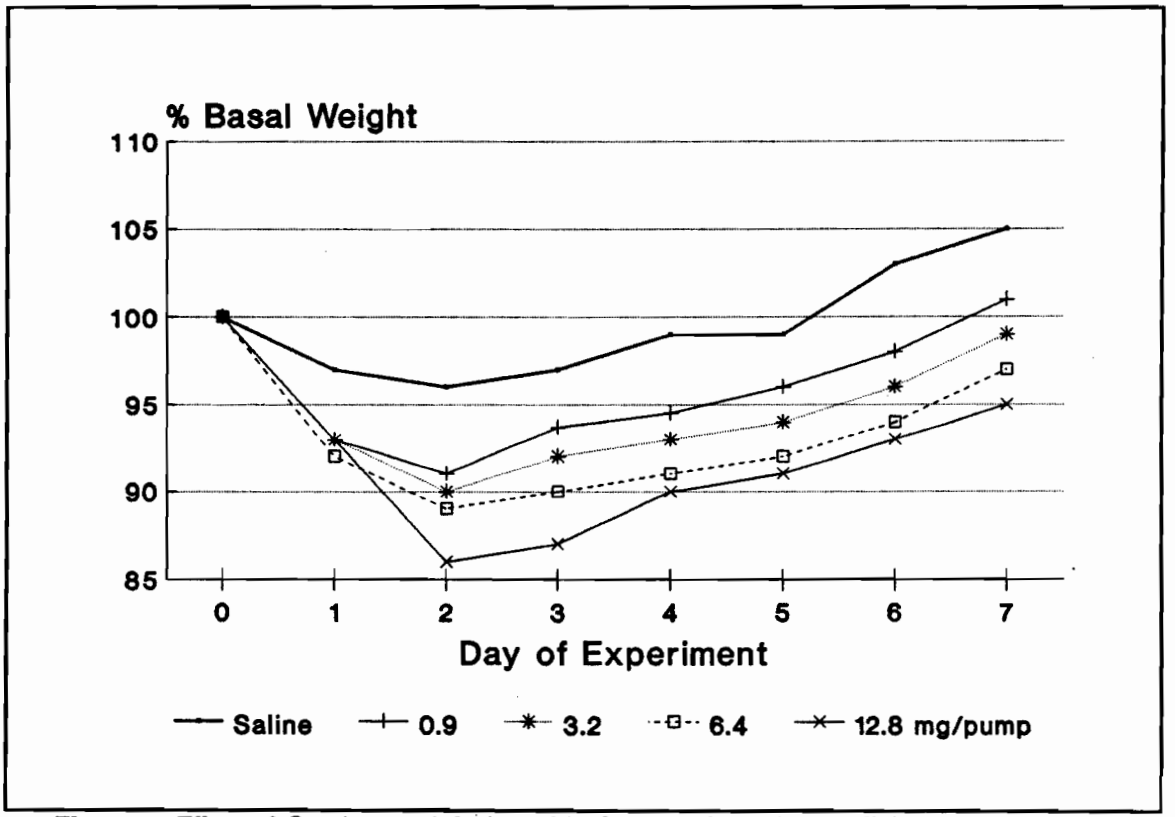

Figure 8. Effect of Continuous Infusion of LPS on Body Weight. Minipumps containing 0.9, 3.2, 6.4 or 12.8 mg per pump of LPS delivering their load over 7 days were implanted ip on Day 0 into 4 groups of rats. Control pumps contained saline solution. Body weight was measured for 7 days.

It is generally accepted that most of the biological effects of endotoxin are not due to a direct action upon the target tissue or cell; instead endogenous mediators generated mainly by cells of the monocyte/macrophage lineage after exposure to LPS are thought to be responsible of the final impact of LPS.

At this point it was unknown if the lack of sensitivity evident in endotoxin tolerance was due to lack of production of the endogenous mediators of LPS such as TNF or if the mediators were still produced but there was indeed a lack of responsiveness to the signal carried by the mediators.

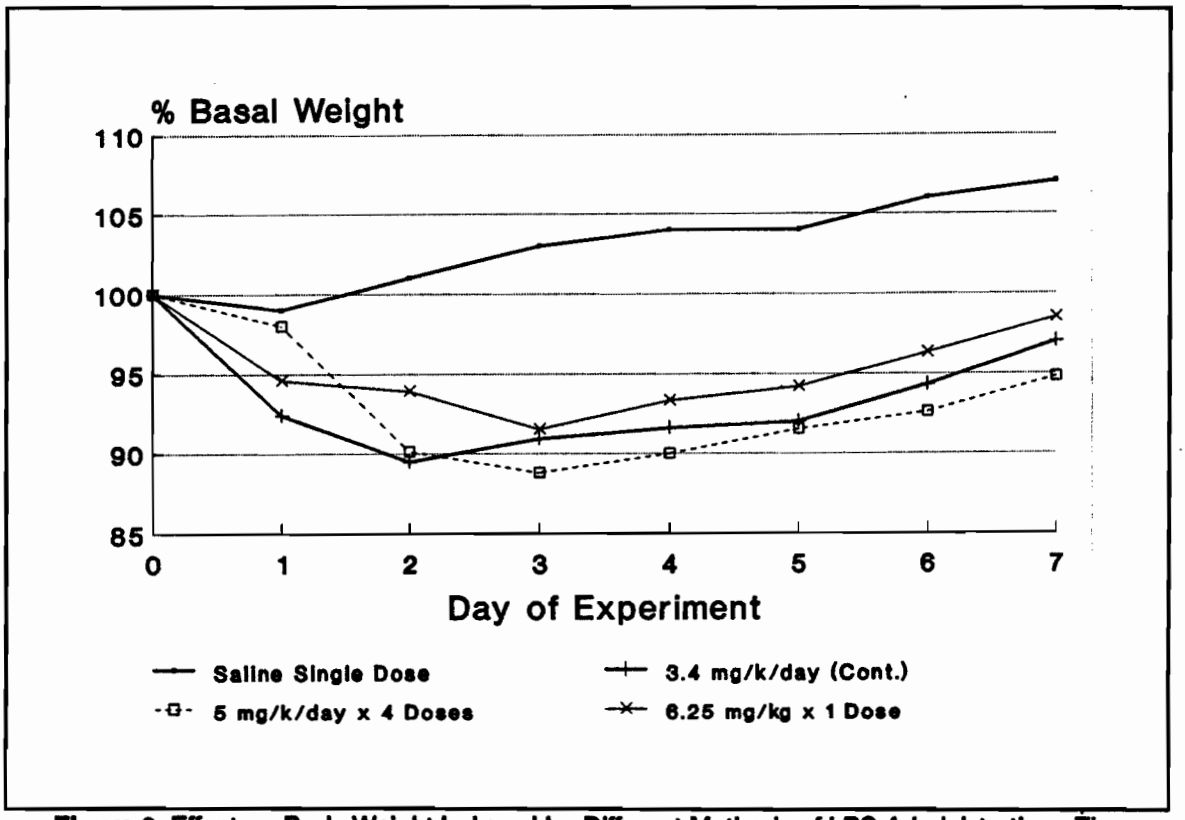

Figure 9. Effect on Body Weight Induced by Different Methods of LPS Administration. The effect of a single ip dose of 6.25 mg/kg of LPS (Day 0), four daily ip doses of 5 mg/kg/day of LPS (Day 0, 1, 2 and 3) continuous ip infusion of 3.4 mg/kg/day of LPS (from Day 0 to Day 7) or a single ip injection of saline solution (Day 0) on body weight are shown.

III. PRODUCTION OF TUMOR NECROSIS FACTOR

As TNF is postulated to be one of the main endogenous mediators of the toxic effects of LPS *in vivo*, the next objective was then to display the relation between LPS administered and the serum levels of TNF generated.

First, the kinetics of serum TNF levels after ip LPS injection were established in two groups of male Wistar rats (6 per group). One group received a single ip bolus injection of 0.5 mg/kg LPS (*E. coli* O127:B8). Rats in the second group were implanted ip with primed osmotic minipumps (primed in order for the pump to start to deliver its content immediately after being installed) loaded with 0.2 mL of a 20 mg/mL solution of LPS. Blood samples were obtained every 30 minutes over 8 hours and serum TNF levels were determined.

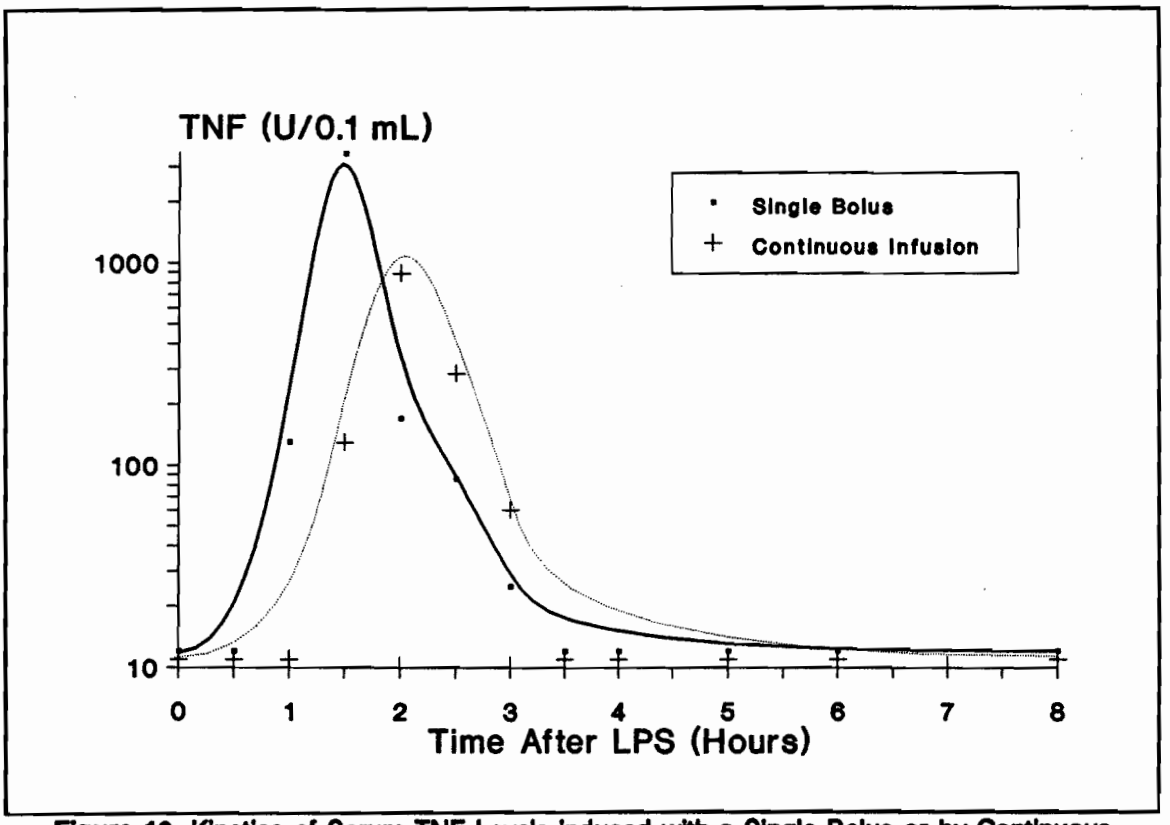

Figure 10. Kinetics of Serum TNF Levels Induced with a Single Bolus or by Continuous Infusion of LPS. Rats were given a single ip bolus injection of 0.5 mg/kg of LPS or continuous ip infusion of 0.57 mg/kg/day of LPS starting at time 0:00 (6 rats/group) . Blood samples were obtained every 30 minutes to measure serum TNF levels.

The serum TNF profile was similar in both groups (Figure 10). TNF was not detectable in the 0 and 30 minutes samples. By 60 minutes in the bolus group TNF was detected with a peak at 90 minutes. In the infusion groups a peak was reached at 2 hours. After the peak, the TNF levels rapidly fell to undetectable levels by 3.5 hours in both groups.

Once we confirmed the peak for serum TNF levels at 90 minutes post-LPS injection, a Dose/Response curve for peak serum TNF levels was generated using different doses of LPS (*E. coli* O127:B8). Male Wistar rats were injected (6-8 rats/dose) with 0.5, 5, 20 or 50 mg/kg, and blood samples were obtained at 90 minutes post LPS injection. This range is the "high" dose range because it was intended to detect the effect using doses in the lethal range.

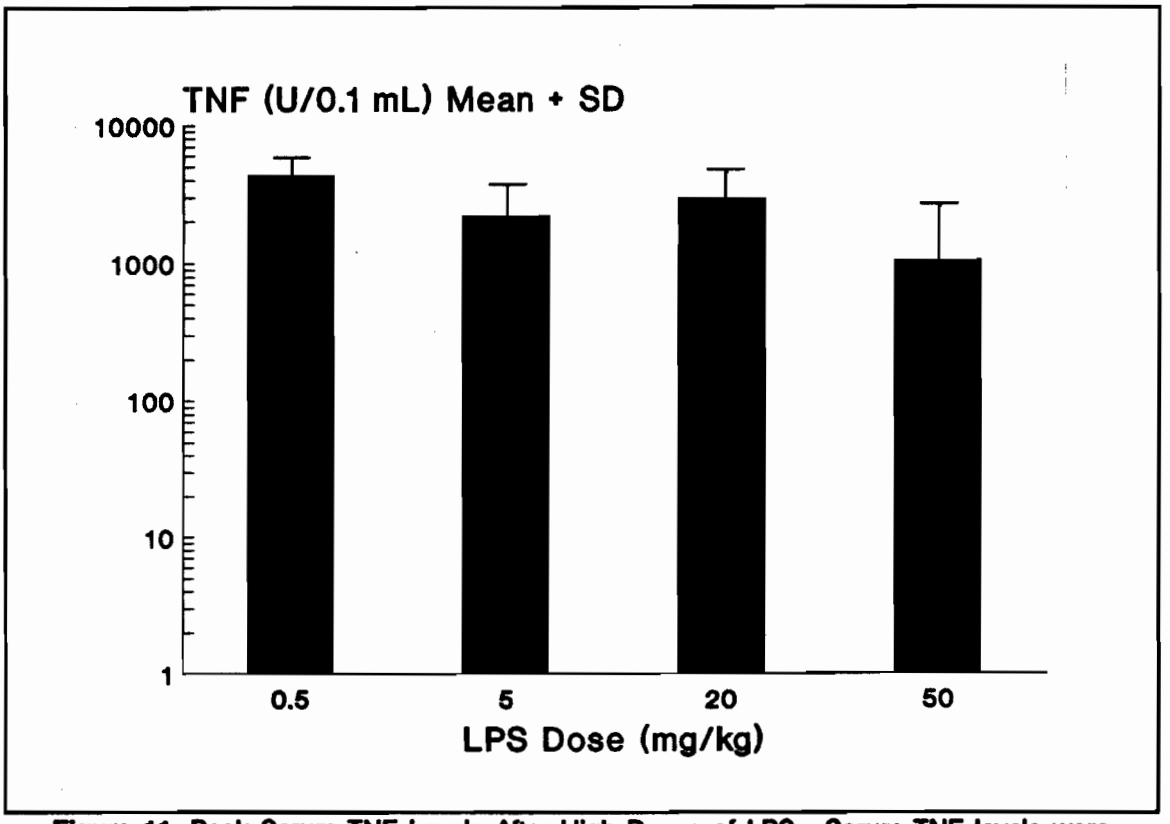

Figure 11. Peak Serum TNF Levels After High Doses of LPS. Serum TNF levels were measured in naive rats 90 minutes after an ip injection of 0.5, 5, 20 or 50 mg/kg LPS (8 rats/dose).

As seen in Figure 11, no direct correlation was found between LPS dose and peak levels of TNF in the "high" dose range (ANOVA $p < 0.0005$). Even more surprising was the observation that the highest peak serum TNF levels were elicited with the non-lethal doses and somewhat lower levels of TNF with the highest dose of 50 mg/kg (LD_{100}). We were expecting high TNF levels with lethal doses and lower or not detectable TNF serum levels after a non-lethal challenge. An inverse correlation (r = -.8020) although not significant was found between peak TNF levels and LPS dose in the range tested.

When we realized that the lowest dose used in the previous experiment was inducing the highest peak levels of TNF it was obvious that a dose/response curve for the elicitation of TNF including much lower doses of LPS should be performed. Ten-fold dilution doses from 5 mg/kg and below ("low doses") were used (Figure 12).

In the "low" dose experiment, the highest peak TNF levels were found in the highest dose group (5 mg/kg) and progressively diminished until they became undetectable at about the 0.5 μg/kg dose group or less. The correlation was positive with an r = .7027 (p = 0.053) (in log scale r = .9461) in the sensitive range (5 mg/kg to 5 μg/kg).

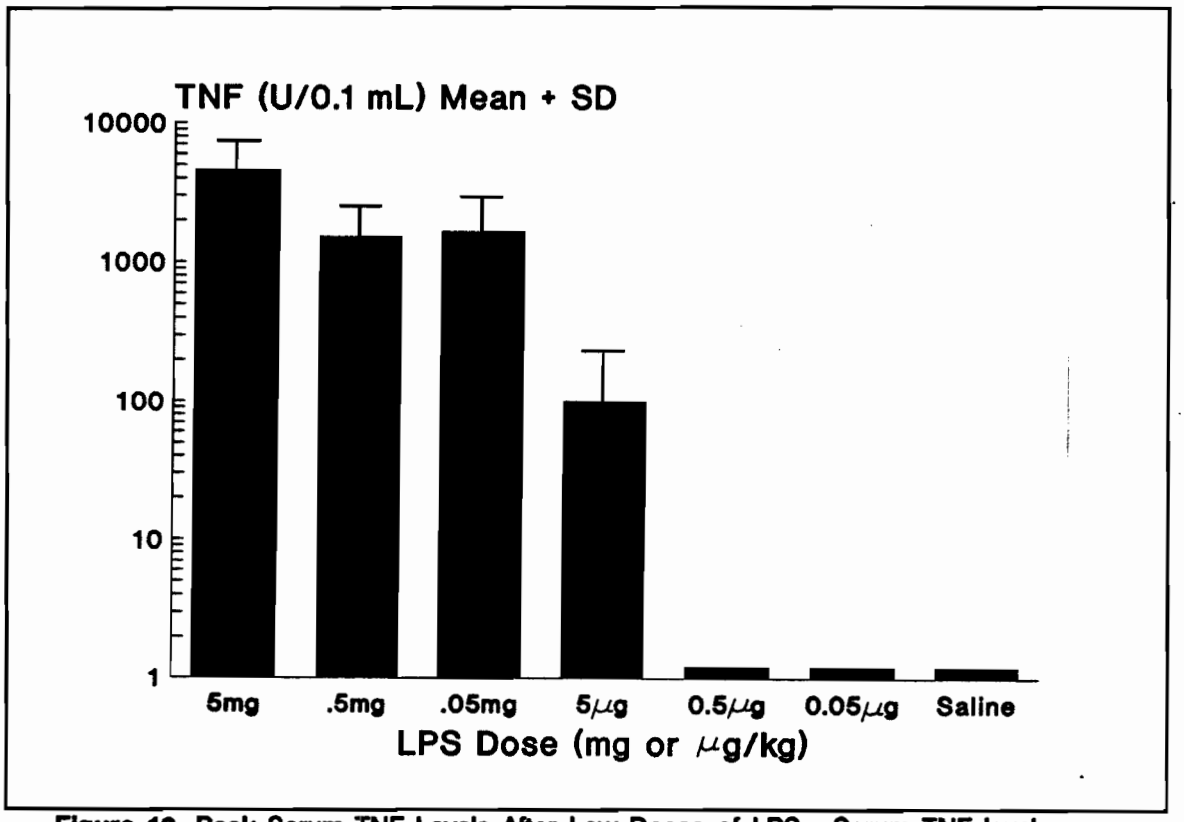

Figure 12. Peak Serum TNF Levels After Low Doses of LPS. Serum TNF levels were measured in naive rats 90 minutes after an ip injection of 5 mg, 0.5 mg, 0.05 mg, 5 μg, 0.5 μg, 0.05 μg/kg LPS or saline solution (6 rats/dose).

The data from figures 11 and 12 has not been presented in a single graph because it was generated in two different experiments and also the TNF levels were measured in two different assays, but is clear that there is a wide plateau of high TNF production after doses from about 0.05 mg/kg to 20 mg/kg of LPS.

It became evident that very similar effects on weight loss were induced by a single dose, four repeated injections or continuous ip infusion of LPS (Figure 9) suggesting that animals responded only to the first exposure to LPS and that once triggered, little or no further effects were provoked by repeated or continued challenges. It was also appreciated that the other signs of toxicity observed in the 48 hours following the first challenge were not provoked by continued administration or subsequent doses of LPS reflecting that a broad insensitivity to LPS effects was established.

MODIFICATIONS OF THE RESPONSE TO LPS DURING THE EARLY PHASE OF ENDOTOXIN TOLERANCE

Animals given LPS became refractory to a second challenge of LPS. Experiments were performed to define in a systematic form how an animal would respond in terms of mortality, body weight loss and serum TNF levels after an LPS challenge while tolerant. These experiments consisted essentially of the administration of one or more non-lethal doses of LPS given to induce tolerance (priming) followed at different times thereafter by one or several other injection of LPS given to test sensitivity during the phase of tolerance (challenge).

According to Beeson four daily doses of LPS seemed sufficient to induce tolerance to LPS, and a waiting period of 3 days was long enough to enable separating the effect of pre-treatment from the effect of the challenge dose avoiding an additive effect but still testing the secondary response while the effect of tolerance was strong (Beeson 1947).

I. LETHALITY.

The ability of tolerant animals to withstand a lethal dose of LPS was first studied. Male F1 Lou/Lewis rats were used; 5 or 6 animals per challenge group. Tolerance was induced by priming with 5 mg/kg LPS on Days -7, -6, -5 and -4. Two groups of control animals were used; animals (naive) injected with saline at the same time, or post-tolerant animals injected with 5 mg/kg LPS but on Days -37, -36, -35 and -34. Animals were then challenged on Day 0 with 1, 5, 15, 30 or 60 mg/kg LPS, the tolerant group with the same doses except 1 mg/kg. Mortality, shown in Figure 13 was registered 72 hours after the challenge.

It was striking to note that rats in the tolerant group survived a dose that proved to be 100% lethal in the other two groups. Sensitivity in the post-tolerant group was similar if not higher than in naive rats indicating that sensitivity to LPS was recovered by 30 days of rest after induction of tolerance. LD_{50} and LD_{100} were estimated by regression analysis and presented on Table 5.

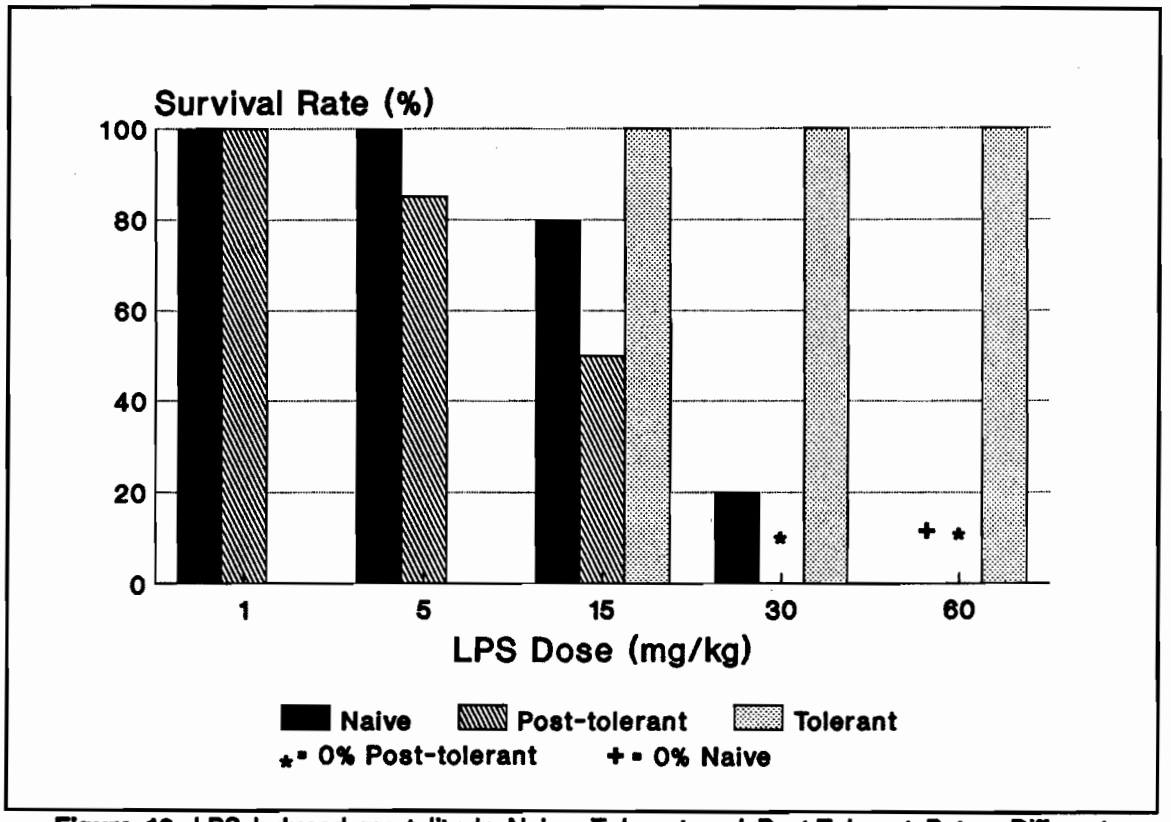

Figure 13. LPS Induced mortality in Naive, Tolerant and Post-Tolerant Rats. Different challenge doses of LPS were administered to 3 groups of rats. Naive rats were challenged with 1, 5, 15, 30 or 60 mg/kg LPS on Day 0. Tolerant rats (primed with 5 mg/kg LPS on Days -7, -6, -5, and -4) were challenged with 15, 30 or 60 mg/kg LPS on Day 0. Post-tolerant rats were primed with 5 mg/kg of LPS on days -37, -36, -35 and -34 and challenged with 1, 5, 15, 30 or 60 mg/kg of LPS on Day 0. Mortality was registered 72 hours after the challenge.

II. BODY WEIGHT.

Body weight was monitored for 7 days after challenge in the 5 mg/kg dose sub-groups in this same preceding experiment. An extra group of controls (6 rats) that was not primed was challenged with saline solution (Day 0) and is included to show the basal body-weight curve.

Table 5. Sensitivity to Endotoxin Lethality

GROUP	LD_{50}	LD_{100}
Naive	21 mg/kg	37.6 mg/kg
Post-tolerant	15 mg/kg	29.8 mg/kg
Tolerant	>60 mg/kg	>60 mg/kg

The weight curves of naive and post-tolerant rats were very alike and so were the curves for the control and tolerant groups (Figure 14). There was no effect of LPS in tolerant animals and continued to gain weight like the controls receiving saline. The recovered responsiveness to LPS after 30 days of rest in the post-tolerant group is evident as the challenge resulted in an effect on body weight loss equal to the consequences experienced by the naive group.

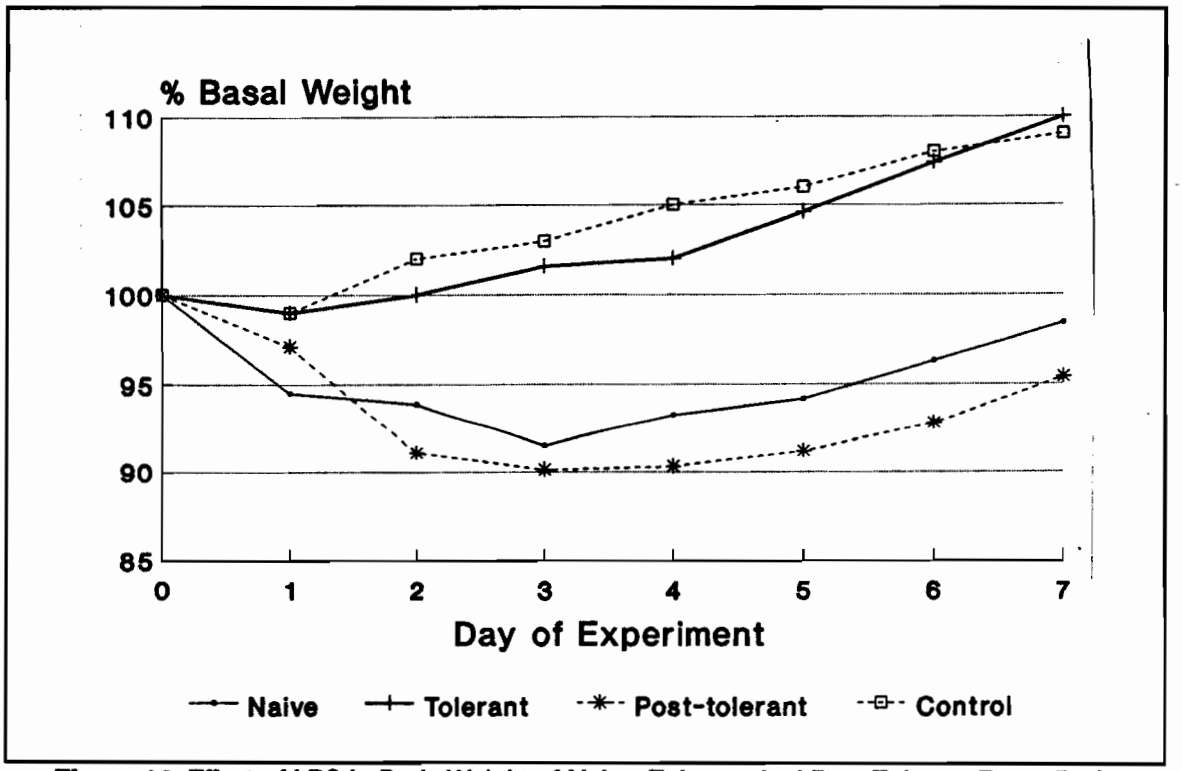

Figure 14. Effect of LPS in Body Weight of Naive, Tolerant And Post-Tolerant Rats. Body weight was daily measured after the ip injection of 5 mg/kg LPS on Day 0. Naive, tolerant and post-tolerant rats received the same pre-treatment as in the previous Figure. Control rats were injected with 10 mL/kg saline solution on Day 0.

III. PRODUCTION OF TUMOR NECROSIS FACTOR.

We wanted to know if the diminished sensitivity to lethality and weight loss was accompanied by a diminished production of TNF during early endotoxin tolerance. We also wanted to know if the transitory span of insensibility noted for the other effects (lethality and weight loss) was also present in the case of TNF production and if full recovery of sensitivity with return to basal conditions would occur if enough time was allowed to pass after induction of LPS tolerance.

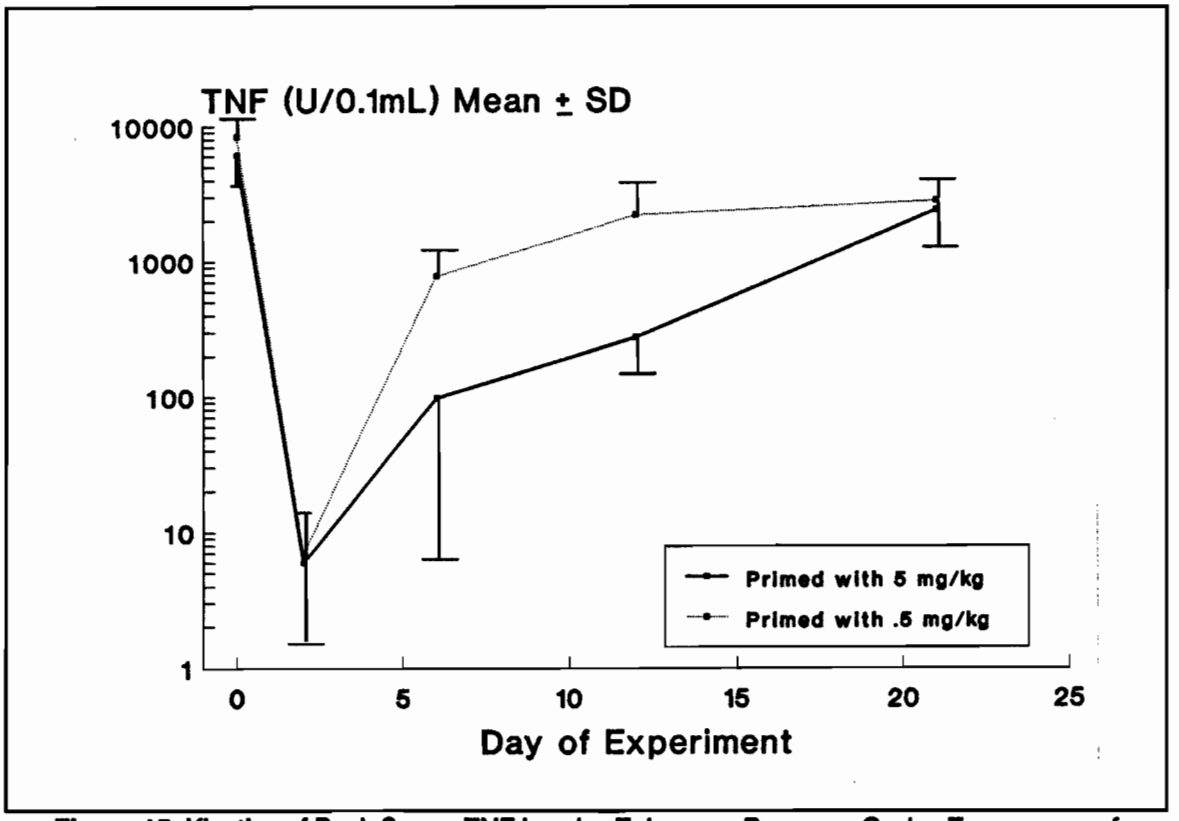

Figure 15. Kinetics of Peak Serum TNF Levels. Tolerance-Recovery Cycle. Two groups of rats were injected ip on Day 0 with 0.5 or 5 mg/kg LPS (Priming Dose). Animals were split into 4 sub-groups and challenged with 5 mg/kg LPS on Days 2, 6, 12 or 21 after the first dose. Blood samples were obtained 90 minutes after each LPS injection to measure peak serum TNF levels and IgG and IgM anti-LPS antibodies.

We followed the course of peak serum TNF levels after LPS injections and anti-LPS antibodies subsequent to inducing early LPS tolerance. Male F1 (Lou/Lewis) rats were injected ip on Day 0 with a single dose of 5 or 0.5 mg/kg LPS (*E. coli* O127:B8) (pre-treatment or induction dose). Each pre-treatment group was then divided into 4 sub-groups (5 rats/sub-group) and challenged with 5 mg/kg LPS at different point times (Day 2, 6, 12 or 21 respectively). Blood samples were obtained 90 minutes

post-injection for determination of TNF levels and anti-LPS antibodies. Peak serum TNF levels are represented in Figure 15.

As shown, the high peak levels of TNF observed after the first dose (Day 0) with either induction or pre-treatment dose are almost completely aborted on Day 2 when challenge elicited only trace amounts of TNF. By Day 6 partial recovery of responsiveness is evident and although it keeps improving, on day 21 peak TNF levels are not completely restored to peak levels induced in the naive animal.

A sooner increased in peak TNF levels by day 6 in the group induced with the low dose are revealed by the earlier recovery of peak TNF levels by day 6 in the group primed with the low dose of LPS (ANOVA $p < 0.05$).

IgG and IgM anti-LPS antibodies, undetectable on Day 0 were still at trace levels by day 2 when tolerance seems to be maximal; and levels became apparent on Day 6 just as TNF production began to recover. The high IgG levels present on day 21 do not seem to be related to the tolerance/recovery cycle (Figure 16).

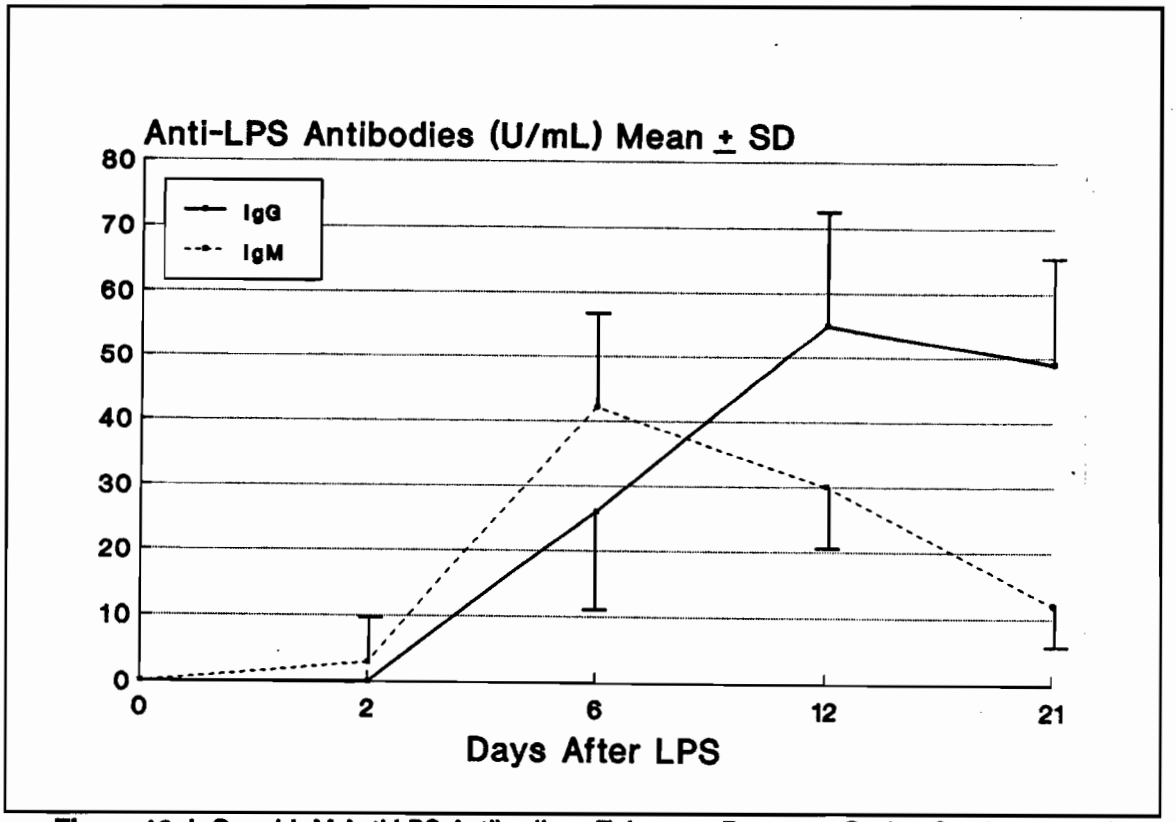

Figure 16. IgG and IgM Anti-LPS Antibodies. Tolerance-Recovery Cycle. See footnote of Figure 15.

IV. GENERATION OF ENDOTOXIN TOLERANCE

Having shown that tolerant animals were refractory to the effects of LPS in terms of lethality, weight loss and TNF production, a series of experiments were initiated to investigate the parameters for induction of tolerance.

The minimal dose of LPS able to induce tolerance was determined. Female F1 Low/Lewis rats were allocated to 6 groups and given 0.25, 0.5, 1, 2 or 4 mg/kg of LPS (priming treatment). Controls received saline solution. On day 3 all groups (3 rats per group) were challenged with 20 mg/kg LPS (LD_{50}). Body weight was measured daily.

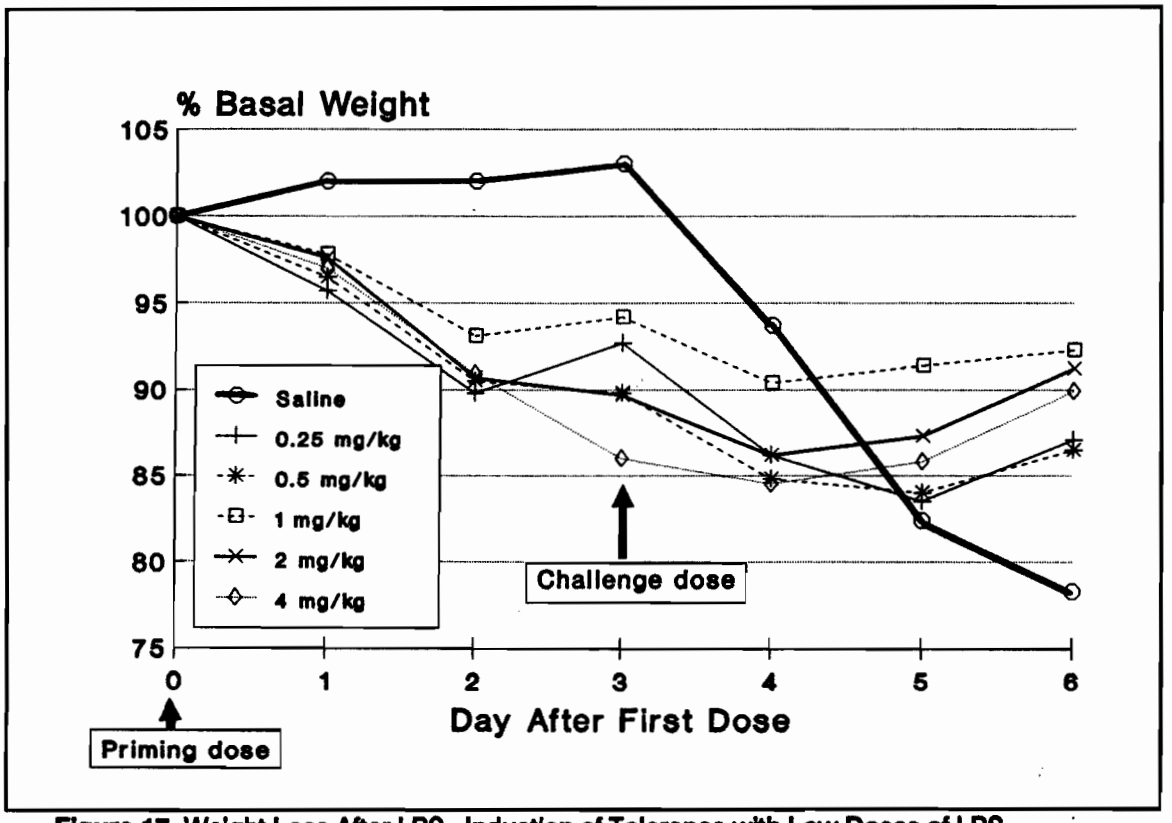

Figure 17. Weight Loss After LPS. Induction of Tolerance with Low Doses of LPS. Different priming doses of LPS (0.25, 0.5, 1, 2 or 4 mg/kg) were injected ip on Day 0 to various groups of naive rats (3 rats per group). Controls were primed with saline solution. On Day 3 after priming dose all groups were challenged with 20 mg/kg LPS. Only one animal survived challenge dose in the control group. All 3 animals in each of the other groups survived. Body weight was measured daily.

All 3 animals in each of the LPS primed groups survived the challenge with LPS but only one in the control group survived the challenge. Body weight loss on day 1 and 2 after the priming dose was similar for the 5 groups that received LPS, as opposed

to the control group in which a steady increase in body weight was seen as expected (Days 1, 2 and 3, Figure 17, $p < 0.001$ for all pre-treated groups compared to controls).

The effect in weight loss after challenge was similar for all groups that received LPS as shown in Figure 17 (Days 4, 5 and 6, differences not significant by ANOVA). The single survivor in the control group had a very important weight loss after challenge which continued on day 6 of the experiment whilst the other groups were regaining weight since Day 4 or 5 of the experiment (second and third day after challenge). These results show that a dose as low as 0.25 mg/kg can confer tolerance even if the challenge dose is within the lethal range. Differences on weight loss on Day 5 compared to body weight on day 3 are significant for all tolerant groups compared to controls ($p < 0.05$).

It was necessary to cover a broader range of low doses in order to reach sub-threshold levels both for the pre-treatment effect on body weight and for the protection conferred from a second challenge. We also wanted to measure serum TNF levels as another way to detect the effect of tolerance so a variant of this experiment was performed as follows.

On Day 0 different doses (5 mg; 0.5 mg; 0.05 mg; 5 μg; 0.5 μg of 0.05 μg per kg) of *E. coli* O127:B8 LPS were injected into male Wistar rats to evaluate induction of early LPS tolerance. A control group received saline (10 mL/kg). Two days later all groups were challenged with an LD_{100} of LPS (50 mg/kg). Body weight was measured daily. Blood samples were obtained 90 minutes after each dose of LPS for peak serum TNF measurement.

Percentage body weight differences between Day 2 and Day 0 are shown in black bars on Figure 18. The higher the priming dose given on Day 0 the more pronounced the weight loss induced on the first two days or the experiment and *vice versa* (ANOVA $p < 0.0001$). The two smallest priming doses (0.5 and 0.05 μg/kg) and the saline injection did not induce any weight loss.

The effect on body weight induced upon challenge (50 mg/kg LPS to all rats) (Day 4 minus Day 2 body weight) is represented in dashed bars in Figure 18. As shown, the higher the priming dose, the smaller the weight loss induced by challenge, or in

other words, the higher the priming dose the deeper the degree of tolerance induced (ANOVA p <0.005). Lethality after challenge was 100% in the 0.05 μg/kg LPS and saline groups (see Table 6) which reflects non tolerance. It is relevant that a dose as small as 0.5 μg/kg which did not provoke weight loss (nor detectable levels of TNF) was enough to induce tolerance to an LD_{100} dose of LPS.

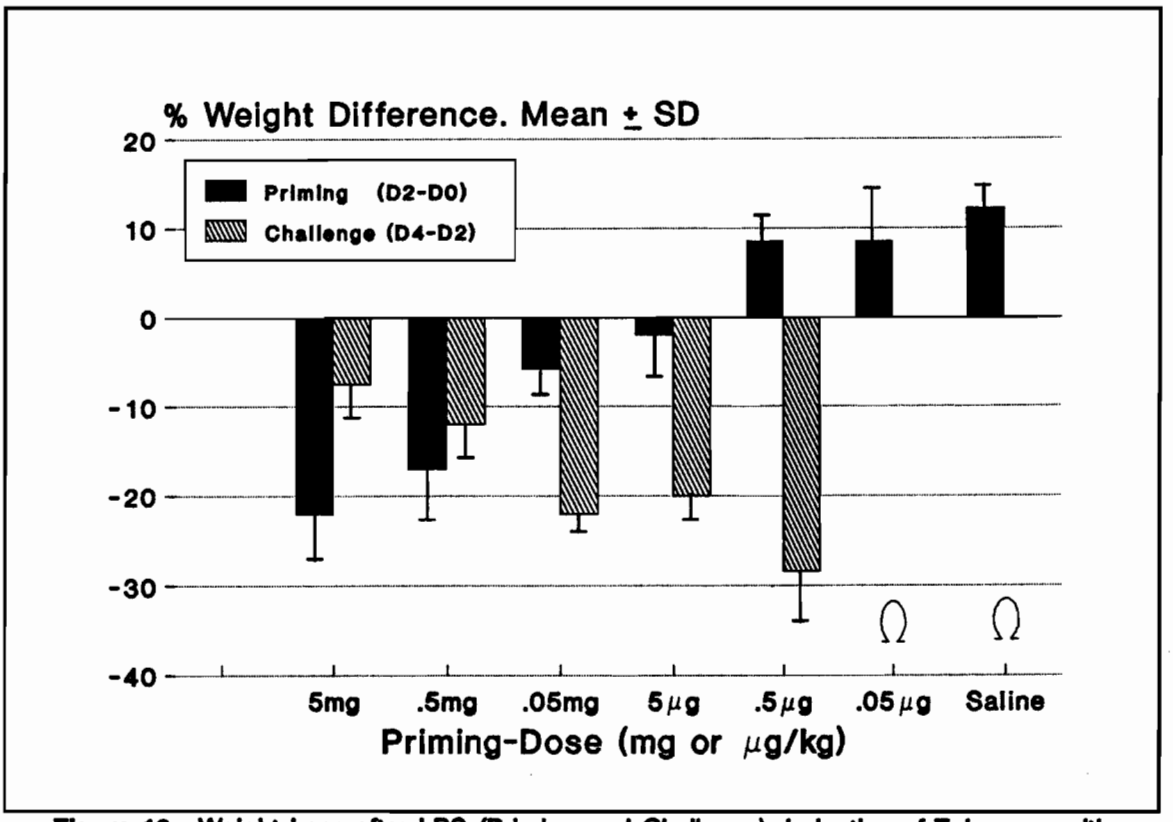

Figure 18. Weight Loss after LPS (Priming and Challenge). Induction of Tolerance with Low Doses of LPS. Doses of 5, 0.5, 0.05 mg/kg and 5, 0.5, 0.05 μg/kg of LPS were injected on Day 0 into naive rats (6 rats/group). Controls received saline. On Day 2 all groups were challenged with 50 mg/kg of LPS (LD_{100}). , All animals died after challenge in the controls and the lowest dose-primed group (Ω). Mean body weight differences between Day 2 and Day 0 (black bars = effect of priming dose) and between Day 4 and Day 2 (hatched bars = effect of challenge) are shown.

Peak serum TNF levels both after priming (black bars) and after challenge doses of LPS (dotted bars) are represented in Figure 19. Priming dose of 5 mg/kg of LPS induced the highest TNF levels (ANOVA p <0.005 considering only the doses that induced detectable levels of TNF). Peak TNF levels were progressively smaller as LPS doses diminished and no TNF was detected after an LPS dose of 0.5 μg/kg or less.

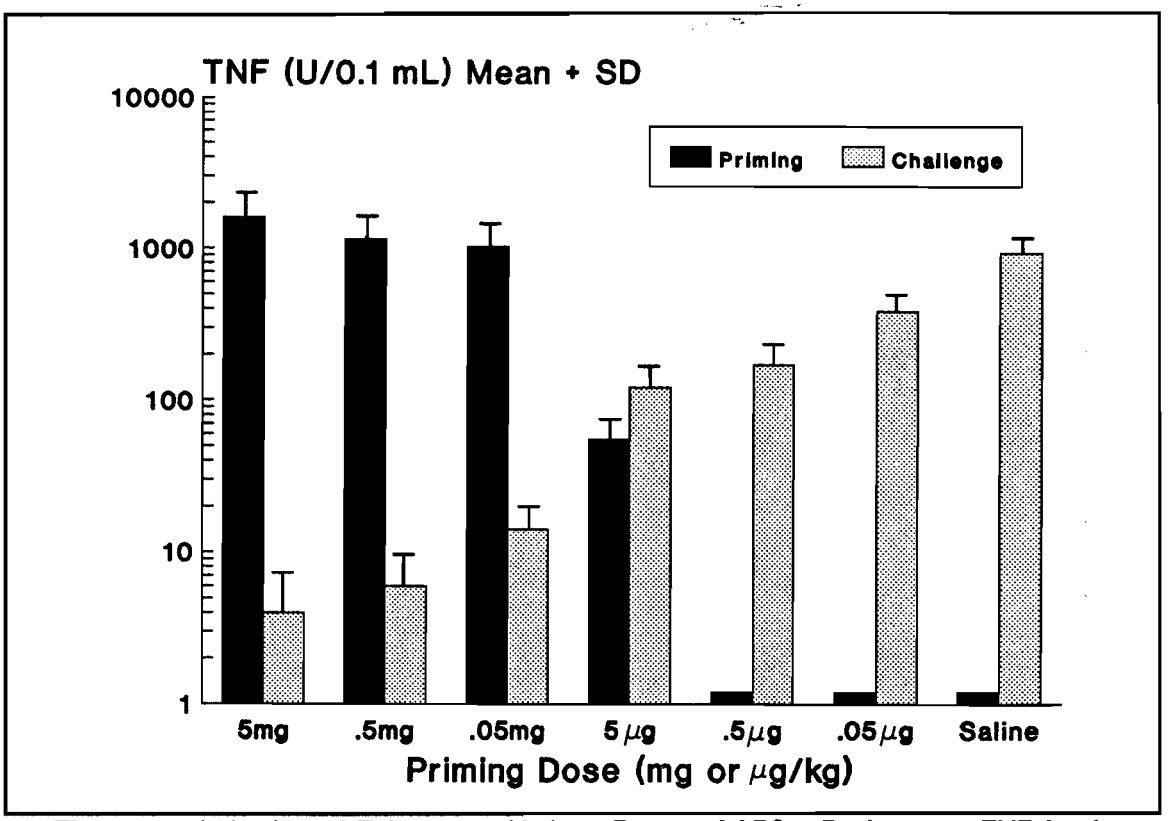

Figure 19. Induction of Tolerance with Low Doses of LPS. Peak serum TNF levels following priming dose (black columns) and following challenge injections (dotted bars) are represented. The priming doses given on Day 0 are shown on the ordinate. All rats were challenged with 50 mg/kg LPS on Day 2. See footnote of Figure 18

After challenge (50 mg/kg LPS), very low peak levels of TNF (< 10 U/0.1 mL) were induced in the 5 mg/kg primed group and progressively higher levels of TNF induced by the challenge were measured as the priming dose was smaller.

As mentioned before, no TNF was detected at 90 minutes after the priming dose in the group that received 0.05 μg/kg. Also, this dose did not induce tolerance to the lethal effect. Nevertheless, TNF levels after challenge were not as high as those induced in naive animals (380 ± 90 U/0.1 mL in the 0.05 μg/kg group v.s. 925 ± 170 U/0.1 mL in the group that received saline solution $p < 0.0001$) and yet this difference did not prevent death.

Peak TNF levels were higher after 5 mg/kg priming dose (1600 ± 280 U/0.1 mL) than in the saline group after a 50 mg/kg challenge dose (925 ± 170 U/0.1 mL) ($p < 0.005$) once again showing an inverse correlation between LPS dose an TNF levels within this range.

Table 6 shows percentage weight loss induced by the challenge in this same experiment. Day 2 body weight is considered the base-line weight and the difference between body weight on Day 4 minus Day 2 as a result of LPS challenge. Survival rates after challenge are also shown.

Table 6. Weight Loss and Survival After LPS (Challenge). Low Dose Tolerance

PRIMING	% WEIGHT LOSS AFTER CHALLENGE (D4-D2)	SURVIVAL/TOT.
5 mg/kg	7.5 ± 3.4	6/6
0.5 mg/kg	12 ± 2.3	6/6
0.05 mg/kg	22 ± 2	6/6
5 μg/kg	20.5 ± 2.8	6/6
0.5 μg/kg	28.4 ± 6.5	5/6
0.05 μg/kg LPS	death	0/6
Saline	death	0/6

The next question addressed was if continuous administration of LPS would prolong the duration of endotoxin tolerance beyond the duration of tolerance induced by a single bolus injection. Male Wistar rats were used in the following experiment. On Day 0 one group (Single Bolus) received a single ip dose of 5 mg/kg LPS *(E. coli* O127:B8). Osmotic minipumps loaded with 0.2 mL of 50 mg/mL LPS to be delivered over 7 days were implanted in the abdominal cavity of a second group of rats (Continuous infusion). The infusion rate was calculated at 7 mg/kg/day. Each group was then subdivided into 4 sub-groups (6 rats each) and challenged with 5 mg/kg LPS on Day 2, 4, 6 or 10 after the priming treatment. Blood samples were obtained 90 minutes after each ip dose (120 min after the implantation of the primed pumps) for TNF levels and body weight was also recorded.

TNF levels are represented on Figure 20. As in a previous experiment (Figure 10), this time higher levels of TNF were also induced after the bolus injection (black columns) than after implantation of the pumps (dashed bars) (Day 0 $p < 0.0001$). Upon challenge on Day 2 both groups produced very low peak levels of TNF although somewhat lower levels were evident in the continuous infusion group ($p < 0.05$). Recovery of TNF production became evident as early as Day 4 in the single

bolus group whereas in the continuous infusion group the state of unresponsiveness was sustained as long as there was infusion of LPS (significance between group differences: Day 4 $p < 0.001$, Day 6 $p < 0.0001$). By Day 10 there was some recovery of TNF production in the infusion group although still low compared with the bolus group ($p < 0.0001$). Blood samples were obtained before each LPS challenge in both groups and undetectable levels of TNF were obtained every time (data not shown).

Figure 21 shows results of maximal weight loss induced by the priming (Day 0) and the challenge treatment (Days 2, 4, 6 or 10) in the same previous experiment. Weights were very consistent (Bolus group = 216.6 ± 10.2 g; Infusion group = 218.5 ± 15.6 g) so the results are expressed as maximal weight loss in grams. Figures correspond to 48 hours weight difference after pre-treatment and 24 hours after challenge which corresponded to the maximal difference. The differences in weight loss between groups were not significant after pre-treatment (Day 0) nor after challenge on Day 2: both groups loss over 25 grams upon priming and between 5 and 10 grams upon challenge on day 2. Thereafter, the single bolus group had a maximal average loss of 3 grams after challenge on Day 4 and 10.3 g on day 6, the continuous infusion group had an average gain of 3 g on day 4 and a gain of 5 g on day 6 demonstrating a lengthened state of tolerance ($p < 0.005$). By day 10 after the pumps were exhausted and no LPS had been infused for 3 days, the animals were again sensitive to LPS challenge showing a weight loss, but less than that seen with the bolus group ($p < 0.0001$).

These results may have relevance in the clinical setting where repeated bolus of LPS are believed to occur in gram-negative infections.

EFFECTS OF UNRELATED TYPES OF LPS DURING EARLY ENDOTOXIN TOLERANCE

The next objective was to determine if tolerance induced with one type O-specific LPS would prevent the effects (including mortality and TNF production) triggered by a different type O-specific LPS. The following experiments were carried on to answer this question.

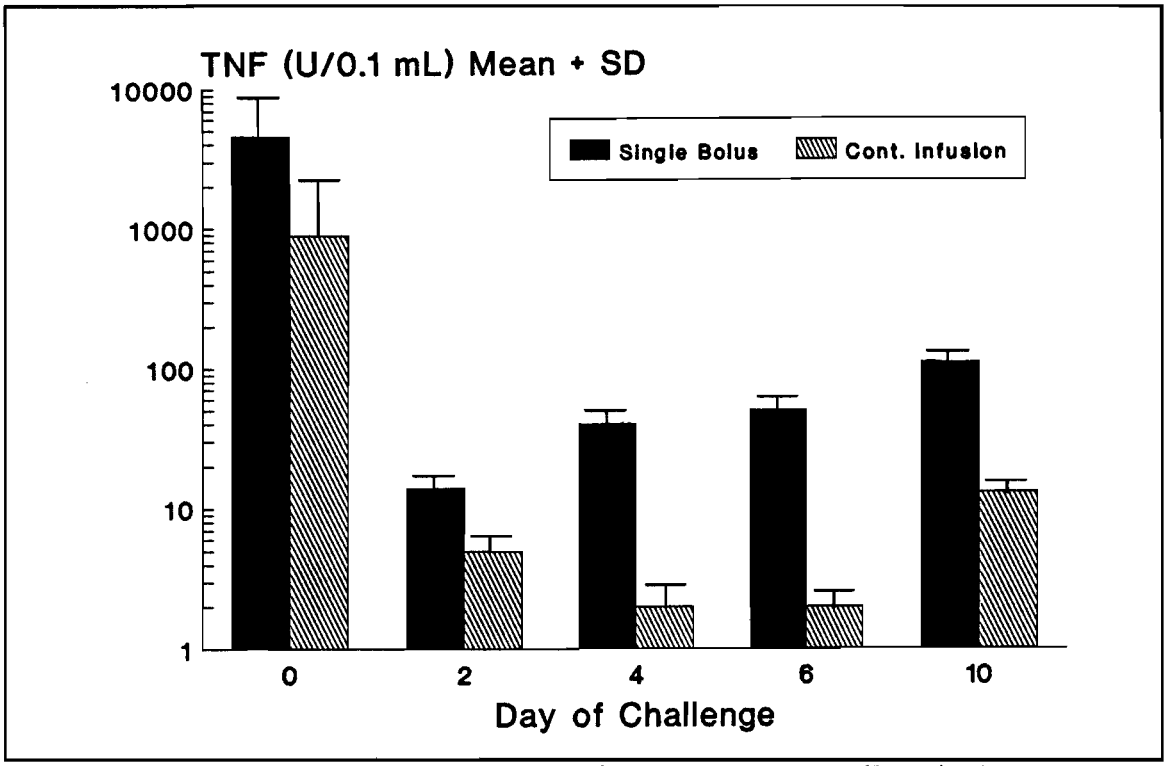

Figure 20. Peak Serum TNF Levels After LPS. Rats were primed (Day 0) with a single bolus injection (black bars) or by continuous infusion of LPS over 7 days (hatched bars). Animals were split into 4 sub-groups and challenged with 5 mg/kg LPS on Days 2, 4, 6 or 10. Peak serum TNF levels after priming dose and after each challenge dose are shown.

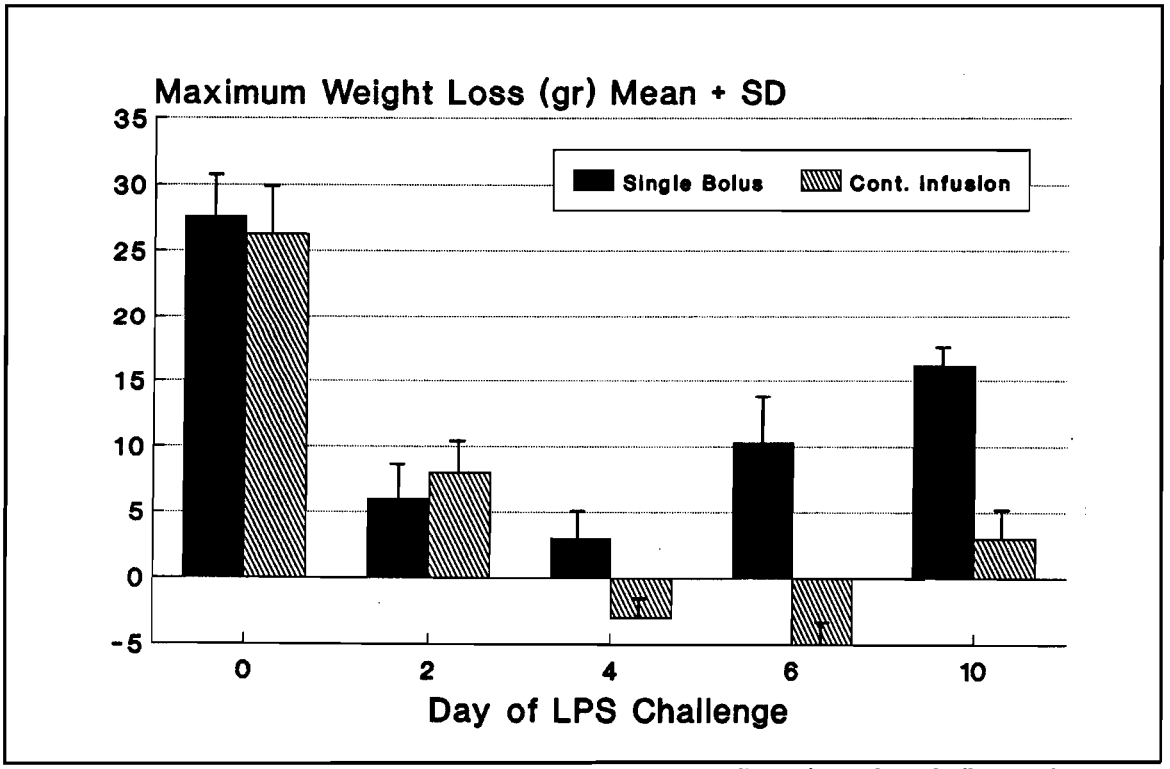

Figure 21. Maximal body weight loss after priming dose (Day 0) or after challenge doses (all others) of LPS are shown (See previous footnote).

I. CONTROL EXPERIMENTS. LETHALITY AND TNF PRODUCTION

A dose/response curve both for lethality and TNF production was first established for each type of LPS. Male Wistar rats (150-220 g body weight) were injected with LPS from different species of the gram-negative bacteria *E. coli* O127:B8, *E. coli* O111:B4, *E. coli* O26:B6, *P. aeruginosa*, and *S. typhosa* and were bled 90 minutes after LPS to measure peak serum TNF, and were monitored for mortality. Rats were initially injected with 6.6 or 20 mg/kg of each type of LPS. Other dose groups were included depending upon the mortality observed for each class of LPS.

The final doses injected (4 rats per group) were:

E. coli O127:B8, 6.6, 13, and 20 mg/kg.
E. coli O26:B6, 6.6, 20, 50, and 100 mg/kg.
E. coli O111:B4, 6.6, 20, and 50 mg/kg.
P. aeruginosa, 6.6, 20, and 50 mg/kg.
S. typhosa, 6.6, 13, and 20 mg/kg.

Survival rates are depicted on Figure 23. From the survival rates a LD_{50} and LD_{100} were calculated for each type of LPS by regression analysis (Table 7).

Table 7. Relative Lethal Potency of LPS from Different Bacteria

TYPE OF LPS	LD_{50}	LD_{100}
E. coli O127:B8	11.1	21
S. typhosa	11.1	21
E. coli O111:B4	14.2	33.8
P. aeruginosa	24.3	45.5
E. coli O26:B6	>100	>100

Potency was highest and identical for *E. coli* O127:B8 and *S. typhosa* LPS. Lethal potency was somewhat less for *E. coli* O111:B4 and *P. aeruginosa*. While the LPS from *E. coli* O26:B6 was not lethal even at the highest dose employed (100 mg/kg).

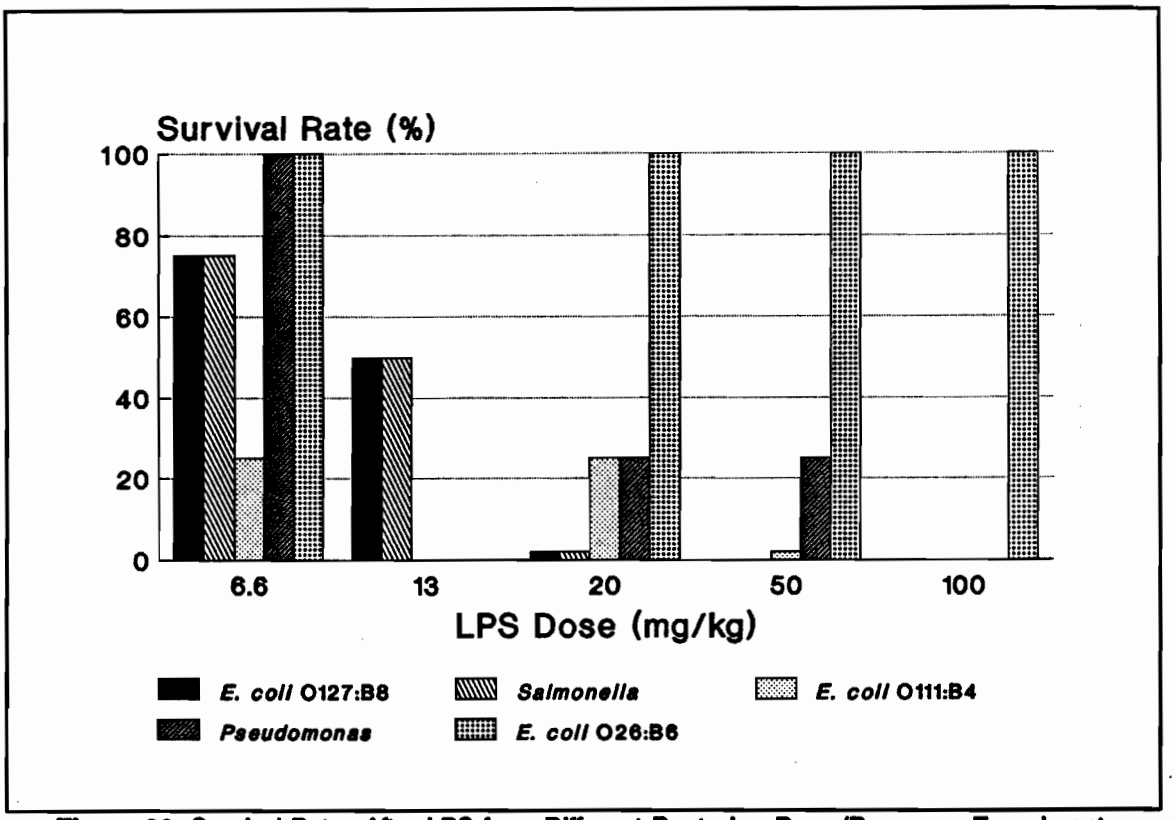

Figure 22. Survival Rates After LPS from Different Bacteria. Dose/Response Experiment. Rats were injected with 6.6 or 20 mg/kg LPS from the different species of gram-negative bacteria shown. Other doses (13, 50 or 100 mg/kg) were included depending upon the mortality observed in order to estimate the LD_{50} and LD_{100} for each type of LPS.

The peak TNF levels (Figure 23) induced by a particular dose of all types of LPS were correlated and were not significantly different in every case (ANOVA). A general tendency to induce diminished peak levels of TNF with doses larger than 20 mg/kg was clear, confirming the impression suggested in Figure 11 although the difference in peak TNF levels induced by the same type of LPS using different doses were statistically significant only for *E. coli* O26:B6 LPS (ANOVA $p = 0.0016$). Once more, no correlation between LPS dose, mortality and peak TNF serum levels was found. Even though the toxic effect of *E. coli* O26:B6 LPS was so different from the others, serum TNF peak levels at equivalent doses were similar to those induced with the more toxic compounds (Not significant at 6.6, 20 or 50 mg/kg ANOVA).

II. SPECIFICITY OF TOLERANCE

The specificity of tolerance induction was then investigated; tolerance was induced with one type of LPS and then challenged with an unrelated class of LPS. A highly

toxic (*E. coli* O127:B8) or the low-toxicity type of LPS (*E. coli* O26:B6) were used to induce tolerance.

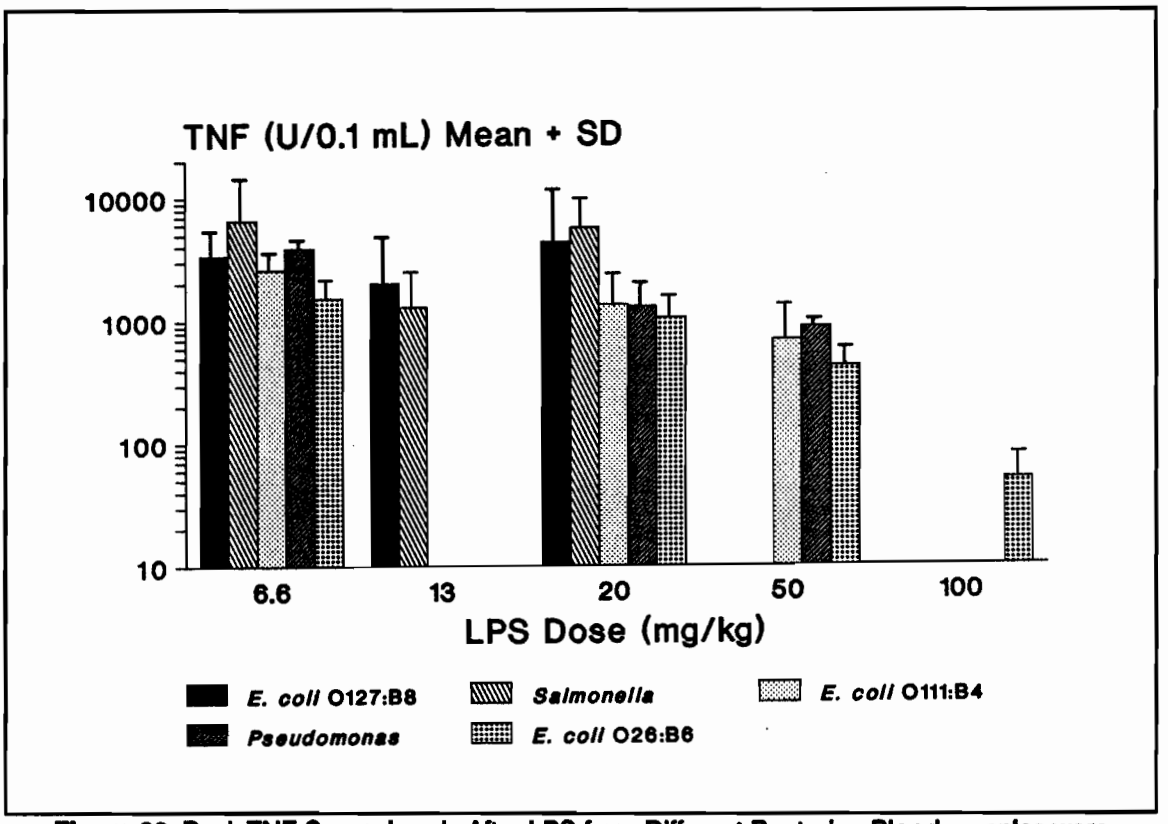

Figure 23. Peak TNF Serum Levels After LPS from Different Bacteria. Blood samples were obtained 90 minutes after injection of the doses of LPS indicated. TNF was measured in the samples as shown.

For the toxic LPS, early tolerance was induced on male Wistar rats with two injections of 4 mg/kg *E. coli* O127:B8 LPS on Day 0 and Day 2. On day 4 animals were challenged with a 100% lethal dose of different types of LPS as follows: *E. coli* O127:B8 (30 mg/kg); *E. coli* O111:B4 (50 mg/kg); *S. typhi* (30 mg/kg) *or P. aeruginosa* (70 mg/kg). Blood samples were obtained in every case 90 minutes after the LPS injection. For the low-toxicity type of LPS (*E. coli* O26:B6), three different schedules of tolerance induction were used: two doses of 10 mg/kg on Day 0 and Day 2; two doses of 25 mg/kg on Day 0 and Day 2, or a single dose of 50 mg/kg on Day 2. On Day 4 all rats were challenged with an LD_{100} of 30 mg/kg of *E. coli* O127:B8 LPS. Table 8 shows the protocols and survival rates. Figures 24 and 25 show peak TNF serum levels.

The results demonstrate that as expected, early LPS tolerance is not specific for the O-antigen of the preparation used to induce tolerance, both in terms of susceptibility to the lethal effect and also for TNF production. As shown in Table 8, tolerance generated by the toxic LPS O127:B8 resulted in protection for all types of LPS. In spite of its low toxicity, *E. coli* O26:B6 LPS was able to induce tolerance to a lethal challenge with the toxic preparation *E. coli* O127:B8 by all 3 different schedules used.

Table 8. Protocol and Survival Rate. Challenge with Unrelated LPS During Early Tolerance

PRE-TREATMENT	CHALLENGE (DAY 4)	SURVIVAL/TOTAL
O127:B8 4 mg/kg	O127:B8 30 mg/kg	8/8
2 doses (D0 & D2)	O111:B4 50 mg/kg	7/8
	Salmonella 30 mg/kg	8/8
	Pseudomonas 70 mg/kg	8/8
O26:B6 10 mg/kg	O127:B8 30 mg/kg	7/8
2 doses (D0 & D2)		
O26:B6 25 mg/kg	O127:B8 30 mg/kg	7/8
2 doses (D0 & D2)		
O26:B6 50 mg/kg	O127:B8 30 mg/kg	4/4
1 dose (D2)		

When *E. coli* O127:B8 LPS was used to induce tolerance, peak levels of TNF induced by the challenge with unrelated types of LPS were actually lower ($p < 0.0001$) than the levels induced by the same LPS (Figure 24). Difference between TNF levels induced by *Salmonella, Pseudomonas* of *E. coli* O111:B4 were non significant (ANOVA). When tolerance was induced with *E. coli* O26:B6 LPS however peak TNF levels were only slightly diminished upon challenge at day 4 with the toxic heterologous LPS although very high protection from lethality was

conferred as 7 out of 8 rats (87%) survived the LD_{100}. It was also observed that when priming with two doses of 10 mg/kg LPS were the challenge induced higher levels of TNF ($p < 0.0001$) implying less efficient tolerance induction than the degree of tolerance induced by two doses of 25 mg/kg LPS. Peak TNF levels after challenge with O127:B8 were significantly higher when priming was done with the low-toxicity type of LPS ($p < 0.01$ ANOVA) than the levels achieved after the same challenge when tolerance was induced by the more toxic compound (*E. coli* O127:B8 LPS).

Serum TNF was not measured in the last protocol in which tolerance was induced by a single dose of 50 mg/kg of O26:B6 LPS which was also shown to be protective (Table 8).

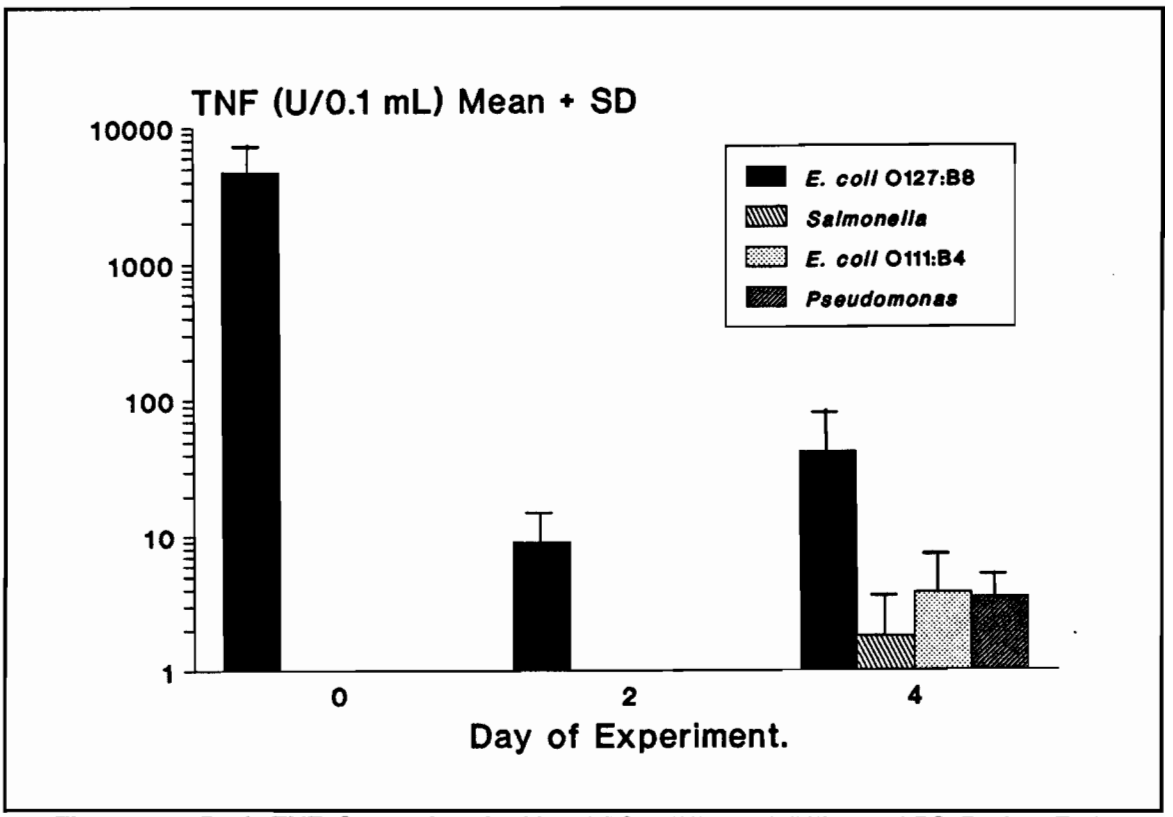

Figure 24. Peak TNF Serum Levels After LPS. Effect of Different LPS During Early Tolerance after priming with toxic LPS. LPS tolerance was induced with two doses of 4 mg/kg *E. coli* O127:B8 LPS on Day 0 and Day 2. On Day 4 animals were challenged with an LD_{100} dose of different types of LPS, *E. coli* O127:B8 (30 mg/kg), *S. typhi* (30 mg/kg), *E. coli* O111:B4 (50 mg/kg) and *P. aeruginosa* (70 mg/kg). Mean peak serum TNF levels after each dose are shown.

production of the post-tolerant state. The purpose of these experiments was to investigate if after recovery of sensitivity from early endotoxin tolerance, animals were as responsive to various effects of LPS administration as their naive counterparts.

I. LETHALITY.

The susceptibility of naive and post-tolerant rats to LPS was first investigated. A dose/response curve for mortality with *E. coli* O127:B8 LPS of naive and post-tolerant rats was generated. Groups of 8-10 rats (post-tolerant) were pre-treated with a single dose of LPS (5 mg/kg) and 21 days later these and age matched naive rats received 1, 5, 10, 25, 40 or 60 mg/kg LPS. Survival rates at 72 hours for both groups were compared.

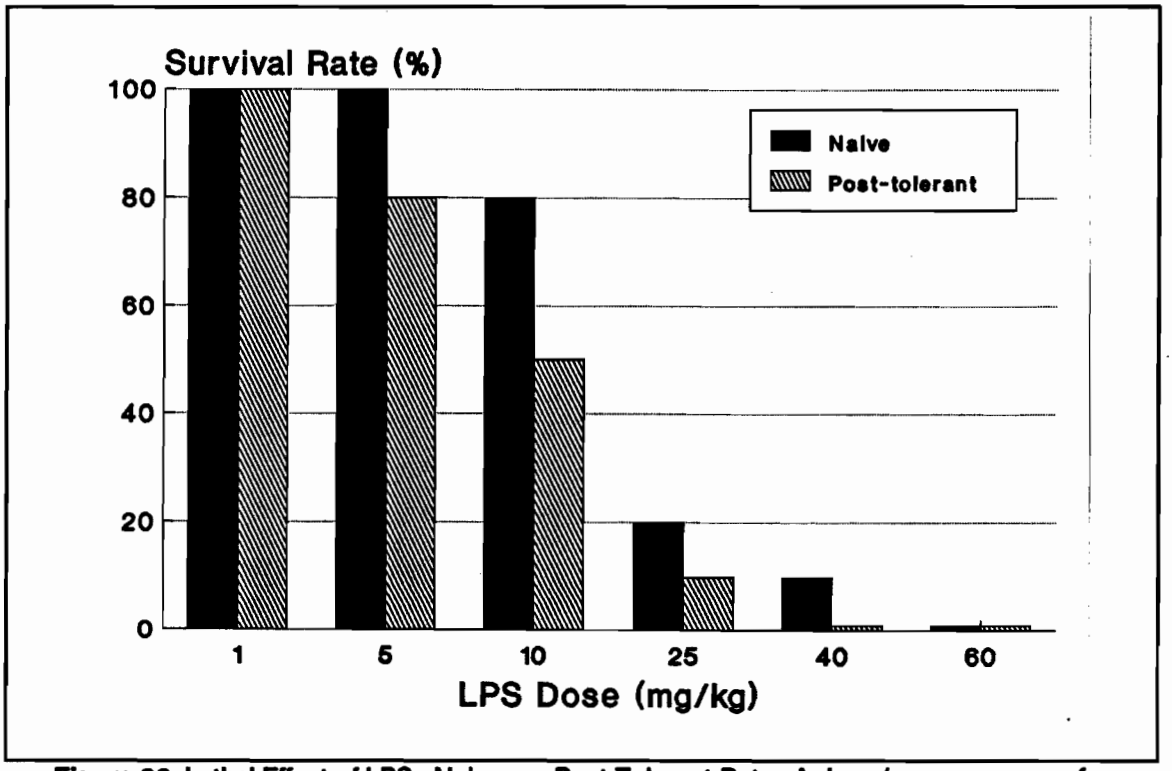

Figure 26. Lethal Effect of LPS. Naive v.s. Post-Tolerant Rats. A dose/response curve for *E. coli* O127:B8 LPS lethal effect was generated. Post-tolerant rats were pre-treated with a single dose of 5 mg/kg on day -21. On Day 0 naive and post-tolerant rats were injected with 1, 5, 10, 25, 40 or 60 mg/kg LPS. Survival rate at 72 hours are compared.

As shown in Figure 27 minimal differences in the survival rate between naive and post-tolerant rats were found in each dose group so it was concluded that recovery of sensitivity to this effect of LPS was complete (LD_{50}: Naive = 24.2 mg/kg, Post-

tolerant 18.8 mg/kg) and that "late endotoxin tolerance" induced by a single dose of LPS 21 days before did not affect lethality induced by LPS.

II. WEIGHT LOSS

The next step was to establish the body wasting effect of LPS during the late phase of endotoxin tolerance. Six male F1 Lou/Lewis rats were given single doses of 5 mg/kg *E. coli* O127:B8 LPS on days 0, 21, 42 and 63. Weight loss was registered for 6 days after each challenge. Results represented in Figure 27 show that the first exposure induced a maximal weight loss of 16% by day 2. This amount of weight loss was not reproduced with subsequent challenges with a maximal loss of 10% induced in every case ($p < 0.0001$ ANOVA). Full recovery of sensitivity to this effect did not seem to occur demonstrating a state of relative insensitivity to the body wasting effect of LPS compatible with "late" endotoxin tolerance ($p < 0.05$ on day 2, ANOVA).

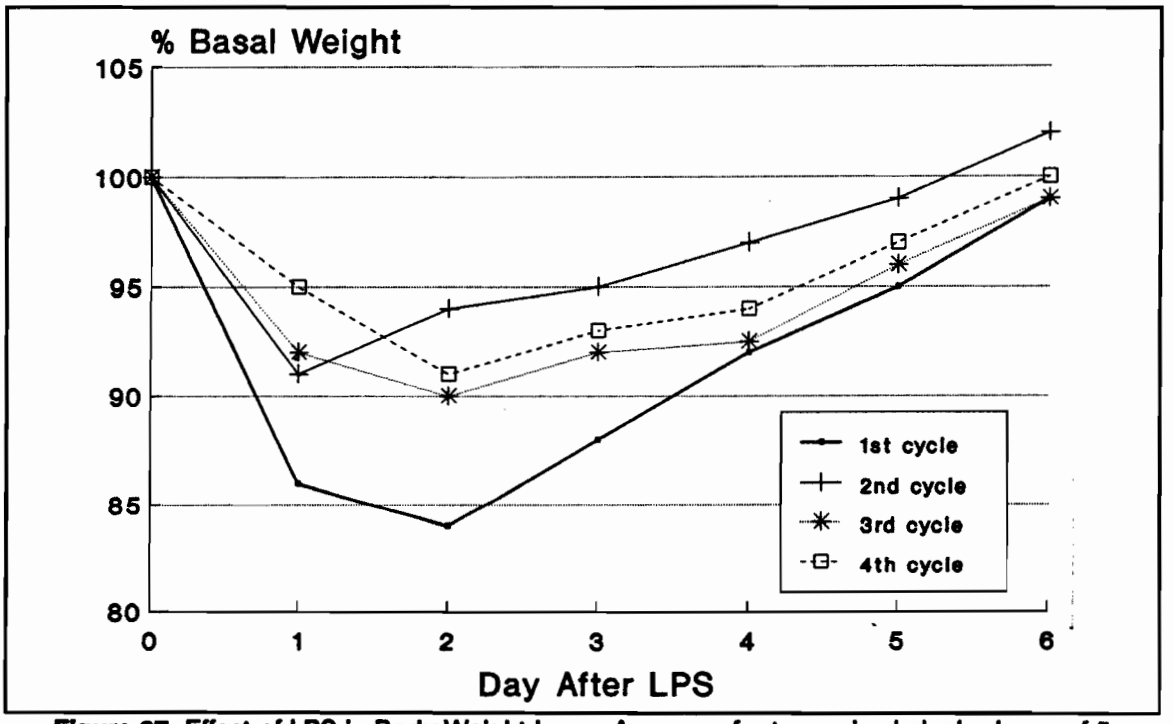

Figure 27. Effect of LPS in Body Weight Loss. A group of rats received single doses of 5 mg/kg *E. coli* O127:B8 LPS on four occasions 21 days apart. Weight loss was registered after each challenge (the day of each challenge is considered Day 0 for that cycle).

The levels of TNF produced in the post-tolerant phase and its correlation with anti-LPS antibodies was then investigated. Lethality and weight loss were also measured to confirm the previous experiments. Male F1 Lou/Lewis rats received 5 mg/kg

LPS on day 0, 21 and 43. On day 66 these post-tolerant rats and age matched naive controls were challenged with LPS at doses of 0.5, 5, 20 or 50 mg/kg of LPS. The survival and total animals per group are shown in Table 9. In this experiment, after 3 cycles of LPS, unlike the previous after only one challenge, the animals were less sensitive to LPS as compared to the naive rats. The relative lethal susceptibility was calculated from the results in Table 8 and the LD_{50} for the naive rats was 18.8 mg/kg and the LD_{100} 35 mg/kg compared to 35.3 mg/kg and 68.3 mg/kg for the post-tolerant animals. Although not striking, there is a clear difference in sensitivity ($p < 0.01$ by X^2 with continuity correction at the 20 mg/kg dose).

Table 9. Survival Rate After LPS. Naive v.s. Post-Tolerant Rats

LPS DOSE	NAIVE		POST-TOLERANT	
(mg/kg)	Survival/tot.	(%)	Survival/totals	(%)
005	6/6	(100)	6/6	(100)
0.5	6/6	(100)	6/6	(100)
5	6/6	(100)	8/8	(100)
20	0/6	(0)	5/7	(71.4)
50	0/6	(0)	2/7	(28.5)

As in the previous experiment, there was only partial recovery of sensitivity to the body wasting effect (Figure 28). At every dose tested, weight loss in the post-tolerant group occurred but it was always greater after the challenge in naive than in post-tolerant rats. In this particular experiment a persistent state of relative insensitivity compatible with late endotoxin tolerance is seen at least during the time span tested.

III. PRODUCTION OF TNF

The impact of LPS tolerance on peak TNF serum levels is shown in Figures 29 and 30. There was a decreasing level of peak serum TNF after each of the 5 mg/kg/dose injections of LPS administered on Days 0, 21, 43 and 66. That is, peak levels obtained after the first dose were never reached again ($p < 0.0001$ ANOVA) with a drop in peak serum TNF levels after successive challenges. By regression analysis the tendency has an $r = -0.9493$ (for the log of TNF value) with a $p = 0.05$.

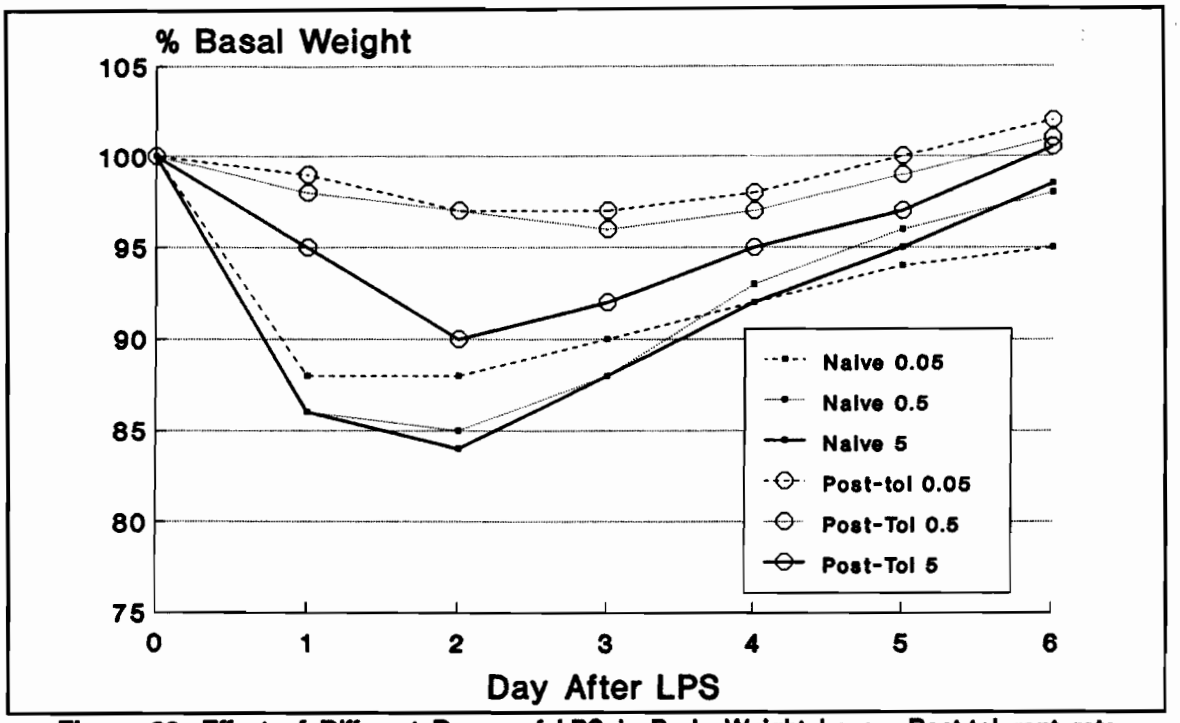

Figure 28. Effect of Different Doses of LPS in Body Weight Loss. Post-tolerant rats received three doses of 5 mg/kg LPS 21 days apart to induce late LPS tolerance. On Day 0 (21 days after the third dose) post-tolerant rats and another group of naive rats received single doses of 0.05, 0.5 or 5 mg/kg LPS. Body weight was daily measured after challenge.

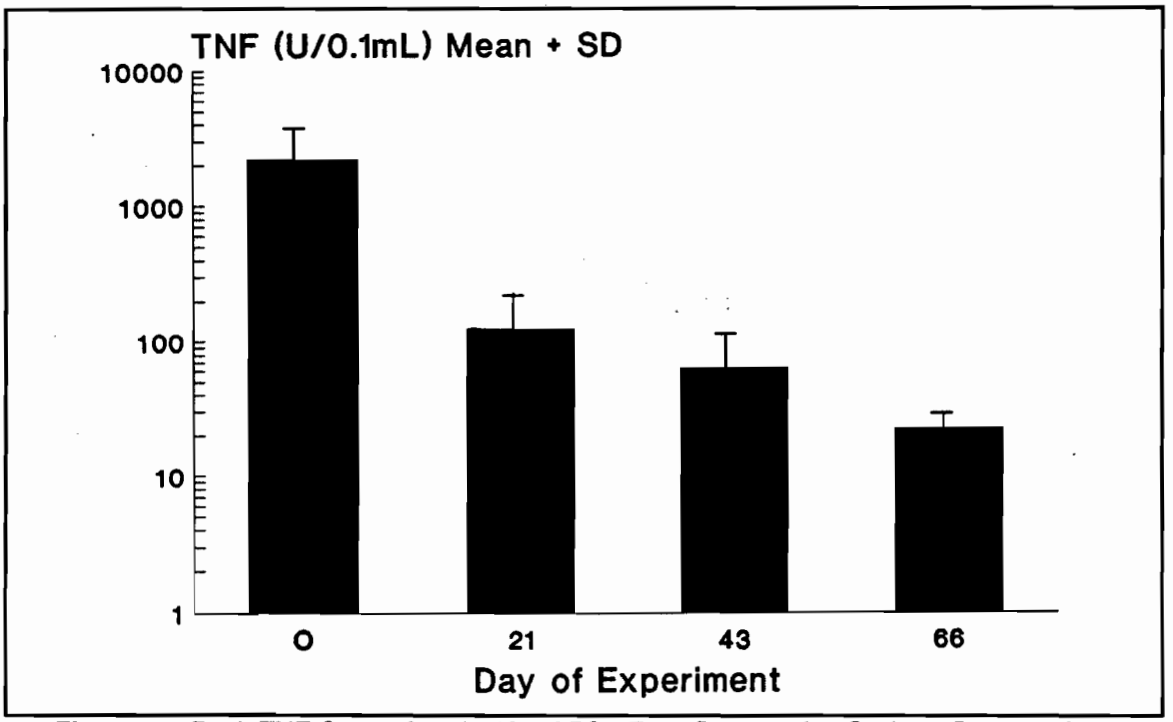

Figure 29. Peak TNF Serum Levels After LPS. Four Consecutive Cycles. Rats received repeated single doses of 5 mg/kg LPS on four cycles 21 days apart. TNF serum levels after each challenge are shown.

The ability of the different doses of LPS to elicit TNF production after the 4th cycle in comparison to the naive animals is shown in Figure 30. At the lower doses of LPS, as seen in previous experiments, only low levels of TNF are produced in comparison to naive animals ($p < 0.001$). However, unlike the naive animals, increasing doses of LPS elicited increasing levels of TNF. At 50 mg/kg the levels in naive and post-tolerant rats were not significantly different. Also the "tolerant" animals had a good correlation between the dose of LPS and peak TNF serum levels, even using lethal doses ($r = 0.9924$ with $p < 0.01$).

In the naive rats, after a low dose of LPS (0.5 mg/kg) over 4300 units of TNF were detected but no lethality in this group. In contrast, in the post-tolerant group, 50 mg/kg LPS resulted in just an average of 300 units of TNF but over 70% of the animals died.

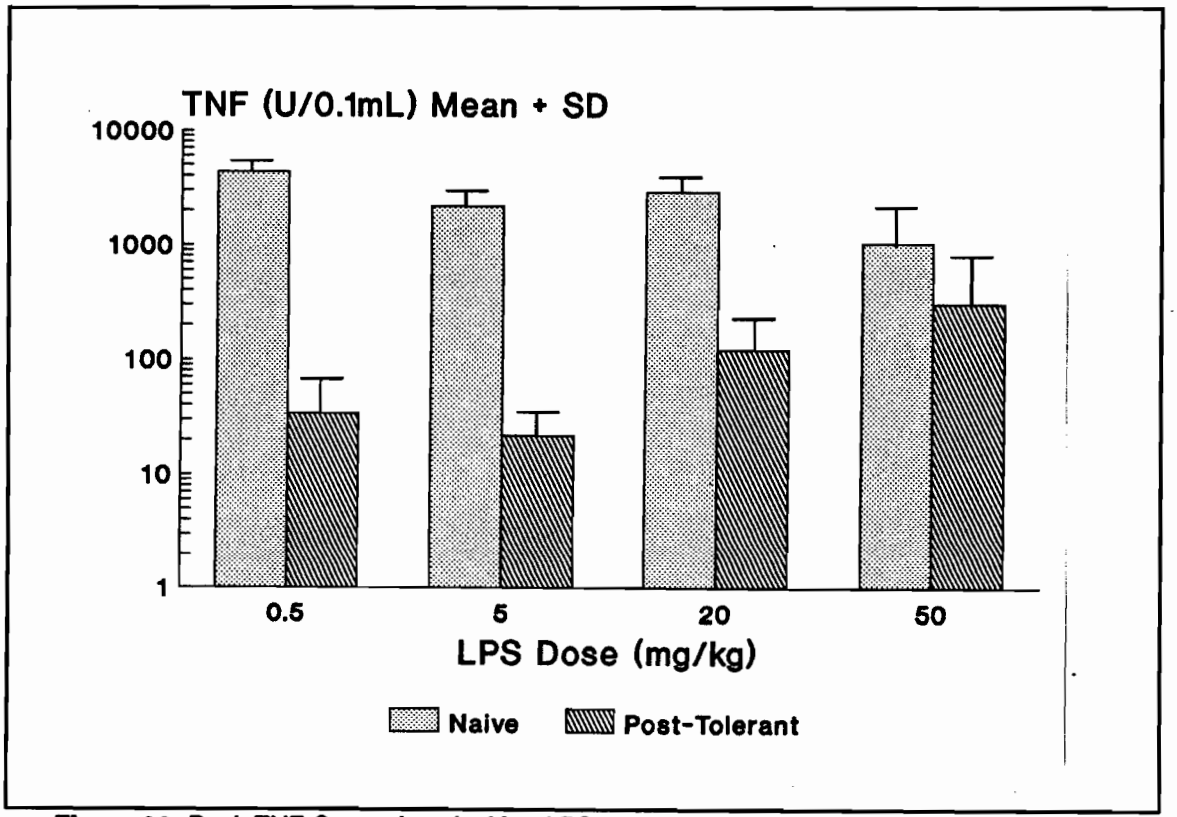

Figure 30. Peak TNF Serum Levels After LPS. Naive v.s. Post-tolerant Rats. A group of naive rats received different single doses of LPS (0.5, 5, 20 or 50 mg/kg). Another group of rats (late-tolerant) received three doses of 5 mg/kg LPS on cycles 21 days apart to induce late LPS tolerance. Twenty one days after the third dose this late-tolerant group was challenged with 0.5, 5, 20 or 50 mg/kg LPS. Peak serum TNF levels were measured in both naive and late-tolerant groups after the challenge dose. Matching survival rates are shown in Table 5. *TNF levels after 0.05 mg/kg in the naive group were not measured and in the late-tolerant were not detectable.

IV. ANTI-LPS ANTIBODY LEVELS

The levels of anti-LPS antibodies present in the serum measured at time of challenge is shown in Figure 31. As expected, IgG and IgM anti-LPS antibody levels constantly increase after each of the 4 cycles. This correlates with the decreasing peak TNF levels. The relationship between Anti-LPS antibody titers and serum TNF levels is plotted (Figure 32), the regression line (r = -.6764 for IgG, $p <$ 0.0001) indicates a correlation between anti-LPS antibody titers and serum TNF levels, thus the ability of the increasing amount of LPS to elicit increasing levels of TNF may be related to antibody neutralizing LPS.

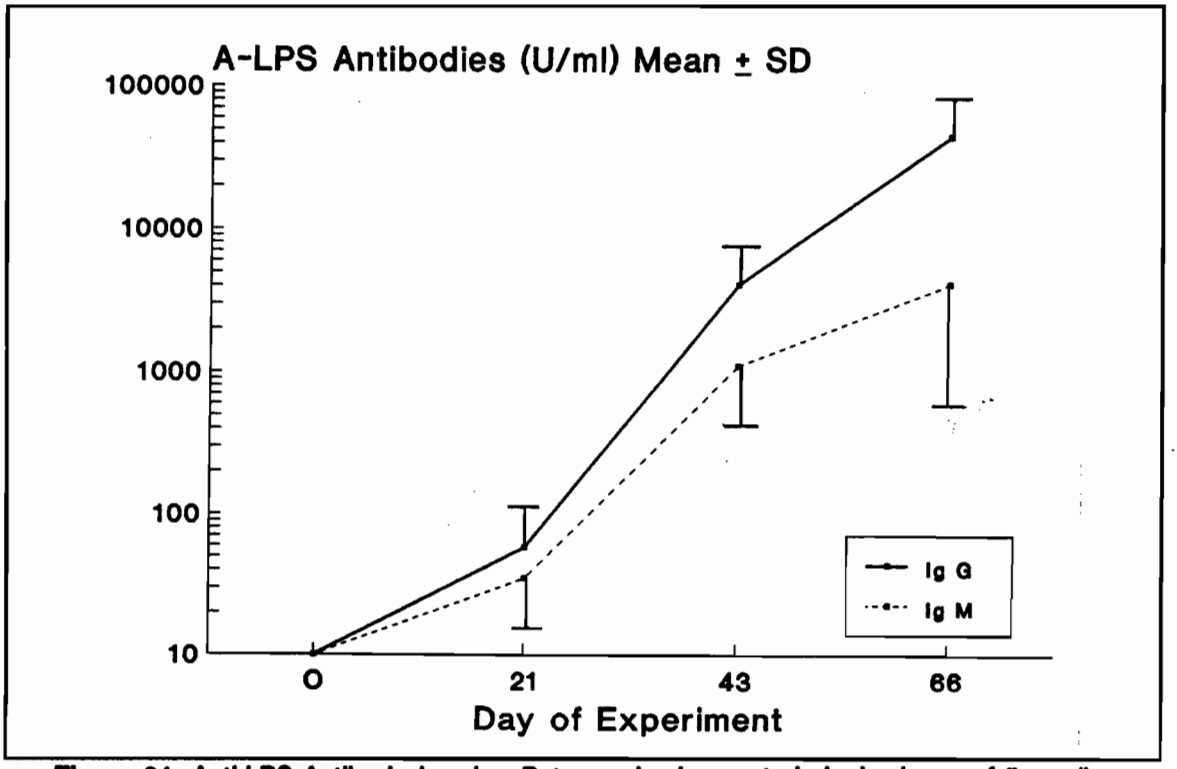

Figure 31. Anti-LPS Antibody Levels. Rats received repeated single doses of 5 mg/kg LPS on four cycles 21 days apart. IgG and IgM anti-LPS antibodies after each dose of LPS are shown.

The high correlation between increasing anti-LPS antibodies and diminishing peak serum levels of TNF after challenge suggests that the tolerance should be specific for LPS; it was important to know if it was specific for either the O-antigen, lipid A or both. If the low production of TNF seen in late tolerance is dependent on the presence of O-specific antibodies which could be the case when the same type of

LPS used for priming was used as challenge, then a challenge with an unrelated type of LPS should induce TNF levels as high as those induced in naive rats.

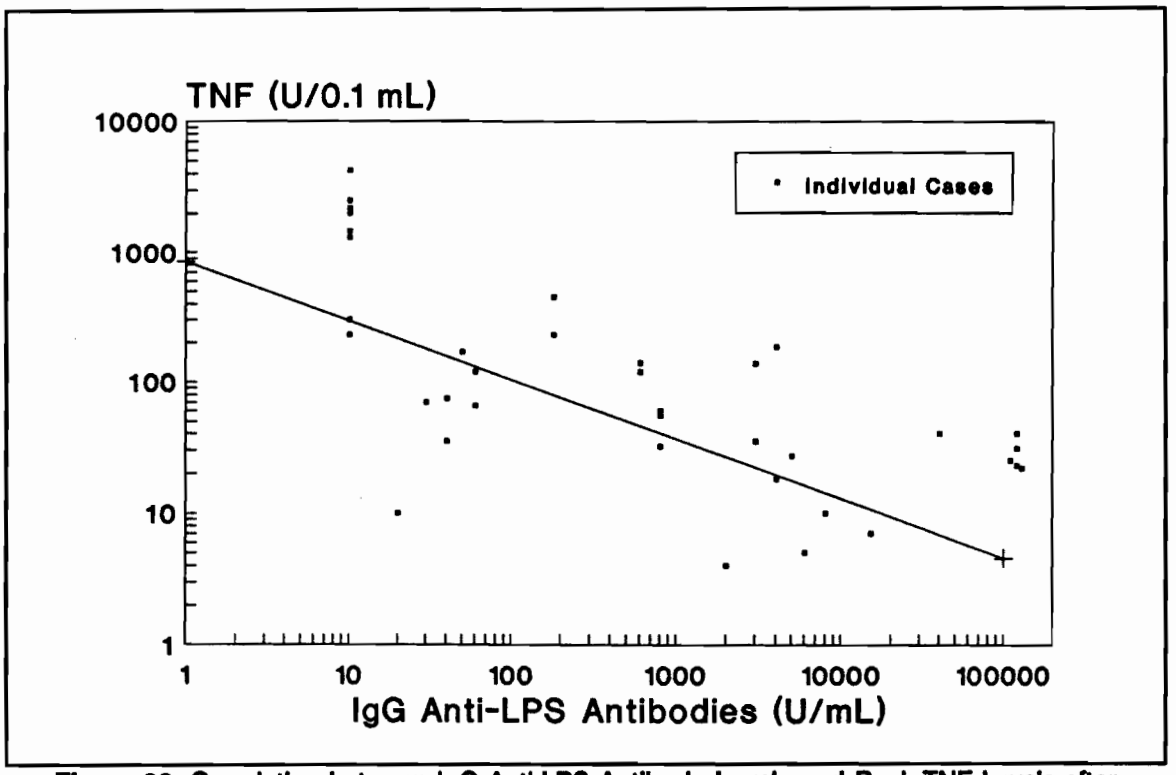

Figure 32. Correlation between IgG Anti-LPS Antibody Levels and Peak TNF Levels after Challenge during Late LPS Tolerance. Regression line (Log) included.

RESPONSE TO DIFFERENT TYPE-O LPS DURING LATE ENDOTOXIN TOLERANCE

The question of specificity was tested with the following experiment. Late endotoxin tolerance was induced with one type of LPS, then LPS from the same or a different gram-negative bacteria was used as challenge. Late-tolerant male Wistar rats received a priming dose of 3 mg/kg *E. coli* O127:B8 LPS on days 0, 23, and 46. Aged matched naive rats were used as controls. On Day 72, both groups were assigned to 4 sub-groups each; the tolerant sub-groups were I through IV and the naive ones V through VIII. Animals were challenged with (a) 25 mg/kg (LD_{100}) or (b) 0.5 mg/kg (non-lethal dose) of *E. coli* O127:B8 or *S. typhosa* LPS. Blood samples were obtained 90 minutes after the challenge dose of LPS and TNF and IgG and IgM anti-*E. coli* LPS and anti-*Salmonella* LPS antibodies were measured. Survival and body weight were monitored. Table 10 shows the type and dose of LPS

used as challenge, the presence (+) or absence (-) of tolerance and the outcome of each sub-group.

The tolerant rats had at best only a weak diminished sensitivity to the lethal effect of LPS both when the same type of LPS (G-I and V) or when an unrelated one were injected (G-III and VII) (X^2 = not significant). Peak serum TNF levels after the challenge (Figure 33) were always higher in the naive than in the tolerant groups ($p < 0.001$). This is particularly striking when *S. typhi* LPS was used to challenge *E. coli* LPS primed rats (Groups III and IV) in clear contradiction with the traditional concept of O-specific dependence for late LPS tolerance.

Table 10. Protocol and Survival Rate. Challenge with Unrelated LPS During Late Tolerance

GROUP	LPS TYPE	TOLERANT	DOSE (mg/kg)	SURVIVE/TOT
I	*E. coli*	+	25	1/7
V	*E. coli*	-	25	0/7
III	*S. typhi*	+	25	2/7
VII	*S. typhi*	-	25	1/7
II	*E. coli*	+	0.5	6/6
VI	*E. coli*	-	0.5	5/6
IV	*S. typhi*	+	0.5	6/6
VIII	*S. typhi*	-	0.5	6/6

As in other experiments, peak TNF serum levels were higher in the tolerant groups after the larger dose of LPS (25 mg/kg) than those induced with a lower dose (0.5 mg/kg Groups I, II, III and IV; $p < 0.001$ ANOVA). No statistically significant differences were found between peak TNF levels induced with either doses or either types of LPS in the naive rats (Groups V, VI, VII and VIII; ANOVA).

Figure 34 shows anti-*E. coli* IgG and IgM antibodies levels for all sub-groups. Markedly higher levels of anti-LPS antibodies of bothe classes were evident the tolerant sub-groups (I to IV) than for the naive sub-groups (V to VIII). The IgM levels (but not IgG) in group I (challenged with 25 mg/kg *E. coli* LPS 90 minutes before the blood sample was obtained) were significantly different ($p < 0.005$ ANOVA) from the other 3 groups that were equally sensitized but challenged with a

low dose of *E. coli* LPS (G-II, 0.5 mg/kg) or with *Salmonella* LPS (G-III 25 mg/kg, G-IV 0.5 mg/kg) which might be related to antibody consumption by the injected LPS. Decreased levels of anti-*E. coli* LPS antibody after challenge with *E. coli* LPS were shown in the naive groups for IgG where significant differences were found between groups ($p < 0.05$ ANOVA).

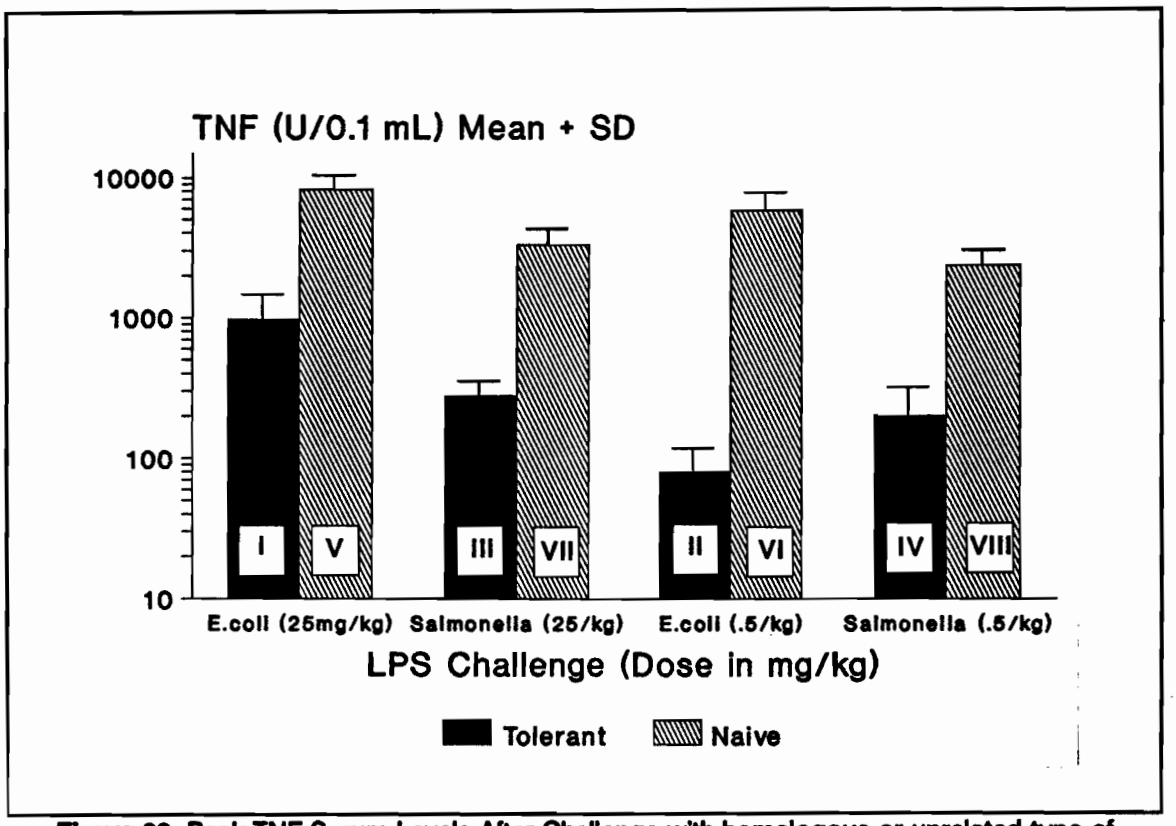

Figure 33. Peak TNF Serum Levels After Challenge with homologous or unrelated type of LPS. Tolerant rats were primed with 3 mg/kg *E. coli* O127:B8 LPS on days 0, 23, and 46. Age matched naive rats were used as controls. On day 72 animals were challenged in one or four different possibilities: 1) With 25 mg/kg (LD_{100}) of *E. coli* O127:B8 LPS; 2) 0.5 mg/kg of *E. coli* O127:B8 (non-lethal dose); 3) 25 mg/kg of *S. typhi* LPS (LD_{100}); or 4) 0.5 mg/kg of *S. typhi* LPS (non-lethal dose). Blood samples were obtained 90 minutes after the challenge dose for TNF measurement.

Results for the anti-*Salmonella* antibodies are shown in Figure 35. There are significantly higher levels of anti-*Salmonella* antibodies in the *E. coli* LPS primed groups (I to IV) (IgG $p < 0.01$; IgM $p < 0.0001$, ANOVA) compared to the levels in naive rats (groups V-VIII). This might be considered to be cross-reactive antibodies targeted towards epitopes common to both *E. coli* and *Salmonella* LPS such as the core or the lipid A fragments, or as non-specific cross reactivity secondary to a general over-production of IgG and IgM antibodies induced by repeated injections

of *E. coli* LPS. The presence of the cross-reactive antibodies may explain the failure of the unrelated *Salmonella* LPS to elicit levels of TNF in the *E. coli* primed post-tolerant animals comparable to those of the naive animals.

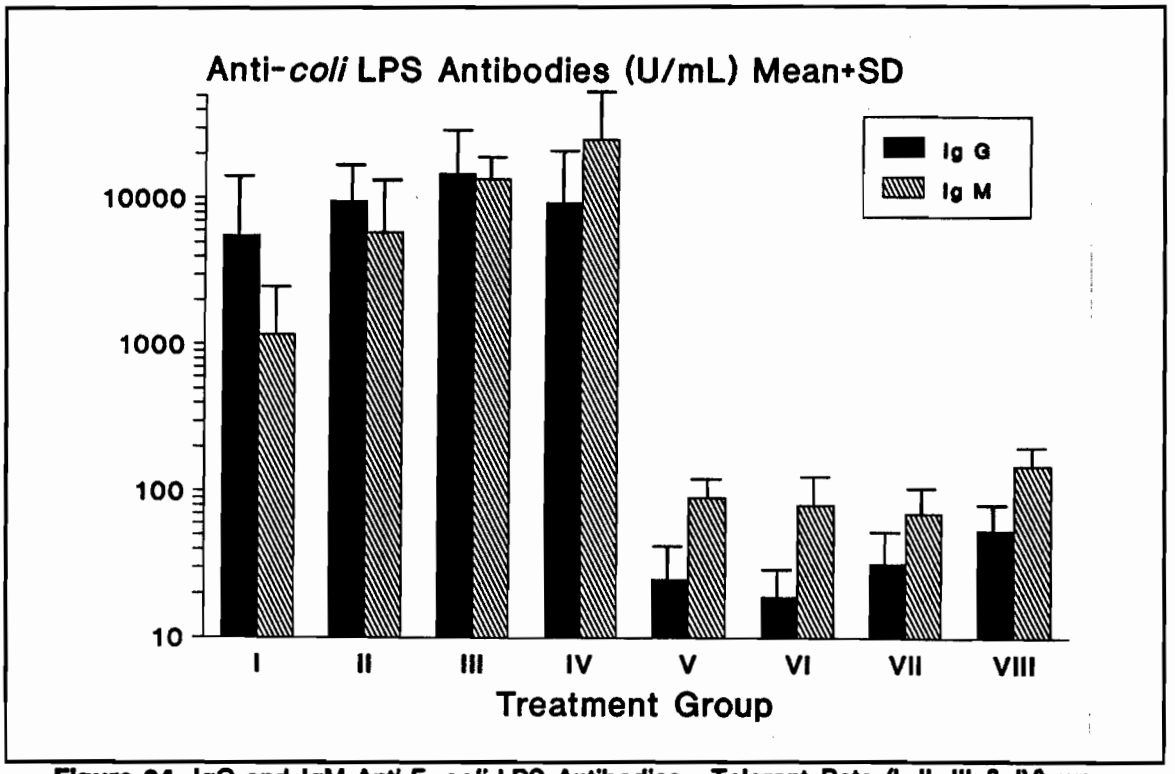

Figure 34. IgG and IgM Anti-*E. coli* LPS Antibodies. Tolerant Rats (I, II, III & IV) were primed with 3 mg/kg *E. coli* O127:B8 LPS on days 0, 23, and 46. Age matched naive rats (V, VI, VII, VIII) were included as controls. On day 72 all rats were challenged: 1) With 25 mg/kg (LD_{100}) of *E. coli* O127:B8 LPS; 2) 0.5 mg/kg of *E. coli* O127:B8 (non-lethal dose); 3) 25 mg/kg of *S. typhi* LPS (LD_{100}); or 4) 0.5 mg/kg of *S. typhi* LPS (non-lethal dose). Blood samples were obtained 90 minutes after the challenge dose to measure IgG and IgM anti-*E. coli* and anti-*Salmonella* antibodies.

Body weight loss of survivors (groups challenged with 0.5 mg/kg LPS) in this same experiment are shown in Figure 36. In the naive animals *E. coli* LPS was more potent than *Salmonella* LPS inducing weight loss ($p < 0.001$). Average percentage weight loss after the challenge with *E. coli* LPS was 4.48 ± 2.7 % for the tolerant and 9.8 ± 1.07 % for naive rats which suggests lower susceptibility to LPS effect in the tolerant ones ($p < 0.05$). The higher titers of anti-*E. coli* antibodies in tolerant animals apparently could account for this difference. Although the challenge with *Salmonella* LPS induced a ten-fold difference in TNF levels between the tolerant (G-IV) and the naive group (G-III) (Figure 33), and not withstanding also the wide difference in anti-*Salmonella* LPS antibody levels found between them ($p < 0.05$ for

IgG and IgM) no difference in weight loss induced by *Salmonella* LPS was detected (G-IV 5.7 ± 1.8 %; G-VIII 5.8 ± 1.0 %).

TNF PRODUCTION AND EFFECT OF ENDOTOXIN TOLERANCE IN MODELS OF SEPSIS OTHER THAN LPS ADMINISTRATION

TNF is considered the mediator of gram-negative sepsis, so it was questioned if TNF levels could be detect in blood in models of sepsis other than pure endotoxin administration. Two models were chosen; (1) Intraperitoneal injection of live bacteria and (2) Caecal Ligation and Puncture.

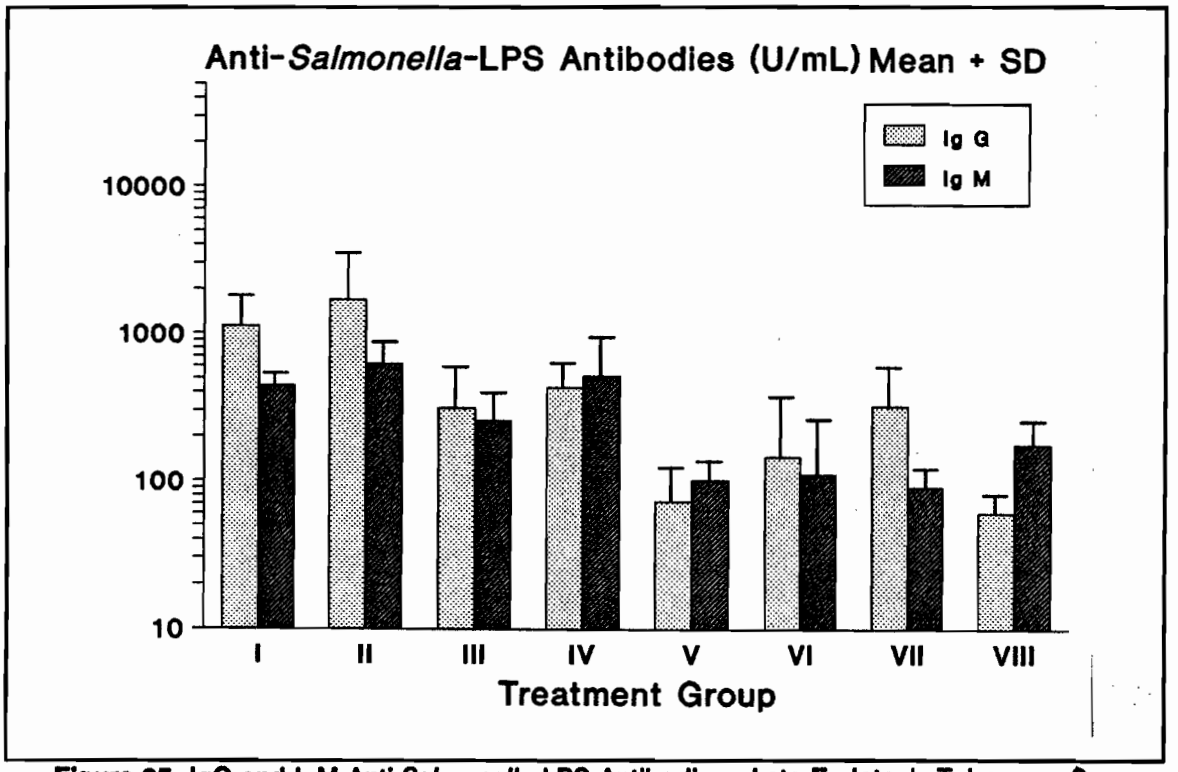

Figure 35. IgG and IgM Anti-*Salmonella* LPS Antibodies. Late Endotoxin Tolerance. See previous footnote.

I. CONTROL EXPERIMENTS.

TNF LEVELS IN 3 ANIMAL MODELS.

In the initial experiment, rats were assigned into 3 groups. The first group (n=6) received a combined suspension of *E. coli* (10^9 CFU/mL) *S. faecalis* (10^9 CFU/mL) and *B. fragilis* (10^7 CFU/mL); the bacteria were mixed in equal proportion just

before their administration. Three mL of the final mixture per 100 g body weight were injected ip. The actual lethality of this particular mixture was not tested but from previous experience in our laboratory (MacPhee, 1989) it was known to be lethal. A second group (Endotoxin, n=3) received 0.5 mg/kg of *E. coli* O127:B8 LPS (non-lethal dose). Caecal Ligation and Puncture with one gauge #18 perforations was practiced in the third group (CLP, n=6). Blood samples for TNF determination were obtained every 30 to 60 minutes after the challenge over a period of 8 hours.

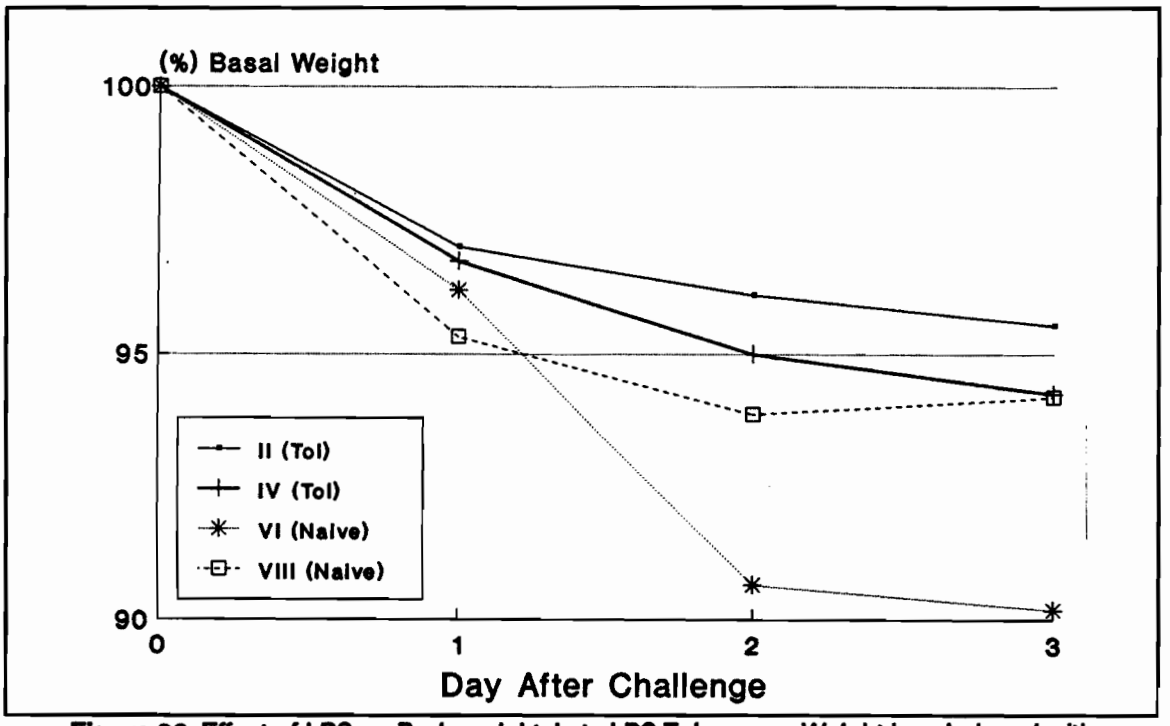

Figure 36. Effect of LPS on Body weight. Late LPS Tolerance. Weight loss induced with 0.5 mg/kg of *E. coli* LPS (II & VI) or *S. typhi* LPS (IV & VIII) in naive or late-tolerant rats.

As expected (Figure 37), LPS challenge induced high peak levels of TNF with a peak at 2 hours returning back to undetectable levels by 3.5 hours. In contrast, TNF could not be detected at any time in the CLP group and only very low levels were shown intermittently at 1, 2, 2.5, 3.5 and 6 hours after the injection in the live bacteria group.

These provocative findings suggested the need for an accurate measurement of lethality with each model, and standardization of the lethality in the three different models to be able to draw conclusions.

LETHALITY INDUCED BY LIVE E. COLI IN BARIUM SULFATE.

Male Wistar rats (150-220 g body weight) were challenged with different concentrations of a pathogenic strain of live *E. coli* (6 rats/dose) prepared as a 50% BHI, 50% saline, 10% (w/v) $BaSO_4$ mixture in a final volume of 2 mL.

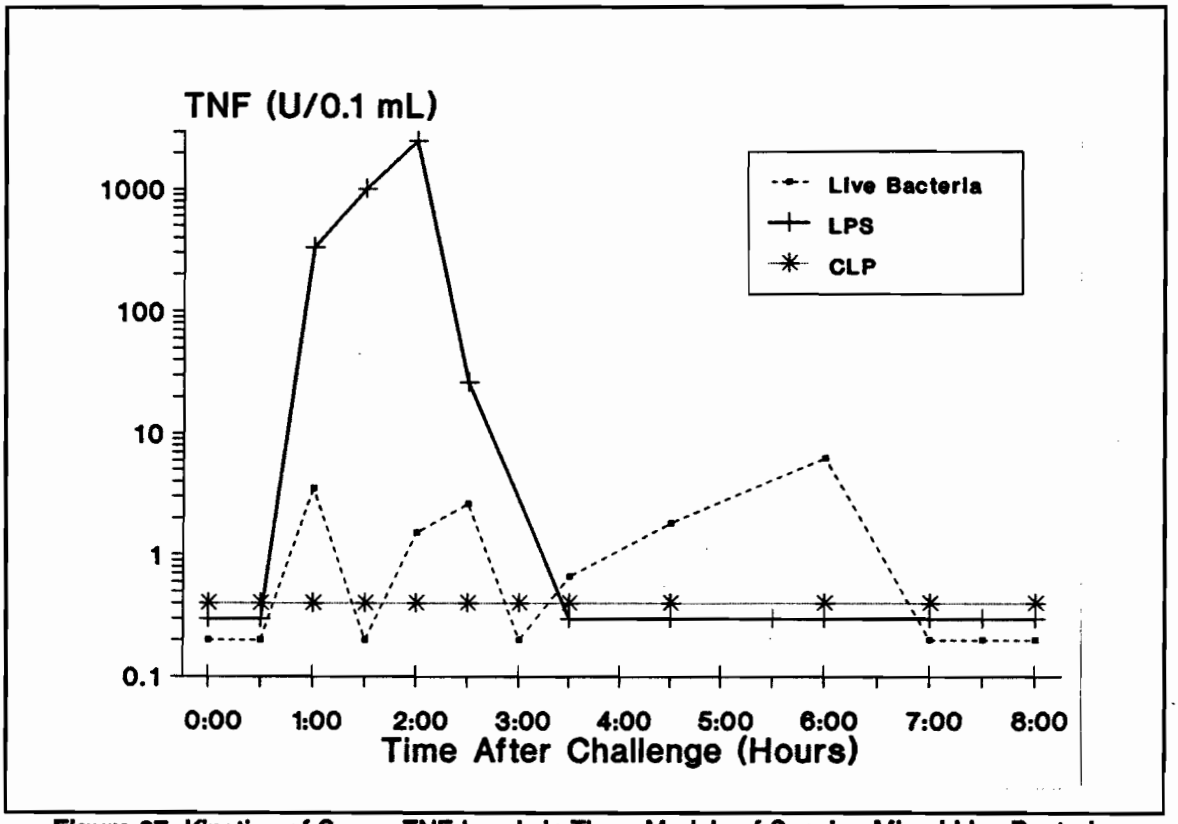

Figure 37. Kinetics of Serum TNF Levels in Three Models of Sepsis: Mixed Live Bacteria, LPS and CLP. Rats were challenged with a combined intraperitoneal suspension of live *E. coli*, *S. faecalis* and *B. fragilis*; a dose of 0.5 mg/kg LPS; or caecal ligation and puncture. After challenge, blood samples were obtained every 30 to 60 minutes over 8 hours for TNF determination. Pilot experiment.

Doses of bacteria injected and survival rate at Day 5 are shown in Table 11. From these data the LD_{50} was estimated by regression analysis as 1.94×10^8 CFU per 100 g body weight.

LETHALITY INDUCED BY CAECAL LIGATION AND PUNCTURE.

Caecal ligation and one or two #18 gauge punctures were performed on 20 male Wistar rats (10 in each group) to define lethality. At 96 hours survival was 20 % for a single puncture and 10 % after two punctures.

Table 11. Lethality Five days After ip Live *E. coli* in 10% Barium Sulfate

GROUP	BACTERIAL DOSE (CFU 100 g body weight)	LETHALITY (%)
I	2.5×10^8	56
II	5×10^8	83
III	10×10^8	100
IV	20×10^8	83
V	40×10^8	100

II. COMPARISON OF TNF LEVELS IN 3 STANDARDIZED ANIMAL MODELS OF SEPSIS.

In order to ensure that failure to detect high levels of TNF in the previous experiments was not due to excessive or insufficient doses of bacteria a variant of the experiment was performed. Male Wistar rats were injected with 5×10^8 CFU/100 g body weight of live *E. coli* in 10% $BaSO_4$ bacteria (n=12; $LD_{75\text{-}85}$); 18 mg/kg *E. coli* O127:B8 LPS (n=6; $LD_{50\text{-}60}$); or with caecal ligation and 2 punctures with a #18 gauge needle (n=12; LD_{90}). Blood samples were obtained at 30 to 60 minutes intervals over 10 hours for TNF measurement. The animals were killed at 11 hours.

The LPS challenge induced a peak level of 2260 ± 500 U/0.1 mL of TNF at 90 minutes that dropped back to base line (sub-threshold) levels at 3.5 hours (Figure 38). In the CLP group, TNF levels remained undetectable until 10 hours at which time trace amounts were detected (at this time one of the animals in this group had already died). In the live bacteria group, low levels were measured at 6 and 10 hours. The highest level of TNF measured in the live *E. coli* group was 22 ± 13 Units/0.1 mL at 10 hours. The highest levels detected in the CLP group were even lower. If TNF was produced in the latter two groups after challenge, it certainly was not reaching the circulation.

III. ENDOTOXIN TOLERANCE AND SURVIVAL AFTER CLP.

Based on the assumption that abolishing the host response to LPS (TNF production included) by inducing endotoxin tolerance, a survival advantage should be expected

in models of infection and sepsis such as CLP or live *E. coli* in 10% $BaSO_4$ in which endotoxin has assumedly a pathophysiologic role. It was asked if early endotoxin tolerance would improve survival in these models.

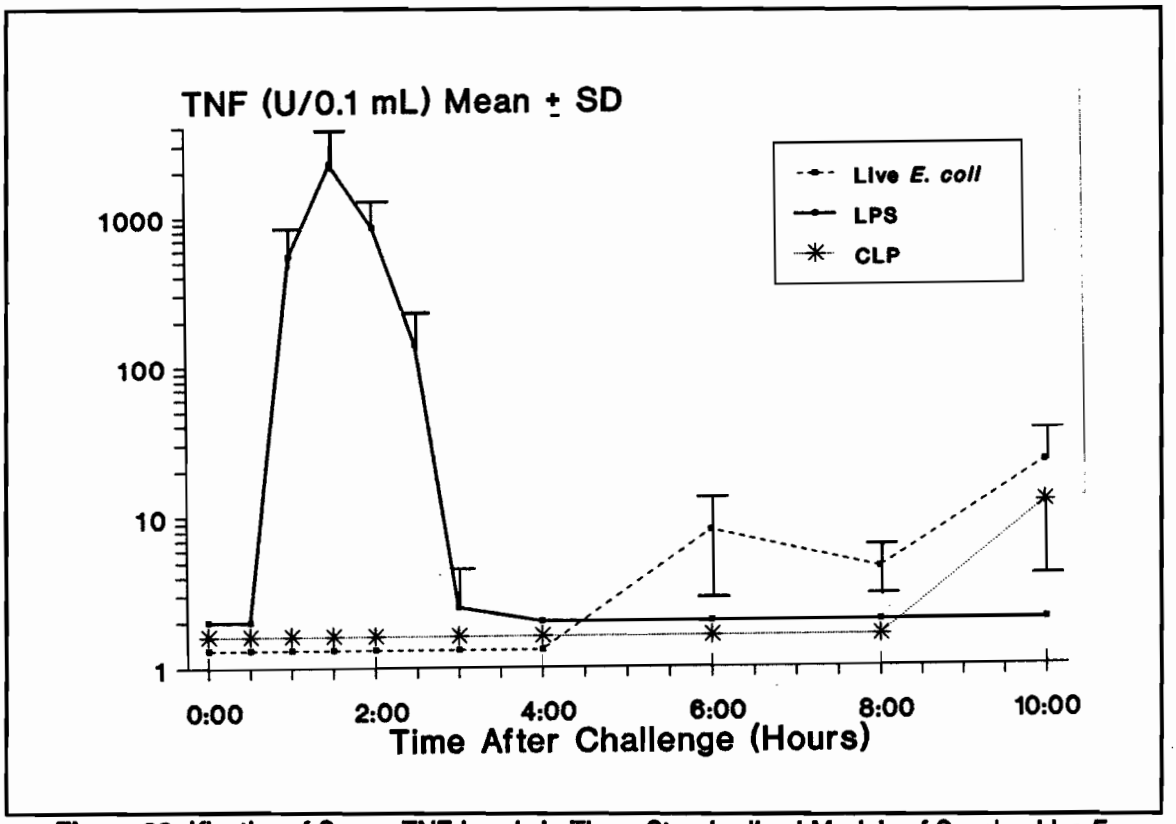

Figure 38. Kinetics of Serum TNF Levels in Three Standardized Models of Sepsis: Live *E. coli* in $BaSO_4$, LPS and CLP. Rats were challenged with Live *E. coli* in 10% $BaSO_4$ (n=12) 18 mg/kg *E. coli* O127:B8 LPS (n=6) or CLP with 2 punctures with a #18 gauge needle (n=12). After challenge, blood samples were obtained every 30 to 60 minutes over 10 hours for TNF determination.

A) CAECAL LIGATION AND PUNCTURE MODEL.

F1 Lou/Lewis rats (32 males [M], 20 females [F]) were used in this experiment. Rats were primed with two injections of 0.5 mg/kg of *E. coli* O127:B8 LPS on Days -2 and -1 to induce LPS tolerance (G-II, 10 M, 10 F). Age matched naive rats (11 M, 10 F) were included as controls. On Day 0 caecal ligation and puncture was performed on both groups. A second control group of male rats was included (G-III, Empty Caecum) when it was realized that the priming dose of LPS in the tolerant group provoked diarrhea resulting in the partially empty caecum and intestines observed during the laparotomy of the tolerant rats. Animals in this

group were naive as well and received the same pre-operative treatment as the other controls. In addition, during the CLP procedure their caecum was emptied by milking out its content into the ascending colon and only a volume of feces equivalent to 10-20% of the full capacity was left in its lumen in order to reproduce what was seen in the LPS primed group. Two #18 gauge punctures were performed. Rectal temperature was registered at 3 and 6 hours post-operative (Figure 39) in the first two groups. Blood and peritoneal fluid for culture were obtained at 10 hours post-operative (Tables 12, 13, and 14) and survival rate was monitored over 8 days in all the groups (Table 15).

Survival rates (Table 12) show the highly protective effect induced both by priming with LPS (80%) and by emptying the caecum (90%) compared with the naive controls (14%) ($p < 0.005$ X^2 with continuity correction). The significance of this improved survival will be addressed in the discussion.

Table 12. Survival Rate after CLP. I-Naive, II-Tolerant and III-Empty Caecum

GROUP	HOURS POST-CLP % Survival					ANIMALS
	12	24	48	72	96	PER GROUP
I. Male	91	9	9	9	9	(11)
Female	90	40	20	20	20	(10)
Total	90	24	14	14	14	(21)
II. Male	100	100	90	80	80	(11)
Female	80	80	80	80	80	(9)
Total	90	90	85	80	80	(20)
III. Male	100	100	90	90	90	(11)

Bacterial cultures (Table 13) were positive in 78% of the tolerant and in 70% of the naive animals (X^2 not-significant), which highly contrasted with the low number of positives in the empty caecum group (G-III 18%) ($p < 0.001$ X^2). Some difference between control G-I and tolerant rats was revealed in the quantitative cultures in which there was showed a four-fold decrease in the average number of bacteria in the tolerant group compared to the naive ones although it was not significant due to the wide variation in the cultures from the individual animals. Both the number of

positives (18%) and the mean counts (1.01 x 10^3 CFU/mL) were very low in the empty caecum control group, and both values were significantly different from the other two groups when the log of bacterial counts (but not when the actual number of bacterial counts) was tested.

Table 13. Blood Cultures Ten Hours after CLP. Number of Positives and Quantitative Results

GROUP	POSITIVE CULTURE	CFU/mL	NUMBER OF ANIMALS
I. Naive	78%	36.7 x 10^3	18
II. Primed	70%	7.04 x 10^3	20
III. Empty caecum	18%	1.01 x 10^3	11

Table 14 shows the distribution of single, multiple and negative blood cultures in each group; the differences were non-significant by X^2. Most of the positive blood cultures (66%) and all peritoneal cultures were polymicrobial (only aerobic cultures were practiced). The varieties of positive cultures (morphologic characterization) are shown in Table 15 and as expected coliform rods were the leading isolates.

Table 14. Blood Cultures Ten Hours after CLP. Single species, Polymicrobial or Negative Cultures

GROUP	ONE SPECIES (%)	POLYMICROBIAL (%)	NEGATIVE (%)
I	3/18 (17)	11/18 (61)	4/18 (22)
II	5/20 (25)	9/20 (45)	6/20 (30)
III	2/11 (18)	0/11 (0)	9/11 (82)

Rectal temperature measurements at 3 and 6 hours for control Group I and tolerant rats are shown in Figure 39. Except for the 3 hour measurement in females, all other groups show a significant difference where the development of hypothermia was prevented by priming treatment with LPS and thus endotoxin tolerance ($p < 0.05$).

Table 15. Blood and Peritoneal Cultures Ten hours after CLP. Morphological Characterization of Colonies

PERITONEUM	BLOOD
E. coli	Coliforms
Proteus sp.	*Lactobacilli*
Pseudomonas sp.	*S. aureus*
Morganella M.	Gram negative coccobacilli
S. aureus	*Enterococcus sp.*
Enterococcus sp.	Gram negative cocci
	Streptococcus D
	Candida

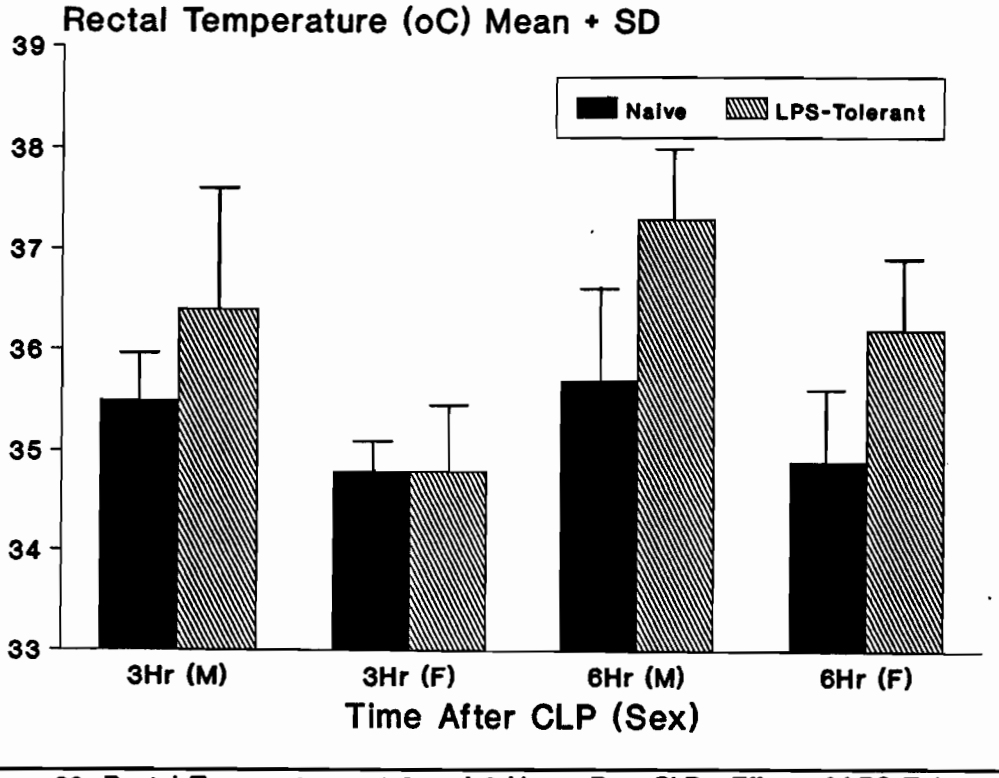

Figure 39. Rectal Temperatures at 3 and 6 Hours Post-CLP. Effect of LPS Tolerance. Male and female rats were assigned into 2 groups: Naive (no pre-treatment n=21) or Tolerant (pre-treated with two doses of *E. coli* LPS 0.5 mg/kg/dose ip on Days -2 and -1 n=20). On Day 0 caecal ligation and puncture was practiced on all groups. Rectal temperature was measured at 3 and 6 hours post-operative.

IV. ENDOTOXIN TOLERANCE AND SURVIVAL AFTER LIVE E.COLI INJECTION.

The diminished (although not statistically significant) bacterial counts in the tolerant group as compared to the control group I suggested that to some extent, survival could depend on the lower bacterial load due to the LPS induced diarrhea and associated bowel emptiness, as was certainly the case in the empty caecum group, so it was imperative to disclose the effect of tolerance induction upon survival rate once the bacterial load was standardized. The following experiment was done to address this problem.

Male Wistar rats were assigned into 2 groups. Tolerant animals were primed with 5 mg/kg *E. coli* O127:B8 LPS on Day 0 and 2 mg/kg on Day 1. Age matched naive rats were controls. On Day 3 rats were challenged with either *E. coli* O127:B8 LPS (30 mg/kg, LD_{80}) (groups I and III) or a suspension of 10×10^8 live *E. coli* in 10% sterile $BaSO_4$ per 100 g body weight (LD_{95} by regression analysis) (groups II and IV). Survival rate on Day 5 and body weigh in the survivors were monitored (Table 16).

Table 16. Survival Rate and Weight Loss after Live *E. coli* in $BaSO_4$. Naive v.s. Endotoxin Tolerant Rats

GROUP	CONDITION	CHALLENGE	SURVIVE/N.	WEIGHT LOSS
I	Tolerant	LPS	9/10	12.6 ± 4.8%
II	Tolerant	*E. coli*	10/10	11.8 ± 7.1%
III	Naive	LPS	0/6	DEATH
IV	Naive	*E. coli*	0/10	DEATH

Results demonstrate a 100% lethality in the naive groups for both the LPS challenge and the bacterial inoculum used. A highly protective effect of LPS pre-treatment was also confirmed in both groups tested ($p < 0.0001$, X^2). No differences were found in weight loss induced by LPS or by live bacteria in the tolerant rats.

DISCUSSION

I. CURRENT CONCEPT.

It has been accepted that in most severe infections it is the host response to gram-negative bacteria and their products rather than the action of bacteria themselves, which ultimately threatens the host's tissues in disseminated infections. As pointed out by Thomas "Our arsenals for fighting off bacteria are so powerful and involve so many different defense mechanisms, that we are in more danger from them than from the invaders." ..."these (endotoxin) macromolecules are read as the very worst of bad news. When we sense LPS, we are likely to turn on every defense at our disposal" (Thomas, 1974). Superficially it might imply that the overproduction of toxic endogenous compounds (particularly TNF) is an imperfection in the host defense against microbial invasion, or that it is an evolutionary adaptation favoring the removal of infected individuals from a population (Beutler 1989).

This last idea cannot be valid because an evolutionary system that eliminates the individual for the sake of the group such as the one proposed by Beutler would not represent a selection advantage because evolutionary pressure can only emerge and take action on an individual bases. I would rather propose more simply, that the mammalian system was probably not meant to survive this septic process, and it is the result of the sophisticated technological support available that we are able to glimpse progressive systemic infection to reach such a state where MSOF can unfold. In some way, our human-developed resources are allowing us to try against the odds, to push the line drawnby nature a bit further. Accordingly, the ultimate therapeutic measure that should be contemplated in this regard probably does not lie in the capacity to counteract the process of MSOF once initiated, but rather to bear in mind that there might be no "magic bullet"; that therapeutic regimens will probably be time dependent and "multiple drug"; and that the only way to avoid the tragic impact of septic shock and MSOF would be to prevent its start in the first place (Stahl 1988; Cerra 1987).

II. DISAGREEMENT.

As soon as I got involved with endotoxin and the mediators of sepsis, serious questions about the validity of some conclusions drawn from the endotoxin models

of sepsis were raised and it was found difficult to make congruent the existence of a phenomenon like endotoxin tolerance with the assumed role of LPS or TNF as mediators of sepsis.

The foundation of my concern rests upon the concept that mammals have the capacity to develop tolerance to LPS's toxic effects. Therefore, in clinical infections, where a rather incremental appearance of LPS is conceivable, one would expect to see that early in the course of an infection, LPS would elicit a powerful inflammatory reaction and synchronous immunologic and phagocytic activation. Later in the course of the infection, a state of endotoxin tolerance could develop which would prevent immunologic damage by avoiding overreaction. At the end the whole response should have proceeded under tight control enabling the damage provoked both by the infectious process itself and by the reaction unfolded to combat it, to be maintained at the very minimum.

Obviously such a smooth course and favorable outcome is not always the case in clinical infections where enough evidence points out to a well defined immunologic participation in sepsis and MSOF. And yet, the *gram-negative infection - endotoxin - TNF and other mediators - tissue damage* model is not entirely satisfactory and several facts seemed to reject this hypothesis.

TNF might be a co-factor in the pathophysiology of sepsis and MSOF with potent synergistic activity however the present results in no way support that TNF has the capacity to induce lethal damage, as demonstrated by the highest TNF levels induced by non-lethal doses. The results also show that TNF is not a requisite for a lethal outcome secondary to LPS as low levels of TNF are present after lethal doses of LPS in post-tolerant animals, and in other models of gram-negative infection such as ip injection of live *E. coli* or CLP, the TNF is barely detectable.

III. THE MODEL OF ENDOTOXIN TOLERANCE.

My intention in this project has been to explore the gap between the pathophysiology of severe bacterial infection and the LPS-induced shock syndrome. The first steps were given to expand the concept of endotoxin tolerance.

During the course of severe clinical infections, progressive deterioration and multisystemic damage are considered secondary to repeated endogenous bolus or continuous leakage of endotoxin coming from the infectious foci or from the gut. Nevertheless, when I tried to reproduce such a system in an animal model, quite different results were obtained. The effect of different schedules of ip administration of LPS upon weight loss was analyzed. Single dose (Figure 6); repeated daily injections (Figure 7); or continuous infusion over 7 days (Figure 8) showed that the induced weight loss and its subsequent recovery was very similar regardless of the schema of LPS administration (Figure 9). This suggested that the animals could respond only to the first exposure to LPS and that once the response was triggered, little or no further effect was provoked by repeated challenges, a process which is not readily compatible with the *infection-endotoxin-TNF-sepsis* theory.

The mechanisms for weight loss in this model is mainly reduction in food intake and diarrhea (Fraker 1988; Socher 1988), although increased catabolic state also occurs (Sacco-Gibson 1989; Spitzer 1989). These responses to LPS including the metabolic one become temporarily refractory and no further reaction is elicited by added amounts of LPS during endotoxin tolerance (Lang 1987).

A similar type of acquired resistance to LPS-induced body weight loss has been identified following repeated administrations of TNF (Fraker 1988; Socher 1988) and IL-1 (Otterness 1989). However, Tracey et al reported sustained low food intake at a 60% level compared to controls under repeated twice daily injections of TNF up to 8 days as long as the dose of TNF was doubled every time they detected the development of tolerance (Tracey 1988). The problem with this model is that increasing TNF levels over several days does not reproduce the sudden and transient levels seen after endotoxin challenge.

The next purpose of the experiments was to define the kinetics of TNF production after a challenge with endotoxin, the results are shown in Figure 10. In 1970 Daniele demonstrated in dogs that 30% or more of the ip injected endotoxin can be recovered from the abdominal cavity 10 hours after a bolus administration (Daniele 1970), thus it was no surprise that there was little difference in the kinetics of serum TNF generation found between the continuous and bolus intra-peritoneal injection of LPS. The main difference most probably was the dose. The pump contained 0.2

mL of a 20 mg/mL solution equivalent to 1.2 μL per hour or 24 μg of LPS equivalent to 120 μg/kg for a 200 g rat. This quantity plus the amount that could have been contaminating the external surface of the pump, minus the amount that was not absorbed from the abdominal cavity was the actual challenge. A dose as low as 5 μg/kg injected ip was later proven to be quite capable of inducing a high peak of serum TNF (Figure 12).

Waage (1987) has also observed that continuous infusion of LPS induced sustained levels of circulating LPS but a single peak of TNF at 1 hour followed by decrease to undetectable levels by 2 hours. The same kinetics of LPS-induced TNF are present in other animal models and in humans. This is the reason why the decision was made to always obtain the blood samples 90 minutes after the LPS injection.

The momentary peak of TNF induced by LPS followed by a rapid disappearance to undetectable levels is another reason why one can question the role of TNF in clinical sepsis and that is why we find futile the search for high serum TNF levels in human sepsis as a prognostic factor which has been deceptive in most cases and although it is claimed to be found a correlation between mortality and higher serum TNF levels, in the few cases that report it, insuficient data has been presented to arrive at definitive conclusions (Damas 1989; Debets 1989).

IV. PEAK TNF LEVELS AND MORTALITY.

Experiments were then performed to define the dose/response relation for peak TNF levels and LPS dose. A lack of correlation was found, on the one hand, between LPS dose and peak TNF levels (Figure 11) and on the other, between peak levels of TNF and lethality, both of which are difficult to explain. Possible explanations are: (1) TNF is not the mediator of the lethal effect of LPS it is actually a protective response like many other ones elicited by gram-negative infection associated-LPS. (2) The kinetics of TNF vary when different doses are used and although peak levels are not as high after higher and more lethal doses of endotoxin, a probable diminished inactivation time will actually maintain damaging levels of TNF for longer period of times than with non-lethal doses. (3) Due to altered hemodynamics and poor tissue perfusion in the lethally challenged animals, TNF is inactivated *in situ* and cannot reach the systemic circulation, thus it is not detectable.

The first argument is a motive for much of the present discussion. The foremost opponent issue to this argument is the protective effect that the administration of anti-TNF antibodies had shown in several experimental models that have been referred to in the review of the literature.

Related to the second argument of different kinetics, Mathison, Wolfson and Ulevitch in a very interesting paper reported that injection of LPS-High Density Lipoprotein (HDL) complexes produced a much better dose/response correlation with peak TNF levels than pure LPS, but once maximal production of TNF is reached with a high enough dose of LPS-HDL as in case of pure LPS administration, no further amounts could be induced neither increasing the dose nor repeating the injections in disregard to the effect upon blood pressure and other parameters of hemodynamic compromise that the different doses or preparations induced. Even if LPS was combined with HDL (which is believed by some to be the more natural condition expected in clinical infections), a peak was detected at 90 minutes followed by a quick reduction and return to base line levels within 3 to 4 hours. Furthermore, the same authors demonstrated that the pharmacokinetics of exogenous TNF injected into naive or into endotoxin-tolerant animals were the same (Mathison 1988). In humans, no dose/response correlation was found between iv administration of TNF and the peak temperature (Hesse 1988) or hypotension induced (Chapman 1987). In non-primed mice, optimal production of TNF was obtained with non-lethal doses of LPS and higher doses were not able to induce higher levels of TNF (Gifford 1987).

Lastly, in relation to the third possibility, a recent publication reported that comparable peak TNF levels were generated upon injection with LPS, or the LPS sub-fractions diphopsphoryl lipid A or monophosphoryl lipid A; while the first two preparations were lethal and the latter was not (Kiener 1988). In our experience,. TNF levels after higher dose of LPS - specifically over 20 mg/kg - elicit lower TNF levels than non-lethal doses. This same decrement was also evident when preparations of LPS with low-toxicity were injected (*E. coli* O26:B6) without being lethal (Figure 23) so the vascular collapse explanation cannot satisfy the apparent conflict. All these arguments, tend to invalidate the proposition that different kinetics and different half lives of TNF are the reason for the lack of correlation between peak LPS induced-TNF serum levels and the ensuing lethality.

V. INTRAPERITONEAL ENDOTOXIN AND SYSTEMIC TNF.

An issue relevant to understand the role of LPS in gram-negative sepsis is to find out how much free endotoxin is actually released into the systemic circulation from a gram-negative bacterial infectious foci or after bowel perforation. Such measurement of LPS in thoracic duct lymph, arterial plasma or portal vein plasma after producing caecal perforation was done by Olofsson and colleagues who estimated that the inflow of endotoxin by way of the portal vein and the thoracic duct carried out of the abdominal cavity most of the endotoxin. The amount calculated never exceeded 300 ng/kg per hour. The same authors also showed that minimal amounts of endotoxin were actually measured at any point in arterial plasma and explained this fact on the bases of an extremely powerful endotoxin clearing capacity of the rat liver reportedly of 1.5 μg/g liver per hour (Olofsson 1986) equivalent to 9-10 μg/hour for a 200 g rat.

In Figure 12 it was shown that peak TNF levels are much lower when less than 0.05 mg/kg LPS are injected ip. Actually, the lowest dose capable of inducing detectable levels of TNF was 5 μg/kg. Considering these amounts of LPS required to generate detectable levels of TNF, it is no surprise that both the injection of live *E. coli* in barium sulphate and caecal ligation and puncture induced only trace amounts of serum TNF (Figure 38) compared to the levels reached after the injection of pure LPS.

VI. TOLERANCE AND RECOVERY OF RESPONSIVENESS. MECHANISMS OF ENDOTOXIN TOLERANCE.

The duration of endotoxin tolerance and its relation with LPS dose and induced TNF levels were then studied. Results confirmed that during early endotoxin tolerance there was no sensitivity to the lethal or the body wasting effects of LPS, but 30 days after, full recovery of responsiveness to LPS ensued (Figure 13 and 14). In fact, as shown in Table 5 and Figure 26, lethal susceptibility in the post-tolerant rats 21 or 30 days after a single priming dose of LPS was higher than in the naive animals but 21 days after three tri-weekly repeated doses of LPS (Table 9) an unequivocal decrease in sensitivity was demonstrated.

In relation to TNF production during and after early endotoxin tolerance some relevant work has been previously done. O'Malley et al (1962) demonstrated that the tumor necrotizing effects of sera from normal mice given *Serratia marsescens* endotoxin was short lived and further production was refractory to repeated endotoxin challenge. This raised the question if there was lack of TNF production after a second injection of LPS during endotoxin tolerance in our model. Naive animals however (Figure 15), generated very high peak levels. Two days later, a second injection of LPS induced minimal TNF levels. Recovery of TNF production was gradual from Day 2 onwards although the tempo or recovery was slower when tolerance was induced with higher than with lower doses of LPS. Compatible results have been found in a rabbit model where injection of a second dose of 10 μg LPS 5 hours after the first one did not induce a detectable TNF release (Mathison 1988).

In Flick's model of mice injected with LPS, tolerance persisted for at least seven days, and even a 10-fold increase in LPS dose was not able to induce detectable levels of TNF when administered 48 hours after priming the animals (Flick 1986a), and according to Waage, when a second injection of LPS is given to rats within 3 days of the priming dose, peak concentration of TNF detected after the second injection was 15% of the concentration detected after the first one (Waage 1987). A similar lack of LPS induced-mediator production during early LPS tolerance has been demonstrated for colony-stimulating factor (Williams 1983; Madonna 1985; Vogel 1987) and for prostaglandins PGE_2 and $PGF_{2\alpha}$ (Rietschel 1982).

Tolerance is not due to an immune mechanism; if antibodies to LPS were involved, one would expect to observe a late developing and long lasting state of refractoriness instead of the early short lived one seen. When anti-LPS antibodies are not present at day 2, sensitivity to LPS is in its nadir, and by day 20 when titers are high (Figure 16), responsiveness is recovered.

VII. INDUCTION OF ENDOTOXIN TOLERANCE. LOW DOSES AND LOW TOXICITY LPS.

The clinical relevance of endotoxin tolerance and the associated refractoriness to the deleterious effects of the endogenous mediators of inflammation is evident, but for this phenomenon to ever be available as a therapeutic tool, the priming mechanism should have low possibilities of inducing adverse effects. For this reason

it was questioned if low doses of LPS that would not induce overt toxicity were capable of inducing tolerance.

In a preliminary experiment (Figure 17) it was probed if doses of LPS much lower than a lethal one would induce LPS tolerance. It was learned that a dose 1/100 of a LD_{50} was capable of preventing death and diminishing LPS induced weight loss after a challenge 2 days later. Even more, a dose of 0.5 μg/kg LPS (1/45000 LD_{50}) while unable to induce body weight loss (Figure 18) or detectable levels of TNF (Figure 19) was still capable of inducing LPS tolerance as shown by markedly improved survival (5 out of 6 rats or 83%) following an LD_{100} dose of LPS (Table 6) and by a diminished production of TNF after a challenge compared with non-tolerant rats (Figure 19).

Several lines of evidence suggest not only that low doses of LPS can induce tolerance but that neither TNF nor toxicity are needed for endotoxin tolerance to develop. First, pre-treatment of rabbits with rHuTNF did not diminish the amount of LPS-induced endogenous TNF reflecting a lack of tolerance (Ulevitch 1989). Second, pre-treatment with anti-TNF globulin resulted in neutralization of LPS-induced serum TNF activity and toxicity after a primary dose, and yet a subsequent injection of LPS failed to induce detectable levels of TNF or lead to clinical signs of toxicity, thus hyporesponsiveness was induced by LPS in the absence of detectable levels of serum TNF (Mathison 1988). Third, in our own experience endotoxin tolerance could be prolonged by continuous infusion of small amounts of endotoxin as shown by hyporesponsiveness to an additional LPS bolus as shown by very low levels of induced-TNF production (Figure 20) and diminished loss of body weight (Figure 21). In this experiment, no detectable levels of TNF were induced by the small amounts of LPS being continuously infused (data not shown). Fourth, when TNF was first induced with double-stranded poly(I:C), tolerance did not develop and either poly(I:C) and LPS could induce more TNF after the second injection. On the other hand, when LPS was used for the first injection, the mice were tolerant and neither LPS nor poly(I:C) given on day 2 would induce TNF synthesis again (Flick 1986).

Having shown that lethal or non-lethal doses of the same endotoxin could induce the same amount of TNF, and that doses of LPS low enough to prevent toxicity or TNF production could still induce endotoxin tolerance, the next problem was to

define if tolerance could be induced with preparations of LPS with low toxicity and if there was correlation between LPS induced-TNF production and lethal potency using LPS of different O-specificities.

It was found that one of the LPS preparations used, namely *E. coli* O26:B6 LPS was remarkably different in toxic potential. Nevertheless, the amount of TNF induced with this preparation was similar to peak TNF levels induced with the same doses of other more toxic preparations (Table 7, Figure 22 and 23).

An explanation for lack of toxicity of LPS may be related to its polysaccharide moieties. Galanos et al has found that preparations of isolated endotoxins, which themselves had no detectable complement consuming activity, nevertheless, contained a lipid A which after isolation by mild acid hydrolysis actively consumed complement. These data suggest that the presence of polysaccharide modulates the full expression of the anti-complementary activity of lipid A. In addition, the study demonstrated that the anti-complementary activity of isolated lipid A was highly dependent on its degree of aggregation and solubility (Galanos 1971).

It has also been reported that polysaccharide-free lipid A obtained from *E. coli* O26:B6 LPS could consume significant amounts of C_1, C_4 and C_2 in human serum. In contrast, the polysaccharide-containing LPS, consumed minimal C_1, C_4 and C_2 but was equally efficient at converting C_3 and Factor B in both normal and C_2-deficient serum (Lachmann 1974) reflecting different biological effects depending on the modulatory effect of the polysaccharide side chains. Finally, in a recent study, using hybrid LPS aggregates (containing polysaccharide-rich or lipid A-rich hybrids) in a mitogenic assay, it was demonstrated that the polysaccharide portion of LPS could negatively regulate the expression of lipid A in LPS micellar aggregates (Vukajlovich 1985). This would explain that a particular type of LPS could be not as toxic as others depending on the content of polysaccharide chains it contained; however the fact that non-toxic preparations induce comparable levels of TNF as the toxic ones is difficult to fit into the traditional concept of *endotoxin - TNF - lethality*.

There is no total agreement on the capacity of non-toxic fragments of lipid A to induce LPS tolerance (Sayers 1987) so it was questioned if a low-toxicity LPS would induce tolerance to other more toxic preparations of endotoxin, and if during LPS

tolerance the injection of a different type of LPS than the one used to prime would be associated with decreased levels of TNF. The results (Table 8 and Figures 24 and 25) corroborated that (1) early LPS tolerance is not O-antigen specific. (2) It was also demonstrated that tolerance could be induced with preparations of LPS with low-toxicity and still conferred protection from preparations with high toxicity. (3) It was shown that even though O26:B6 LPS-induced tolerance did not completely prevent TNF production induced with a lethal challenge of the toxic type O127:B8 LPS (Figure 25, Day 4) it did prevent mortality.

This is compatible with published data. Golenbock's group found that natural lipid X had little toxicity, low pyrogenicity, provoked low grade pulmonary hypertension, and prevented lethality (but not neutropenia or moderate hypotension) form a lethal dose of LPS in sheep (Golenbock 1987). Proctor (1986) found natural lipid X non-lethal even at doses of 5000 μg ip in mice which was capable of inducing LPS tolerance even when given up to 6 hours after an LD_{100} of LPS.

VIII. LATE ENDOTOXIN TOLERANCE. MODIFIED RESPONSE TO LPS.

The next problem addressed was the modified response to LPS once sensitivity to endotoxin was recovered after early endotoxin tolerance. The response to endotoxin was evaluated in terms of weight loss, mortality and TNF production and for obvious reasons, the relation of the response to anti-LPS antibody levels was analyzed.

The eventual long-lasting decrement in sensitivity to the lethal effect of LPS after repeated priming doses has been discussed above.

Sensitivity to weight loss was not fully recovered 21 days after a single priming dose (Figure 27). Actually, repeated injections did not further decrease sensitivity to this effect (Figure 28). Results also show that tolerance after 3 cycles was more readily evident when low (0.5 or 0.05 mg/kg) rather than high (5 mg/kg) doses of LPS were used to challenge the rats which suggest a dose-dependent degree of tolerance.

TNF production did not fully recover either after a single or after repeated priming doses of LPS. In fact, peak levels of TNF decreased progressively over successive cycles of booster doses of LPS (Figure 29). Interestingly, after 3 cycles of

LPS the dose/response curve showed that LPS dose and peak TNF levels did correlate in contrast with what was shown in the naive rats (Figure 30). Nevertheless, levels of 4344 U of TNF per 0.1 mL of serum seen after 0.5 mg/kg LPS in naive animals associated with no mortality on the one hand, and a mean peak level of 306 U of TNF per 0.1 mL after 50 mg/kg LPS associated with 71% mortality in the post-tolerant group on the other are disturbing (Table 9 and Figure 30).

An approach to bring this into terms with the general concept of TNF as the mediator of sepsis is to assume that a more efficient system for TNF action is achieved and a lesser amount of the cytokine is capable of inducing quantitatively the same effect after repeated exposure to endotoxin and TNF. This could be due to higher numbers or higher affinity of the TNF receptors; no evidence however exists for either case, and actually, it is known that exposure to LPS or IL-1 down-regulates specific surface receptors at least *in vitro* (Mizel 1987; Matsushima 1986).

Alternative explanations of this lack of correlation between TNF levels and lethal outcome are:

(1) TNF is not the mediator of the lethal effect of LPS, and the actual mediator (if one ought to exist) is still being release in high enough amount to kill.

(2) Serum levels of TNF are actually irrelevant but the presence of TNF in extravascular tissues is what matters.

There is considerable evidence against this simplification. Nine day old Meth A tumors but not earlier ones, are at the most sensitive time for induction of necrosis by LPS. These tumors have the highest concentrations of neutrophils at the time of treatment not in the tumor mass but in the blood vessels reflecting that is in the vascular compartment, most probably the endothelium, where the actual effect of endotoxin is taking place. Also by 9 days, the tumor's vascular bed has been developed, and before this happens, tumors are resistant to the action of TNF (Palladino 1987).

It is not clear what properties allow certain vascular beds to rupture in response to TNF and not others. In the implanted tumor, the endothelium is immature (approximately eight days), in contrast to the endothelium of the adult rat caecum,

which is reportedly, also highly reactive to TNF. In both tissues there is rapid cell division. Since the GI tract has been shown to accumulate approximately 90% of the total injected TNF in mice after eight minutes (Beutler 1985), perhaps the number of endothelial TNF receptors is particularly rich in these region and thus, the GI tract becomes particularly sensitive to a systemic inflammatory reaction. In any case, the relevant factor seems to be once more within the vascular compartment.

Another argument that favors the intravascular space as the arena where events leading to the development of sepsis has been presented by Gollan et al who were unable to prevent mortality in dogs given endotoxin intravenously and exchange transfusion 5 minutes later (Gollan 1979). Greisman et al had similar result in a rabbit model doing the exchange transfusion 20 minutes after the injection of endotoxin (Greisman 1983).

(3) Anti-LPS antibodies have dampened the TNF-inducing capacity of LPS. This is actually the mostly favored opinion explaining pyrogenic tolerance during late LPS tolerance as was presented in the review, but, as Milner warns us, extrapolation of these results (on pyrogenic tolerance in rabbits) to other effects or other animals should be avoided (Milner 1973).

Figure 31 shows progressively increased titers of both IgG and IgM anti-LPS antibodies as expected. This very nicely correlates (Figure 32) with the decreasing peak TNF serum levels induced by the same LPS dose at that same time. The idea was further explored.

IX. RESPONSE TO LPS OF DIFFERENT TYPE-O IN LATE LPS TOLERANCE.

Rats were repeatedly primed with one type of LPS and 21 days after the last dose were challenged with the same type or with another O-specific type of LPS (Table 10). The rationale for this experiment was that if late tolerance to LPS was exclusively due to the presence of O-specific anti-LPS antibodies, if tolerance was induced with an LPS of one type of O-specificity, the response to a challenge with another O-specific LPS would be the same in the primed as in the naive animals. Milner did a similar experiment using rabbits and testing for pyrogenic tolerance. In Milner's experiment, rabbits were given single priming doses of endotoxin from

three types of bacteria of different O-specificities and all groups were challenged seven days later with endotoxin from one type. Only those primed homologously were shown to be tolerant (Milner 1973).

In our experiment (Table 10), priming did not confer any significant survival advantage to the post-tolerant rats (G-I and III) over the naive ones (G-V and VII) neither to the group that was challenge with the homologous type of LPS used to prime (G-I) nor to the group challenged with the non-related type of LPS (G-III). We found again that high levels of TNF in naive animals or low levels in tolerant rats were not proportional to the likelihood for survival between the groups injected with 25 mg/kg of LPS and the groups injected with 0.5 mg/kg (Figure 33).

In terms of body weight loss (Figure 36), our results are compatible with Milner's. In naive rats *E. coli* LPS showed a higher potency to induce body weight loss than *S. typhi* LPS. When challenged with *E. coli* LPS, tolerant rats (G-II) lost 4% of body weight by day 2 and 4.5 % by day 3. On the other hand, the naive rats lost a mean of 9.5% by day 2 and 9.8% by day 3. The body wasting effect of *S. typhi* LPS upon late-tolerant or naive animals was not different (Tolerant 5.7 ± 1.8% vs Naive 5.8 ± 1%).

The fact that *E. coli* challenged tolerant rats in our experiment produced much lower levels of TNF than the naive counterparts (Figure 34), would seem easily explained by the high levels of anti-*E. coli* LPS measured (Figure 34), but as shown in Figure 35, although anti-*Salmonella* antibodies were barely elevated above control levels in the tolerant rats, TNF production was precluded as well in this group (Figure 33).

These results generate to paradoxical conditions: First, if pre-treated animals have titers of anti-LPS antibodies that were high enough to diminish the body wasting effect and levels of TNF produced, why it did not prevent a lethal outcome? Secondly, why did not the late-tolerant rats challenged with a non-related LPS type produce as much TNF as the naive ones? We propose three alternative explanations:

1) The pyrogenic, the body wasting effect, the TNF-inducing capacity and the lethal effects of LPS can be dissociated.

Some evidence supports this idea:

a) In humans, for unknown reasons, despite the administration of equipyrogenic doses of LPS, nausea and emesis are consistently more pronounced with some endotoxins (*E. coli* O127:B8) than with others (*Pseudomonas* sp.) (Greisman 1973).

b) In rabbits, during LPS tolerance, administration of TNF is not lethal but still induces fever which shows that the febrile response (TNF and IL-1 mediated ?) can be dissociated from the lethal response (Fraker 1988; Greisman 1966).

c) The C3H/HeJ mouse is refractory to the toxic effect of LPS but sensitive to toxicity by TNF, nevertheless, LPS tolerance can be induced with sublethal doses of TNF and it confers resistance to lethality from further challenge with higher doses of TNF (Cerami 1985, Socher 1988).

2) The TNF-producing capacity of the cells exposed to LPS is altered for a longer period of time than the 21 days that passed since the last dose, thus the low TNF levels that were obtained might be the result of early LPS tolerance still present but that will eventually vanish allowing TNF levels to be high after challenge with an unrelated LPS.

3) The low amount of cross reacting anti-LPS antibodies that were induced by the *E. coli* LPS (Figure 35) were capable of preventing release of TNF. The cross reacting antibodies could have been generated by common epitopes shared by *E. coli* and *S. typhi* LPS. This could be the case with anti-core or anti-lipid A antibodies where high degree of common antigens are present between different enterobacterial species (Lüderitz 1966). The higher levels of anti-*Salmonella* antibodies in the primed groups could be just an enhanced content of natural antibodies that were amplified as part of the general B cell mitogenic stimuli of LPS.

X. INTRAPERITONEAL BACTERIA, TNF LEVELS AND ENDOTOXIN TOLERANCE.

When it first became evident that ip injection of live bacteria and CLP were not inducing high levels of TNF (Figure 37) different possibilities were invoked

including wrong timing for blood sampling, wrong doses of LPS or bacteria and wrong number of perforations in the CLP model, or other inadverted factors whose randomness might prevent these same results from happening again. The models were improved; the sampling time extended until animals first start to die; the estimated lethality was standardize and adjusted to be fairly similar in the 3 models and the experiment was repeated. Results were almost identical (Figure 38).

New explanations were sought. Some of them had been discussed when dealing with the previous conflicting findings such as the lack of correlation between LPS dose, mortality and TNF levels in the naive animal and the lack of proportion between TNF levels and mortality in naive vs. tolerant rats. Other alternatives were also proposed such as the low amounts of free LPS that might actually be available in the abdominal cavity in this models; the formation of less toxic LPS-HDL complex; different kinetics of TNF production, and others that have also been addressed. Even though any particular one or all of them could have certain degree of participation, most of them could actually lead to the possibility of septic shock developing whithout much participation of TNF or even LPS.

We certainly have arrived to an area plagued with uncertainties. It is likely that LPS is shed from the bacteria *in vivo* as constituents of bacterial membrane fragments. Alternatively disruption of bacterial membranes by plasma or tissues might release LPS from other membrane components, making "free" LPS available. On the other hand, it is also possible that artificial extraction of bacterial membranes with the various procedures in use alters the physical state of the LPS (by removing the lipid moieties from the outer membrane, for example) so that extracted LPS have properties that the native LPS do not.

Evidence has been presented suggesting that during gram negative sepsis LPS-HDL complexes may form as a consequence of interaction between bacteria and plasma (Munford 1982). Mathison and others showed that exposure of LPS to serum or plasma results in formation of LPS-HDL complexes. Difference between the administration of the original LPS preparation vs plasma modified (HDL complexed) LPS include a much lower toxicity and peak TNF levels induced by HDL-LPS and a better D/R relation between HDL-LPS complex dose and peak TNF levels. Maximal peak TNF levels were nevertheless comparable between the

two preparations, provided a higher dose of the HDL-LPS complex was injected (Mathison 1988).

In a report already quoted, the net inflow of endotoxin after caecal perforation never exceeded 300 ng/kg per hour and minimal amounts reached the arterial system (Olofsson 1986), so if as Mathison reports, peak TNF levels induced by HDL-LPS reach the minimal detectable production of TNF and provoked no hypotension after 10 μg of HDL-LPS (while TNF production reached maximal capacity with the same amount of non-treated LPS), then the 300 ng/kg per hour are some 30 times under threshold levels for TNF production and hemodynamic consequences.

Even more, the histopathologic and lethal effect of systemic venous injection of endotoxin are abrogated if the same amount of the endotoxin is administered through the intraportal route (Mori 1973), then again, if fair amounts of LPS are drained out of the abdominal cavity through the portal route, the consequences of LPS gaining access to different organs should not be assumed as totally interchangeable.

Our last two experiments show that priming the animals with LPS conferred protection from a lethal challenge with LPS, ip injection of live *E. coli* in $BaSO_4$ and finally, from caecal ligation and puncture. What we call inducing LPS tolerance in fact is, as described in the review, the activation of most of the body defense capabilities through the provocation of an acute phase response. Energy conserving metabolic resources, endocrine status, functions procuring hydro-electrolitic homeostasis, inflammatory and immune capabilities, hepatic and hematopoietic activities, all have been set for optimal performance through the effect of LPS-induced endogenous mediators (including TNF).

It is also relevant to remember that the agents that have shown to prime mice for increased TNF production (BCG, zymosan, *C. parvum*, etc.) also increase resistance to bacterial infection inducing macrophage activation and proliferation, in spite of causing heightened susceptibility to endotoxin lethality (Green 1977, Benacerraf 1959).

Rothstein & Schreiber propose an interesting explanation. Having shown that TNF causes hemorrhagic necrosis in normal skin, only in conjunction with bacteria or their products, they suggest that this combination may represent a primitive defence reaction against infectious organisms or their products since this response effectively eliminates the infecting organisms and may limit the diffusion of toxic products by cutting off the blood supply to the infected region. In order for this necrotizing response to be protective and not damaging, it must be tightly controlled. Three requirements should be fulfilled to provide the host with significant protection against a damaging, destructive self-reaction. First, since the response lacks a distinction between self and non-self, it must rely on a cell that has been already damaged by the infection as an indication of invasion. Secondly, the response will occur only if the pathogen or its product is present. Thirdly, TNF will cause necrosis of the tissue only if both the other components are present simultaneously. Thus, TNF appears to cause hemorrhagic necrosis and lethal shock only when the other components are also present and, in the absence of infections, TNF may be utilized by the host for other functions without causing injury (Rothstein 1987).

CONCLUSIONS

Studying a rat model of endotoxin tolerance and two models of intra-peritoneal gram-negative bacterial infections we gathered data that made questionable the direct translocation of the pathophysiology of endotoxin induced shock and the mechanisms involved in sepsis and Multiple System Organ Failure syndrome. The presumption that TNF is "the" mediator of clinical sepsis and MSOF because TNF has seemingly an outstanding role in endotoxin induced shock was particularly challenged. Too many pieces of the puzzle are still poorly understood and such statements are of no service to the understanding of the pathophysiology of sepsis.

We obtained the following conflictive results:

In rats, the early phase of endotoxin tolerance is associated with diminished serum TNF levels after a subsequent challenge with the same or a different type O-specific endotoxin.

In naive rats, there is no correlation between peak TNF serum levels and mortality after an intra-peritoneal injection of different lethal and non-lethal doses of the same type of LPS. The injection of similar doses of low or high toxicity preparations of LPS also induce similar peak TNF serum levels.

In spite of markedly decreased LPS-induced peak TNF serum levels, rats in the late phase of endotoxin tolerance have similar susceptibility to the lethal effects of LPS as the naive animals.

Exposure to LPS and TNF confers a very important survival advantage after a challenge with intra-peritoneal injection of lethal amounts of live *E. coli* in barium sulfate or caecal ligation and puncture.

Lethal challenge with ip live *E. coli* or caecal ligation and puncture are not associated with significant levels of circulating TNF as in the model of pure endotoxin administration.

FINAL REMARKS

Stephen Gould suggested that nature is not a fully accomplished design, but newly appearing conditions continue to influence its shape and modify its patterns of response (Gould 1980). To what extent are the present technological condition and their effects upon the macro and also the micro environment (the ICU for example) going to inflict selective pressure now that previously lethal pathologies are more and more curable is still a speculative matter.

What is indisputable is that gram-negative bacteria and most probably endotoxin were a factor of evolutionary pressure long before the first proto-gene for cytokine synthesis was around. The inflammatory response and the immune system developed from their beginnings always under the pressure of bacteria and LPS, and under this light the unfolding pattern of host-bacteria interaction should be interpret.

From a practical point of view, our next steps will be to study the behavior of other LPS induced-mediators and other biological effects of endotoxin during early endotoxin tolerance such as PAF, complement system activation, IL-1, IL-6 and thromboxanes.

On the long run the challenge is to understand what to be honest we barely perceive behind the fog.

*... I am better off than he is, for he knows nothing and thinks he knows; I neither know nor think I know. In this latter particular, then, I seem to have slightly the advantage over him....*Socrates

BIBLIOGRAPHY

Aasen AO, Smith-Erichsen N, Amundsen E. Plasma kallikrein-kinin system in septicemia. **Arch Surg 1983;118:343.**

Abernathy RX. Homologous and heterologous resistance in mice given bacterial endotoxins. **J Immunol 1957;78:387-394.**

Aderem AA, Cohen D, Wright SD, et al. Bacterial lipopolysaccharides prime macrophages for enhanced release of arachidonic acid metabolites. **J Exp Med 1986;164:165.**

Aderka D, Holtmann H, Toker L, Hahr T, Wallach D. Tumor necrosis factor induction by Sendai virus. **J Immunol 1986;136:2938-2942.**

Aggarwal BB, Eessalu TE, and Hess PE. Characterization of receptros for human tumor necrosis factor and their regulation by γ-interferon. **Nature 1985;318:665.**

Aguayo SM, Miller YE. Neuropeptides in inflammation and tissue repair. In: **Handbook of Inflammation, Volume 6: Mediators of the Inflammatory Process.** Henson PM, Murphy RC editors. Elsevier, New York, **1989:219-244.**

Aldridge MC, Chadwick SJD, Cheslyn-Curtis S, Rapson N, Dudley HAF. Antibody to endotoxin core glycolipid reverses reticuloendothelial system depression in an animal model of severe sepsis and surgical injury. **J Trauma 1987;27:1166-1172.**

Alexander JW, Babcock GF, Waymack JP. Immunotherapeutic approaches to the prevention and treatment of infection in the surgical patient. In. **Surgical Infectious Diseases.** edited by Howard RJ, Simmons RL. Appleton & Lange, **1988:307-317.**

Almdahl SM, Brox JH, Osterud B. Mononuclear phagocyte thromboplastin and endotoxin in patients with secondary bacterial peritonitis. **Scand J Gastroenterol 1987;22:914-918.**

Alzet osmotic pumps. **Technical Applications File,** Alza Corp. **1986,** Palo Alto, CA.

Amano F, Nishijima M, Akamatsu Y. A monosaccharide precursor of *Escherichia coli* lipid A has the ability to induce tumor-necrosis factor production by a murine macrophage-like cell line, J774.1. **J Immunol 1986;136:4122-4127.**

Anderson FL, Jubiz W, Kralios AC, et al. Plasma prostaglandin levels during endotoxin shock in dogs. **Circulation 1972;46 (Suppl 2):124.**

Andus T, Geiger T, Hirano T, Kishimoto T, Heinrich PC. Action of recombinant human interleukin 6, interleukin 1β and tumor necrosis factor α on the mRNA induction of acute-phase proteins. **Eur J Immunol 1988;18:739-746.**

Apte RN, Ascher O, Pluznik DH. Genetic analysis of generation of serum interferon by bacterial lipopolysaccharide. **J Immunol 1977;119:1898-1902.**

Archer CB, MacDonald DM, Morley J, Page CP, Paul W, and Sanjar S. Effects of serum albumin, indomethacineand histamine H1-antagonists on PAF-acether-induced inflammatory responses in the skin of experimental animals and man. **Br J Pharmacol 1985;85:109-113.**

Arenza-Seisdedos F, Virelizier JL, Fiers W. Interferons as macrophage activating factors. III. Preferential effect of interferon-γ on the interleukin 1 secretory potential of fresh or aged human monocytes. **J Immunol 1985;134:2444-2448.**

Armitage P, Berry G. **Statistical Methods in Medical Research.** Blackwell Scientific Publications, Oxford, **1987.**

Atkins E. Pathogenesis of fever. **Physiol Rev 1960;40:580-646.**

Atkins E. Fever. The old and the new. **J Infect Dis 1984;149:339-48.**

Bach RD. **Johathan Livingston Seagull.** A story. The MacMillan Co. New York, **1970. p,14.**

Balk RA, Bone RC. The Septic Syndrome. Definition and Clinical Implications. **Critical Care Clinics 1989;5:1-8.**

Balkwill F, Burke F, Talbot D, Tavernier J, Osborne R, Naylor S, Durbin H, Fiers W. Evidence for Tumor Necrosis Factor/Cachectin production in Cancer. **Lancet 1987;2:1229-1232.**

Ball HA, Cook JA, Wise WC. Role of thromboxane, prostaglandins and leukotrienes in endotoxic and septic shock. **Intensive Care Med 1986;12:116.**

Bartlett JG, Onderdonk AB, Louie T, et al. Lessons from an animal model of intra-abdominal sepsis. **Arch Surg 1978;113:853.**

Basham TY, Merigan TC. Recombinant interferon-τ increases HLA-DR synthesis and expression. **J Immunol 1983;130:1492.**

Baue AE. Multiple Progressive or sequential systems failure. A syndrome of the 1970s. **Arch Surg 1975;110:779-781.**

Baue AE, Chaudry IH. Prevention of Multiple Systems Failure. **Surg Clin North Am 1980;60:1167-1178.**

Baumgartner JD, Glauser MP, McCutchan JA, Ziegler EJ, van Melle G, Klauber MR, Vogt M, Muehlen E, Luethy R, Chiolero R, Geroulanos S. Prevention of gram-negative shock and death in surgical patients by antibody to endotoxin core glycolipid. **Lancet 1985;2:59-63.**

Becker EL, Showell HJ. The ability of chemotactic factors to induce lysosomal enzyme release. II The mechanism of release. **J Immunol 1974;112:2055-2062.**

Beeson PB. Tolerance to bacterial pyrogens. I. Factors influencing its development. **J Exp Med 1947;86:29-44.**

Benacerraf B. Thorbecke GJ, Jacoby D. Effects of zymosan on endotoxin toxicity in mice. **Proc Soc Exp Biol Med 1959;100:796-799.**

Benedict CR, Grahame-Smith DG. Plasma noradrenaline and adrenaline concentrations and dopamine-B-hydroxylase activity in patients with shock due to septicaemia, trauma and hemorrhage. **Q J Med 1978;47:1.**

Bereiter DA, Zaid AM, Gann DS. Adrenocorticotropin response to graded blood loss in the cat. **Am J Physiol 1984;247:E398.**

Bereiter DA, Zain AM, Gann DS. The effect of rate of hemorrhage on sympathoadrenal catecholamine release in the cat. **Am J Physiol 1985;250:E69.**

Bernton EW, Long JB, Holaday JW. Opioids and neuropeptides: mechanisms in circulatory shock. **Federation Proc 1985;44:290-299.**

Bersten A, Sibbald WJ. Acute Lung Injury in Septic Shock. **Critical Care Clinics 1989:49-79.**

Bertele V, Falanga A, Tomasiak M, Chiabrando C, Cerletti C, de Gaetano G. Pharmacologic inhibition of thromboxane synthetase and platelet aggregation: modulatory role of cyclooxygenase products. **Blood 1984;63:1460-1466.**

Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. **Nature 1986;319:516.**

Besedovsky H, del Rey A, Sorkin E, Dinarello CA. Immunoregulatory feedback between Interleukin-1 and glucocorticoid hormones. **Science 1986;233:652-654.**

Bessin P, Bonet J, Apffel D, Soulard C, Desgroux L, Pelas I, Benveniste J. Acute circulatory collapse caused by platelet-activating factor (PAF-acether) in dogs. **Eur J Pharmacol 1983;86:403-413.**

Beutler B. Orchestration of septic shock by cytokines: The role of cachectin (Tumor Necrosis Factor). **Prog Clin Biol Res 1989;286:219-235.**

Beutler B, Cerami A. Cachectin and tumor necrosis factor as two sides of the same biological coin. **Nature 1986(a);320:584-588.**

Beutler BA, Cerami A. Recombinant Interleukin 1 supressess lipoprotein lipase activity in 3T3-L1 cells. **J Immunol 1985(b);135:3969-3971.**

Beutler B, Krochin N, Milsark IW, Luedke C, Cerami A. Control of cachectin (Tumor Necrosis Factor) synthesis: Mechanisms of endotoxin resistance. **Science 1986(b);232:977-980.**

Beutler BA, Mahony J, Le Trang N, Pekala P, Cerami A. Purification of cachectin, a lipoprotein lipase suppressing hormone secreted by endotoxin induced RAW 264.7 cells. **J Exp Med 1985(c);161:984-995.**

Beutler B, Milsark IW, Cerami AC. Cachectin/Tumor Necrosis Factor: Production, distribution, and metabolic fate *in vivo*. **J Immunol 1985(a);135:3972-3977.**

Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/Tumor necrosis factor protects mice from lethal effect of endotoxin. **Science 1985(c);229:869-871.**

Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA. Interleukin-1 induced biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. **J Exp Med 1984;160:618.**

Björk J, Hugli TE, Smedagard, G. Microvascular effects of anaphylatoxins C3a and C5a. **J Immunol 1985;134:1115-1119.**

Bjornson AB, Altmaier WA, Bjornson C. Changes in humoral components of host defense following burn trauma. **Ann Surg 1977;186:88.**

Blick M, Sherwin SA, Rosenblum M, Gutterman J. Phase I study of recombinant Tumor Necrosis Factor in Cancer patients. **Cancer Res 1987;47:2986-2989.**

Bocci B. The physiological interferon response. In: **The Interferon System,** edited by Baron S, Dianzani F, Stanton GJ, Fleishmann WR. University of Texas Press, Austin, **1987:177.**

Boivin A, Mesrobeanu L: Recherches sur les antigènes somatiques et sur les endotoxines des bactéries. I. Considérations générales et exposé des techniques utilisée. **Rev Immunol 1935;1:553-569.**

Bond RF, Green HD. Peripheral circulation. In: **Handbook of shock and trauma, Vol 1, Basic science.** Altura BM, Lefer AM, Schumer W, editors. Raven Press, New York. **1983:29-49.**

Bone RC, Fischer CJ Jr, Clemmer TP, Slotman GJ, Metz CA, Balk RA, and the Methylprednisolone Severe Sepsis Study Group. A controlled clinical trial of high-dose methylprednisolone in the treatment of severe sepsis and septic shock. **N Eng J Med 1989;317:653-658.**

Bone RC, Fischer CJ Jr, Clemmer TP, Slotman GJ, Metz CA, Balk RA, and the Methylprednisolone Severe Sepsis Study Group.Sepsis syndrome: A valid clinical entity. **Crit Care Med 1989;17:389-393.**

Boraschi D, Censini S, Bartalini M, Tagliabue A. Regulation of arachidonic acid metabolism in macrophages by immune and nonimmune interferons. **J Immunol 1985;135:502-505.**

Border JR. Hypothesis: Sepsis, multiple systems organ failure, and the macrophage. **Arch Surg 1988;123:285-286.**

Borzotta AP, Polk HC. Multiple System Organ Failure. **Surg Clin North Am 1983;63:315-336.**

Braquet P, Hosford D. The potential role of Platelet-activating Factor (PAF) in shock, sepsis and adult respiratory distress syndrome (ARDS). **Prog Clin Biol Res 1989;308:425-439.**

Braquet P, Rola-Pleszczynski M. Platelet-activating Factor and cellular immune responses. **Immun Today 1987;8:345-352.**

Braude AI, Douglas H. Passive immunization against the local Shwartzman reaction. J Immunol 1972;108:505-512.

Braude AI, Douglas H, Davis CE. Treatment and prevention of intravascular coagulation with antiserum to endotoxin. J Infect Dis. 1973;128(Suppl):S157-S164.

Braude AI, Douglas H, Jones J. Experimental production of lethal *Escherichia coli* bacteremia of pelvic origin. J Bacteriol 1969;98:979-991.

Bridenbaugh GA, Flynn JT, Lefer AM. Arachidonic acid in splanchnic artery occlusion shock. Am J Physiol 1976;231:112.

Brockmann DC, Stevens JH, O'Hanley P, et al. The effects of prostaglandin E_1 on the adult respiratory distress syndrome in septic primates. Am Rev Respir Dis 1986;134:885.

Browne MK, Leslie GB. Animal models of peritonitis. Surg Gynecol Obstet 1976;143:738-740.

Brunson KW, Watson DW. *Limulus* amoebocyte lysate reaction with streptococcal pyrogenic exotoxin. Infect Immun 1976;14:1256.

Butler T, Bell WR, Levin J, Linh NN, Arnold K. Typhoid fever. Studies of blood coagulation, bacteremia, and endotoxemia. Arch Intern Med 1978;138:407-410.

Calandra T, Glauser MP, Schellekens J, Verhoef J, and the Swiss-Dutch J5 Immunoglobulin Study Group. Treatment of gram-negative septic shock with human IgG antibody to *Escherichia coli* J5:A prospective, double-blind, randomized trial. J Infect Dis 1988;158:312-319.

Canadian Council on Animal Care. Guide to the care and use of experimental animals. Vol. 1. Addendum, Otawa, 1984, pp 74, 97.

Caridis DT, Reinhold RB, Woodruff PWH, Fine J. Endotoxemia in man. Lancet 1972;1:1381-1386.

Carmona RH, Tsao TC, Trunkey DD. The role of prostacyclin and thromboxane in sepsis and septic shock. Arch Surg 1984;119:189.

Carrico CJ, Meakins JL, Marshall JC, Fry D, Maier RV. Multiple-Organ-Failure Syndrome. Incidence and Problems of MOF Syndrome. Arch Surg 1986;121:196-206.

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 1975;72:3666-3670.

Carvalho EM, Badaro R, Reed SG, Jones TC, Johnson WD. Absence of γ interferon and interleukin 2 production during active visceral leishmaniasis. J Clin Invest 1985;76:2066-2069.

Casey LC, Fletcher JR, Zmudka MI, et al. The role of thromboxane in primate endotoxin shock. J Surg Res 1985;39:140.

Cavender DE, Haskard DO, Joseph B, Ziff M. Interleukin 1 increases the binding of human B and T lymphocytes to edothelial cell monolayers. J Immunol 1986;136:203-207.

Cavender D, Saegusa Y, Ziff M. Stimulation of endothelial cell binding of lymphocytes by tumor necrosis factor. J Immunol 1987;139:1855-1860.

Center DM, Cruikshank WW, Berman JS, Beer DJ. Functional characteristics of histamine receptor-bearing mononuclear cells. I. Selective production of lymphocyte chemoattractant lymphokines with histamine used as a ligand. J Immunol 1983;131:1854-1859.

Cerami A, Ikeda Y, LeTrang N, Hotez PJ, Beutler B. Weight loss associated with an endotoxin-induced mediator from peritoneal macrophages: the role of cachectin (tumor necrosis factor). Immunol Lett 1985;11:173.

Cerra FB. Hypermetabolism, organ failure, and metabolic support. Surgery 1987;101:1-14.

Chapman PB, Lester TJ, Casper ES, Gabrilove JL, Wong GY, Kempin SJ, Gold PJ, Welt S, Warren RS, Starnes HF, Sherwin SA, Old LJ, Oettgen HF. Clinical pharmacology of recombinant human tumor necrosis factor in patients with advanced cancer. **J Clin Oncol 1987;5:1942-1951.**

Charo IF, Yuen C, Perez HD, Goldstein IM. Chemotactic peptides modulate adherence of human polymorphonuclear leukocytes to monolayers of cultured endothelial cells. **J Immunol 1986;136:3412-3419.**

Chedid L, Parrant M, Boyer F. A proposed mechanism for natural immunity to enterobacterial pathogens. **J Immunol 1968;100:292.**

Chen AR. McKinnon KP, Koren HS. Lipopolysaccharide (LPS) stimulates fresh human monocytes to lyse actinomycin D-trteated WEHI-164 target cells via increased secretion of a monokine similar to Tumor Necrosis Factor. **J Immunol 1985;135:3978-3987.**

Chen BDM, Najor R. Macrophage activation by interferon α and β is associated with a loss of proliferative capacity. **Cell Immunol 1987;130:1210-1214.**

Chenoweth D, Cooper SW, Hugli TE, Stewart RW, Blackstone EH, Kirklin JW. Complement activation during cardiopulmonary bypass: Evidence for generation of C3a and C5a anaphylatoxins. **N Engl J Med 1981;304:497-503.**

Chouaib S, Chatenoud L, Klatzmann D, Fradelizi D. The mechanisms of inhibition of human IL-2 production. II. PGE_2 induction of suppressor T Lymphocytes. **J Immunol 1984;132:1851-1857.**

Clark IA, Hunt NH, Butcher GA, Cowden WB. Inhibition of murine malaria *(Plasmodium chabaudi)* in vivo by recombinant interferon-τ or tumor necrosis factor, and its enhancement by butylated hydroxyanisole. **J Immunol 1987;139:3493-3496.**

Claybaugh JR, Share L. Vasopressin, renin and cardiovascular responses to continuous slow hemorrhage. **Am J Physiol 1973;224:519.**

Cochrane CG, Griffin JH. The biochemistry and pathophysiology of the contact system of plasma. **Adv Immunol 1982;33:241-304.**

Colditz IG. Kinetics of tachyphylaxis to mediators of acute inflammation. **Immunology 1985;55:149-156.**

Colditz IG, Movat HZ. Desensitization of acute inflammatory lesions to chemotaxins and endotoxin. **J Immunol 1984a;133:2163-2168.**

Colditz IG, Movat HZ. Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxins and chemotaxinigens. **J Immunol 1984b;133:2169-2173.**

Collart MA, Belin D, Vassali JD, de Kossodo S, Vassali P. τ-Interferon enhances macrophage transcription of the tumor necrosis facotr/cachectin, interleukin 1, and urokinase genes which are controlled by short lived repressors. **J Exp Med 1986;164:2113-2118.**

Collier JR, Herman AB, Vane JR. Appearance of prostaglandins in renal venous blood of dogs in response to acute systemic hypotension produced by bleeding or endotoxin. **J Physiol 1973;230:14P.**

Collins T, LaPierre LA, Fiers W, Strominger JL, Pober JS. Recombinant human tumor necrosis factor increases factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts *in vitro*. **Proc Natl Acad Sci USA 1986;83:446.**

Colton T. Statistical Inference, In: **Statistics in medicine.** Little, Brown and Co. Boston, **1974:99-218.**

Cook JA, Wise WC, Halushka PV. Elevated thromboxane levels in the rat during endotoxic shock: Protective effects of imidazole, 13-azaprostanoic acid or essential fatty acid deficiency. **J Clin Invest 1980;65:227.**

Cross AS, Sadoff JC, Kelly N, Bernton E, Gemski P. Pretreatment with recombinant murine tumor necrosis factor α/cachectin and murine interleukin 1 α protects mice from lethl bacterial infection. J Exp Med 1989:169:2021-2027.

Curtis T, Lefer A. Protective actions of naloxone in hemorrhagic shock. Am J Physiol 1980;239:H416.

Cuturi MC, Murphy M, Costa Giomi MP, Weinmann R, Perussia B, Trinchieri G. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. J Exp Med 1987;165:1581.

Cybulsky MI, Colditz IG, Movat HZ. The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. Am J Pathol 1986;124:367-372.

Dahlen S-E, Bjork J, Hedqvist P, Arfors K-E, Hammarstrom S, Lindgren JA, Samuelsson B. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: *In vivo* effect with relevance to the acute inflammatory response. Proc Natl Acad Sci USA 1981;78:3887-3891.

Damas P, Reuter A, Gysen P, Demonty J, Lamy M, Franchimont P. Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. Crit Care Med 1989;17:975-978.

Damonneville M, Wietzerbin J, Pancre V, Joseph M, Delanoye A, Capron A, Auriault C. Recombinant tumor necrosis factor mediates platelet cytotoxicity to *Schistosoma* mansoni larvae. J Immunol 1988;140:3962.

Daniele R, Singh H, Appert HE, Pairent FW, Howard JM. Lymphatic absorption of intraperitoneal endotoxin in the dog. Surgery 1970;67:484-487.

Danner RL, Parrillo JE. The role of endotoxin in human septic shock: Therapeutic potential of lipid A analogs. Prog Clin Biol Res 1989;286:183-200.

Davies CL, Newman RJ, et al. The relationship between plasma catecholamines and severity of injury in man. J Trauma 1984;29:99.

Davies CE, Ziegler EJ, Arnold KF. Neutralization of meningococcal endotoxin by antibody to core glycolipid. J Exp Med 1978;147:1007-1017.

Dayer JM, Beutler B, Cerami A. Cachectin/Tumor Necrosis Factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. J Exp Med 1985;162:2163.

Debets JMH, Buurman WA, van der Linden CJ. The role of tumor necrosis factor/cachectin in septic shock. Prog Clin Biol Res 1989;308:463-466.

Debets JMH, Kampmeijer R, van der Linden MPMH, Buurman WA, van der Linden CJ. Plasma tumor necrosis factor and mortality in critically ill septic patients. Crit Care Med 1989;17:489-494.

Deby-Dupont G, Braun M, Lamy M, et al. Thromboxane and prostacyclin release in adult respiratory distress syndrome. Intensive Care Med 1987;13:167.

Desiderio JV, Kiener PA, Lin P-F, Warr GA. Protection of mice against *Listeria monocytogenes* infection by recombinant human tumor necrosis factor α. Infect Immun 1989;57:1615-1617.

Desiderio JV, Kiener PA, Marek F, Rodgers GH. Dissociation of TNF production from acute lethality in mice following dministration of toxic and non-toxic forms of Lipid A. J Leuk Biol 1987;42(Abstract):421.

Dinarello CA. Interleukin 1 and the pathogenesis of the acute-phase response. N Eng J Med 1984;311:1413-1418.

Dinarello CA. Biology of interleukin 1. FASEB J 1988;2:108-115.

Dinarello CA, Bodel PT, Atkins E. The role of the liver in the production of fever and in pyrogenic tolerance. J Exp Med 1968;81:334-344.

Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA, O'Connor JV. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. **J Exp Med 1986;163:1433-1450.**

Dinarello CA, Wolff SM. Molecular basis of fever in humans. **Am J Med 1982;72:799-819.**

Dinarello CA. Okusawa S, Gelfand JA. Interleukin-1 induces a Shock-like state in rabbits: Synergism with Tumor Necrosis Factor and the effect of Cyclooxygenase inhibition. **Prog Clin Biol Res 1989;286:243-263.**

Djeu J, Blanchard DK, Halkias D, Friedman H. Growth inhibition of Candida albicans by human polymorphonuclear neutrophils. Activation by interferon-γ and tumor necrosis factor. **J Immunol 1986;137:2980.**

Dobber TW, Wu MS, Robbins JC, Choy BM, Chang MN, Shen TY. Platelet activating factor (PAF) involvement in endotoxin-induced hypotension in rats. Studies with PAF receptor antagonist kadsurenone. **Biochem Biophys Res Commun 1985;127:799-808.**

Duchateau J, Hass M, Schreyen H, Radoux L, Sprangers I, Noel FX, Braun M, Lamy M. Complement activation in patients at risk of developing the adult respiratory distress syndrome. **Am Rev Resp Dis 1984;130:1058-1064.**

Dunn DL, Ferguson RM. Immunotherapy of gram-negative bacterial sepsis: Enhanced survival in a guinea pig model by use of rabbit antiserum to *Escherichia coli* J5. **Surgery 1982;92:212-219.**

Dunn DL, Bogard WC, Cerra FB. Efficacy of type-specific and cross-reactive murine monoclonal antibodies directed against endotoxin during experimental sepsis. **Surgery 1985;98:283-289.**

Durum SK, Oppenheim JJ. Macrophage-Derived mediators: Interlukin 1, Tumor Necrosis Factor, Interleukin 6, Interferon, and related cytokines. In: **Fundamental Immunology. Paul WE (editor),** Raven Press Ltd. New York **1989:621-638.**

Elias JA, Gustilo K, Baeder W, Freundlich B. Synergistic stimulation of fibroblast prostaglandin production by recombinant interleukin 1 and tumor necrosis factor. **J Immunol 1987;138:3812-3816.**

Elin RJ, Knowles R, Barth WF, Wolff SM. Lack of specificity of the *Limulus* lysate test in the diagnosis of pyrogenic arthrits. **J Infect Dis 1978;137:507-**

Elin RJ, Robinson RA, Levine AS, Wolff SM. Lack of clinical usefulness of the *limulus* test in the diagnosis of endotoxemis. **N Eng J Med 1975;293:521-524.**

Elin RJ, Sandberg AI, Rosenstreich DL. Comparison of the pyrogenicity, *Limulus* activity, mitogenicity, and complement reactivity of several bacterial endotoxins and related compounds. **J Immunol 1976;117:1238.**

Elin RJ, Wolff SM. Nonspecificity of the *Limulus* amoebocyte lysate test: Positive reaction with polynucleotides and proteins. **J Infect Dis 1973;128:349.**

Emerson TW. Participation of endogenous vasoactive agents in the pathogenesis of endotoxic shock. **Adv Exp Med Biol 1974;23:25.**

England WC, Byrnes GJ, Gann DS. The pituitary adrenocortical response to hemorrhage depends on the time of day. **Endocrinology 1982;110:1856.**

England WC, Bereiter DF, Gann DS. Sympathetic control of adrenal secretion after hemorrhage in awake dogs. **Am J Physiol 1986;251:R341.**

van Epp DE, Kutvirt SL. Modulation of human neutrophil adherence by β-endorphin and met-enkephalin. **J Neuroimmunol 1987;15:219.**

Etienne A, Hecquet F, Soulard C, Spinnewyn B, Clostre F, Braquet P. *In vivo* inhibition of plasma protein leakage and *Salmonella enteritidis*-induced mortality in the rat by a specific PAF acether antagonist BN 52021. **Agents Actions 1985;17:368-370.**

Fast DJ, Schlievert PM, Nelson RD. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. **Infect Immun 1989;57:291-294.**

Feist W, Ulmer AJ, Musehold J, Brade H, Kusumoto S, Flad H-D. Induction of Tumor Necrosis Factor-α release by Lipopolysaccharide and defined Lipopolysaccharide partial structures. **Immunobiol. 1989;179:293-307.**

Feurestein G, Hallengeck JM. Leukotrienes in health and disease. **FASEB J 1987;1:186-192.**

Fink MP. Role of Prostaglandins and related compounds in the pathophysiology of endotoxic and septic shock. **Sem Resp Med 1985;7:17-23.**

Fisch H, Gifford GE. In vitro production of rabbit macrophage tumor cell cytotoxin. **Int J Cancer 1983;32:105-112.**

Flegel WA, Wolpl A, Männel DN, Northoff H. Inhibition of Endotoxin-induced activation of human monocytes by human lipoproteins. **Infect Immun 1989;57:2237-2245.**

Flick DA, Gifford GE. Production of tumor necrosis factor in unprimed mice: mechanism of endotoxin-mediated tumor necrosis. **Immunobiology 1986a;171:320-328.**

Flick DA, Gifford GE. Pharmacokinetics of murine tumor necrosis factor. **J Immunopharmacol 1986b;8:89-97.**

Flynn JT, Bridenbaugh Ga, Lefer AM. Release of prostaglandin $F_{2\alpha}$ during splanchnic artery occlusion shock. **Am J Physiol 1976;230:684.**

Ford-Hutchinson AW. Leukotrienes: their formation and role as inflammatory mediators. **Federation Proc 1985;44:25-29;1985.**

Fox CC Jr, Lasker SE. Protection by histamine and metabolites in anaphylaxis, scalds and endotoxin shock. **Am J Physiol 1962;202:111-113.**

Fraker DL, Langstein HN, Norton JA. Passive immunization against tumor necrosis factor partially abrogates interleukin 2 toxicity. **J Exp Med 1989;170:1015-1020.**

Fraker DL, Stovroff MC, Merino MJ, Norton JA. Tolerance to tumor necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. **J Exp Med 1988;168:95-105.**

Freudenberg MA, Bog-Hansen T, Back U, Galanos C. Interaction of lipopolysaccharides with plasma high-density lipoprotein in rats. **Infect Immun 1980;28:373-380.**

Freudenberg MA, Galanos C. Induction of tolerance to lipopolysaccharide (LPS)-D-Galactosamine lethality by pretreatment with LPS is mediated by macrophages. **Infect Immun 1988;56:1352-1357.**

Freudenberg MA, Kleine B, Galanos C. The fate of lipopolysaccharide in rats: Evidence for chemical alteration in the molecule. **Rev Infect Dis 1984;6:483-487.**

Friedman RM. Interferons. In: **Textbook of Immunology.** Edited by Oppenheim JJ, Shevach EM. Oxford Press, New York, 1988.

Fry DE, Pearlstein L, Fulton RL, Polk HC. Multiple System Organ Failure. The role of uncontrolled infection. **Arch Surg 1980;115:136-140.**

Fulton RL, Jones CE. The cause of post-traumatic pulmonary insufficiency in man. **Surg Gynecol Obstet 1975;140:179.**

Gahhos FN, Chiu RCJ, Hinchey EJ, Richards GK. Endorphins in septic shock. Hemodynamic and endocrine effects of an opiate receptor antagonist and agonist. **Arch Surg 1982;117:1053-1057.**

Gahring LC, Daynes RA. Desensitization of animals to the inflammatory effects of ultraviolet radiation is mediated through mechanisms which are distinct from those responsible for endotoxin tolerance. **J Immunol 1986;136:2868-2874.**

Galanos C, Freudenberg MA, Jay F, Nerkar D, Veleva K, Brade H, Strittmatter W. Immunogenic properties of Lipid A. **Rev Infect Dis 1984;6:546-552.**

Galanos C, Freudenberg MA, Matsura M, Coumbos A. Hypersensitivity to endotoxin and mechanisms of host-response. **Prog Clin Biol Res 1989;272:295-308.**

Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of endotoxin. **Proc Natl Acad Sci USA 1979;76:5939-5943.**

Galanos C, Lehmann V, Lüderitz O, Rietschel ET, Westphal O, Brade H, Brade L, Freudenberg MA, Hansen-Hagge T, Lüderitz T, McKenzie G, et al. Endotoxic properties of chemically synthesized lipid A part structures. Comparison of synthetic lipid A precursor and synthetic analogues with biosynthetic lipid A precursor and free lipid A. **Eur J Biochem 1984;140:221-227.**

Galanos C, Rietschel ET, Lüderitz O, Westphal O. Interaction of lipopolysaccharides and lipid A with complement. **Eur J Biochem 1971;19:143-152.**

Gamble JR, Harlan JN, Klebanoff SJ, Vadas MA. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. **Proc Natl Acad Sci USA 1985;82:8667.**

Gann DS, Amaral JF. Endocrine and metabolic responses to injury. In: **Principles of Surgery.** Schwartz SI, Shires GT, Spencer FC (editors) McGraw-Hill, New York, **1989:1-68.**

Gardner R, Chater BV, Brown DL. The role of complement in endotoxin shock and disseminated intravascular coagulation. Experimental observations in the dog. **Br J Hematol 1974;28:393-401.**

Garskill HV. Continuous infusion of tumor necrosis factor: mechanisms of toxicity in the rat. **J Surg Res 1988;44:664-671.**

Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. Interferon $\beta 2$/B cell stimulatory factor, Type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. **Proc Natl Acad Sci USA 1987;84:7251-7255.**

Gauldie J, Richards C, Northermann W, Fey G, Baumann H. IFN$\beta 2$/BSF2/IL-6 is the monocyte-derived HSF that regulates receptor-specific acute phase gene regulation in hepatocytes. **Ann NY Acad Sci 1989;557:46-59.**

Gifford GE, Lohmann-Matthes ML. Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharides. **J Natl Can Inst 1987;78:121.**

Gilbert VE, Braude AI. Reduction of serum complement in rabbits after injection of endotoxin. **J Exp Med 1962;116:477-490.**

Gillis S. T-Cell derived Lymphokines. In: **Fundamental Immunology,** Paul WE (editor), Raven Press Ltd. New York **1989:621-638.**

Ghebrehiwet B, Randazzo BP, Dunn JT, Silverberg M, Kaplan AP. Mechanisms of activation of the classical pathway of complement by Hageman factor fragment. **J Clin Invest 1983;71:1450-1456.**

Goetzl EJ, Brindley LL, Goldman DW. Enhancement of human neutrophil adherence by synthetic leukotriene constituents of the slow-reacting substance of anaphylaxis. **Immunology 1983;50:35-41.**

Golenbock DT, Will JA, Raetz CRH, Proctor RA. Lipid X ameliorates pulmonary hypertension and protects sheep from death due to endotoxin. **Infect Immun 1987;55:2471-2476.**

Gollan F, McDermott J. Total hypothermic blood exchange in acute endotoxin shock. **Resuscitation 1979;7:229-236.**

Goodman MG, Chenoweth DE, Weigle WO. Induction of interleukin 1 secretion and enhancement of humoral immunity by binding of human C5a to macrophage surface C5a receptors. **J Exp Med 1982;156:912-917.**

Gould SJ. *The flamingo's* smile. **Reflections in Natural History.** Norton and Co. New York, 1985.

Gowen M, Wood DD, Ihrie EJ, McGuire MKB, Russel RGG. An interleukin-1 like factor stimulates bone resorption in vitro. **Nature 1983;306:378.**

Grant JA, Settle L, Whorton EB, Dupree E. Complement-mediated release of histamine from human basophils. II. Biochemical characterization of the reaction. **J Immunol 1976;117:450-456.**

Green HD, Bond RF, Papela CE, et al. Competition between intrinsic and extrinsic controls of resistance vessels of major vascular beds during hemorrhagic hypotension and shock. In: **Advances in shock research, vol. 3.** Lefer AM, Saba TM, Mela LM. editors. Allan Liss, New York, 1980:77-104.

Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines. **J Immunol Methods 1984, 70:257-268.**

Green S, Dobrjansky A, Chiasson MA, Carswell E, Schwartz MK, Old LJ. *Corynebacterium parvum* as the priming agent in the production of tumor necrosis factor in the mouse. **J Natl Cancer Inst 1977;59:1519-1522.**

Greisman SE. Mechanism of endotoxin tolerance: effect of exchange transfusion [abstract]. **J Clin Invest 1972;51:39a.**

Greisman SE, Hornick RB. Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. **Proc Soc Exp Biol Med 1969;131:1154-1158.**

Greisman SE, Hornick RB. Endotoxin tolerance. In: **The role of immunological factors in infectious, allergic, and autoimmune processes.** Beers RF, Bassett EG (editors), New York, Raven Press, 1976, pp 43-63.

Greisman SE, Hornick RB. Mechanisms of endotoxin tolerance with special reference to man. **J Infect Dis 1973;128(Suppl.):S265-S276.**

Greisman SE, Hornick RB, Wagner HN, Woodward WE, Woodward TE. The role of endotoxin during typhoid fever and tularemia in man. IV. The integrity of the endotoxin tolerance mechanisms during infection. **J Clin Invest 1969;48:613-629.**

Greisman SE, Wagner HN, Iio M, Hornick RB. Mechanisms of endotoxin tolerance. II. Relationship between endotoxin tolerance and reticuloendothelial system phagocytic activity in man. **J Exp Med 1964;119:241-265.**

Greisman SE, Woodward WE. Mechanisms of endotoxin tolerance. III. The refractory state during continuous intravenous infusions of endotoxin. **J Exp Med 1965;121:911-933.**

Greisman SE, Young E, Woodward WE. Mechanisms of endotoxin tolerance. IV. Specificity of the pyrogenic refractory state during continuous intravenous infusion of endotoxin. **J Exp Med 1966;124:983-1000.**

Greisman SE, Young EJ, Carozza FA. Mechanisms of endotoxin tolerance. V. Specificity of the early and late phase of pyrogenic tolerance. **J Immunol 1969;103:1223-1236.**

Greisman SE, Hornick RB, Wagner HN, Woodward WE, Woodward TE. The role of endotoxin during typhoid fever and tularemia in man. IV. The integrity of the endotoxin tolerance mechanisms during infection. **J Clin Invest 1969;48:61.**

Greisman SE, Young EJ, Dubuy B. Mechanisms of endotoxin tolerance. VIII. Specificity of serum transfer. **J Immunol 1973;111:1349-1360.**

Groeger JS, Carlon GC, Howland WS. Naloxone in septic shock. **Crit Care Med 1983;2:650.**

Halushka PV, Reines D, Barrow, et al. Elevated plasma 6-keto-prostaglandin $F_{1\alpha}$ in patients in septic shock. **Crit Care Med 1985;13:451.**

Hamilton TA, Somers SD, Becton DL, Celada A, Scheiber RD, Adams DO. Analysis of deficiencies in IFN-τ-mediated priming for tumor cytotoxicity in peritoneal macrophages from A/J mice. **J Immunol 1986;137:3367-3371.**

Hammerschmidt DE, Hudson LD, Weaver LJ, Craddock PR, Jacob HS. Association of complement activation and elevated plasma-C5a with adult respiratory distress symdrome: Pathophysiological relevance and possible prognostic value. **Lancet 1980;1:947-949.**

Hansch GM, Seitz M, Martinotti G, Betz M. Rautenberg EW and Gemsa D. Macrophages release arachidonic acid, prostaglandin E_2 and thromboxane in response to late complement components. **J Immunol 1984;133:2145-2150.**

Hase S, Rietschel ET. Isolation and analysis of the lipid A backbone: Lipid A structure of lipopolysaccharides from various bacterial groups. **Eur J Biochem 1976;63:101-107.**

Havell EA. Production of tumor necrosis factor during murine listeriosis. **J Immunol 1987;139:4225.**

Havell EA. Evidence that tumor necrosis factor has an important role in antibacterial resistance. **J Immunol 1989;143:2894-2899.**

Heflgott DC, May LT, Sthoeger Z, Tamm I, Sehgal PB. Bacterial lipopolysaccharide (endotoxin) enhances expression and secretion of β2 interferon by human fibroblasts. **J Exp Med 1988;66:1300-1309.**

Heflin AC, Brigham KL. Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. **J Clin Invest 1981;68:1253-1260.**

Hesse DG, Tracey KJ, Fong Y, Manogue KR, Palladino MA, Cerami A, Shires GT, Lowry SF. Cytokine appearance in human endotoxemia and primate bacteremia. **Surg Gynecol Obstet 1988;166:147-153.**

Hinshaw LB, Vick JA, Carlson CH, Fan YL. Role of histamine in endotoxin shock. **Proc Soc Exp Biol Med 1960;104:379-381.**

Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. **Nature 1986;324:73.**

Hoffman M, Weinberg JB. Tumor necrosis Factor-α induces increased hydrogen peroxide production and Fc receptor expression, but not increased Ia antigen expression by peritoneal macrophages. **J Leuk Biol 1987;42:704-707.**

Holaday JW, Faden AI. Naloxone reversal of endotoxin hypotension suggests role of endorphins in shock. **Nature 1978;275:450-451.**

Holtman H, Wallach D. Down regulation of the receptors for tumor necrosis factor by interleukin 1 and 4β-phorbol-12-myristate-13-acetate. **J Immunol 1987;139:1161-1167.**

Homma JY, Matsuura M, Kanegasaki S, et al. Structural requirements of Lipid A responsible for the functions: A study with chemically synthesized lipid A and its analogues. **J Biochem 1985;98:395-406.**

Hong SC, Levine L. Stimulation of prostaglandin synthesis by bradykinin and thrombin and their mechanisms of action on MC5-5 fibroblasts. **J Biol Chem 1976;251:5814-5816.**

Honn KV, Busse WD, Sloane BF. Prostacyclin and thromboxanes: implications for their role in tumor cell metastasis. **Biochem Pharmacol 1983;32:1-11.**

Hoover RL, Briggs RT, Karnovsky MJ. The adhesive interaction between polymorphonuclear leukocytes and endothelial cells *in vitro*. **Cell 1978;14:423-428.**

Hornick RB, Greisman SE. On the pathogenesis of typhoid fever. **Arch Intern Med 1978:138:357-359.**

Howard RJ. Microbes and their pathogenicity. In. **Surgical Infectious Diseases**. Howard RJ, Simmons RL, (editors). Appleton & Lange, **1988:1-13.**

Huber M, Beutler B, Keppler D. Tumor necrosis factor α stimulates leukotriene production in vivo. **Eur J Immunol 1988;18:2085-2088.**

Hurme M. Both interleukin 1 and tumor necrosis factor enhance thymocyte proliferation. **Eur J Immunol 1988;18:1303-1306.**

Inarrea P, Gómez-Cambronero J, Pascual J, Ponte MC, Hernando L, Sánchez-Crespo M. Synthesis of PAF-acether and blood volume changes in gram-negative sepsis. **Immunopharmacology 1985;9:45-52.**

Issekutz AC, Movat KW, Movat HZ. Enhanced vascular permeability and hemorrhage inducing activity of rabbit C5a des Arg: Probable role of PMN-leukocyte lysosomes. **Clin Exp Immunol 1980;41:512-520.**

Issekutz TB, Stoltz JM. Stimulation of lymphocyte migration by endotoxin, tumor necrosis factor, and interferon. **Cell Immunol 1989;120:165-173.**

Jablons DM, McIntosh KJ, Mule JJ, Nordan RP, Rudikoff S, Lotze MT. Induction of Interferon-β2/Interleukin-6 (IL-6) by cytokine administration and detection of circulating interleukin-6 in the tumor-bearing state. **Ann NY Acad Sci 1989;557:157-161.**

Jacob AI, Goldberg PK, Bloom N, Degenshein GA, Kozinn PJ. Endotoxin and bacteria in portal blood. **Gastroenterology 1977;72:1268-1270.**

Jacobson ED, Mehlman B, Kalas JP. Vasoactive mediators as the "trigger mechanism" of endotoxin shock. **J Clin Invest 1964;43:1000-1013.**

Jaffe BM, LaRosa CA, Kimura K. Prostaglandins and surgical diseases: Part I and II. **Curr Prob Surg 1988;October-November:679-747.**

Jelinek DF, Lipsky PE. Enhancement of human B cell proliferation and differentiation by tumor necrosis factor-α and interleukin 1. **J Immunol 1987;139:2970-2976.**

von Jeney N, Gunther E, Jann K. Mitogenic stimulation of murine spleen cells: Relation to susceptibility to *Salmonella* infection. **Infect Immun 1977;15:26.**

Johns MA, Sipe JD, Melton LB, Strom TB, McCabe WR. Endotoxin-associated protein: interleukin-1-like activity on serum amyloid A synthesis and T-lymphocyte activation. **Infect Immun 1988;56:1593-1601.**

Jones CE, Alexander JW, Fisher M. Clinical evaluation of pseudomonas hyperimmune globulin. **J Surg Res 1973;14:87-96.**

Joyce LD, Hau T, Hoffman R, Simmons RL, Lillehei RC. Evaluation of the mechanism of zymosan-induced resistance to experimental peritonitis. **Surgery 1978;83:717-725.**

Kass EH, Porter PJ, McGill MW, Vivaldi E. Clinical and experimental observations on the significance of endotoxemia. **J Infect Dis 1973;128(Suppl):s299-s302.**

Kawakami M, Cerami A. Studies of endotoxin-induced decrease in lipoprotein lipase activity. **J Exp Med 1981;154:631-639.**

Kawasaki H, Moriyama M, Ohtani Y, Naitoh M, Nariuchi H, Tanaka A. Restoration of normal febrile response to endotoxin in pyrogen-tolerant rabbits by injection with human β interferon. **Infect Immun 1987;55:2574-2578.**

Kawasaki H, Moriyama M, Ohtani Y, Naitoh M, Tanaka A, Nariuchi H. Analysis of endotoxin fever in rabbits by using a monoclonal antibody to tumor necrosis factor (cachectin). **Infect Immun 1989;57:3131-3135.**

Keahey TB, Indrisano UJ, Kaliner MA. Measurement of cutaneous vascular permeability. **J Allergy Clin Immunol 1985;75:129.**

Kenzora JL, Perez JE, Bergmann SR, Lange LG. Effects of acetyl glyceryl ether of phosphorylcholine (platelet-activating factor) on ventricular preload, afterload, and contractility in dogs. **J Clin Invest 1984;74:1193-1203.**

Kettelhut IC, Fiers W, Goldberg AL. The toxic effects of tumor necrosis factor *in vivo* and their prevention by cyclooxygenase inuhibitors. **Proc Natl Acad Sci USA 1987;84:4273-4277.**

Kidahl-Andersen O, Nissen-Meyer J. Production and characterization of cytostatic protein factors released from human monocytes during exposure to lipopolysaccharide and muramyl dipeptide. **Cell Immunol 1985;93:375-386.**

Kiener PA, Marek F, Rodgers G, Ling PF, Warr G, Desiderio J. Induction of Tumor Necrosis Factor, IFN-τ, and acute lethality in mice by toxic and non-toxic forms of Lipid A. **J Immunol 1988;141:870-874.**

Kim YB, Watson DW. Biologically active endotoxins from *Salmonella* mutants deficient in O- R-polysaccharides and heptose. **J Bacteriol 1967;94:1320-1326.**

Kimball HR, Melmon KL, Wolff SM. Endotoxin-induced kinin production in man. **Proc Soc Exp Biol Med 1972;139:1078-1082.**

Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal grnulomas during BCG infection. **Cell 1989;56:731-740.**

Kirkland TN, Ziegler EJ. An immunoprotective monoclonal antibody to lipopolysaccharide. **J Immunol 1984;132:2590-2592.**

Kirchner H. Interferons, A group of Multiple Lymphokines. **Springer Semin Immunopathol 1984;7:347-374).**

Kishimoto T, Hirano T. B lymphocyte activation, proliferation, and immunoglobulin secretion. In: **Fundamental Immunology. Second Edition.** Paul DE, editor. Raven Press, New York. **1969:385-411.**

Kitzmiller JL, Lucas WE, Yelenosky PF. The role of complement in feline endotoxin shock. **Am J Obstet Gynecol 1972;112:414-421.**

Koj A. The role of Interleukin-6 as the hepatocyte stimulating factor in the network of inflammatory cytokines. **Ann NY Acad Sci 1989;557:1-7.**

Kotani S, Takada H, Tsukimoto M, Ogawa T, Mori Y. Lipophilic Muramyl Peptides and Synthetic Lipid A analogs as Immunomodualtors. **Prog Immunol 1983;5:1359-1368.**

Kozin F, Cochrane CG. The contact activation system of plasma: Biochemistry and pathophysiology. In: **Inflammation: Basic Principles and Clinical Correlates.** Edited by Gallin JI, Goldstein IM, Snyderman R. Raven Press, New York, **1988:101-120.**

Krane SM, Goldring SR, Dayer J-M. Interactions among lymphocytes, monocytes and other synovial cells in the rheumatoid synovium. In: Pick E, Landy M, editors. **Lymphokines.** Vol. 7, Academic Press, New York. **1982:75-136.**

Krausz MM, Utsunomiya T, Dunham B. Inhibition of permeability edema with imidazole. **Surgery 1982;92:299.**

Kreger BE, Craven DE, Carling PC, McCabe WR. Gram-negative Bacteremia. III. Reassessment of Etiology, Epidemiology and Ecology in 612 Patients. **Am J Med 1980;68:332-343.**

Kreger BE, Craven DE, McCabe WR. Gram-negative Bacteremia. IV. Re-evaluation of clinical features and treatment in 612 Patients. **Am J Med 1980;68:344-355.**

Kunkel SL, Wiggins RC, Chensue SW, Larrick J. Regulation of macrophage tumor necrosis factor by prostaglandin E2. Biochem **Biophys Res Commun 1986;137:404-410.**

Kupper TS, Min K, Sehgal P, Mizutani H, Birchall N, Ray A, May L. Production of IL-6 by keratinocytes. **Ann NY Acad Sci 1989;557:454-465.**

Kurtz RS, Young KM, Czuprynski CJ. Separate and combined effects of recombinant interleukin-1α and τ interferon on antibacterial resistance. **Infect Immun 1989;57:553-558.**

Lachman E, Pitsoe SB, Gaffin SL. Anti-lipopolysaccharide immunotherapy in management of septic shock of obstetric and gynaecological origin. **Lancet 1984;1:981-983.**

Lachmann PJ, Nicol PAE. Studies on the C3b feedback cycle. **Advances in the Biosciences,** Vol 12. Edited by G Raspe, S Bernhard. Pergammon Press, Vieweg, **1974, pp 262-269.**

Lang CH, Spitzer JA. Glucose Kinetics and development of endotoxin tolerance during long-term continuous endotoxin infusion. **Metabolism 1987;36:469-474.**

Larsen NE, Sullivan R. Interaction between endotoxin and human monocytes: Characteristics of the binding of 3H-labeled lipopolysaccharide and ^{51}Cr-labeled lipid A before and after the induction of endotoxin tolerance. **Proc Natl Acad Sci USA 1984;81:3491-3495.**

Lefer AM. Blood-borne humoral factors in the pathophysiology of circulatory shock. **Circ Res 1973;32:129.**

Lefer AM. Eicosanoids as mediators of ischemia and shock. **Federation Proc 1985;44:275-280.**

Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nureddin N. Macrophage-induced angiogenesis is mediated by tumor necrosis factor-α. **Nature 1987;329:630-632.**

Leive L. Release of lipopolysaccharide by EDTA treatment of *E. coli.* **Biochem Biophys Res Commun 1965;21:290-296.**

Lengyel P. Biochemistry of interferons and their actions. **Annu Rev Biochem 1982;51:251.**

Lilly MP, England WC, Gann DS. Responses of cortisol secretion to repeated hemorrhage in the anesthetized dog. **Endocrinology 1983;112:681.**

Loos M, Bitter-Suermann D, Dierich M. Interaction of the first (C_1), the second (C_2) and the fourth (C_4) component of complement with different preparations of bacterial lipopolysaccharides and with lipid A. **J Immunol 1974;112:935-940.**

Lüderitz O, Staub AM, Westphal O. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. **Bacteriol Rev 1966;30:192-255.**

Lugler TA, Charon JA, Colot M, Micksche M, Oppenheim JJ. Chemotactic properties of partially purified human epidermal cell-derived thymocyte activating factor (ETAF) for polymorphonuclear and mononuclear cells. **J Immunol 1983;131:816-820.**

Lundberg JM. Airways responses to tachykinins. In: **The airways in Health and Disease.** Edited by M. Kaliner and PJ Barnes. Marcel Dekker, New York, **1987.**

MacPhee MJ. Cellular and soluble mediators of Delayed-Type Hypersensitivity and their use in correcting anergy. **PhD Thesis,** McGill University, **Montreal 1989:6.4.**

Madonna GS, Peterson JE, Ribi EE, Vogel SN. Early-phase endotoxin tolerance: induction by a detoxified lipid A derivative, monophosphoryl lipid A. **Infect Immun 1986;52:6-11.**

Madonna GS, Vogel SN. Early endotoxin tolerance is associated with alterations in bone marrow-derived macrophage precursor pools. **J Immunol 1985;135:3763-3771.**

Madonna GS, Vogel SN. Induction of Early-phase endotoxin tolerance in athymic (nude) mice, B-cell-deficient (xid) mice, and splenectomized mice. **Infect Immun 1986;53:707-710.**

de Man P, van Kooten C, Aarden L, Engberg I, Linder H, Svanborg Eden C. Interleukin-6 induced at mucosal surfaces by gram-negative bacterial infection. **Infect Immun 1989;57:3383-3388.**

Männel DN, Falk W. Optimal induction of Tumor Necrosis Factor production in human monocytes requires Complete S-Form lipopolysaccharide. **Infect Immun 1989;57:1953-1958.**

Männel DN, Moore RN, Mergenhagen SE. Macrophage as a source of tumoricidal activity (tumor necrotizing factor). **Infect Immun 1980;30:523.**

Marcus AJ. Eicosanoids: Transcellular metabolism. In: **Inflammation: Basic Principles and clinical correlates.** Edited by Gallin JI, Goldstein IM, Snyderman R. Raven Press, Ltd, New York **1988:129-137.**

Markley K, Horakova Z, et al. The role of histamine in burn, tourniquet and endotoxic shock in mice. **Eur J Pharmacol 1975;33:255.**

Marks MI, Ziegler EJ, Douglas H, Corbeil LB, Braude AI. Induction of immunity against lethal *Haemophilus influenzae* type b infection by *Escherichia coli* core lipopolysaccharide. **J Clin Invest 1982;69:742-749.**

Marshall JC, Christou NV, Horn R, Meakins JL. The microbiology of Multiple Organ Failure. **Arch Surg 1988;123:309-315.**

Marshall J, Sweeney D. Microbial Infection and the Septic Response in Critical Surgicall Illness. **Arch Surg 1990; 125:17-23.**

Mathison JC, Ulevitch RJ. The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. **J Immunol 1979;123:2133-2143.**

Mathison JC, Wolfson E, Ulevitch RJ. Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. **J Clin Invest 1988;81:1925-1937.**

Matsushima K, Yodoi J, Tagaya Y, Oppenheim JJ. Down-regulation of interleukin 1 (IL-1) receptor expression by IL-1 and fate of internalized ^{125}I-labeled IL 1β in a human large granular lymphocyte cell line. **J Immunol 1986;137:3183-3188.**

McCabe WR, Bruins SC, Craven DE, Johns M. Cross-reactive antigens: Their potential for immunization-induced immunity to gram-negative bacteria. **J Infect Dis 1977;136(Suppl):161-166.**

McCabe WR, Greely A. Immunization with R mutants of *S. minessota*. I. Protection against challenge with heterologous gram-negative bacilli. **J Immunol 1972;108:6010610.**

McCabe WR, Greely A, DiGenio T, Johns MA. Humoral immunity to type-specific and cross-reactive antigens of gram-negative bacilli. **J Infect Dis 1973;128(Suppl):S284-S289.**

McCabe WR, Kreger BE, Johns M. Type specific and cross-reactive antibodies in gram-negative bacteremia. **N Eng J Med 1972;287:261-267.**

McCabe WR, Olans RN. Shock in gram-negative bacteremia: predisposing factors, pathophysiology, and treatment. In: **Current Clinical Topics in Infectious Diseases.** Remington JS, Swartz MN editors. McGraw-Hill, New York, **1981:121-150.**

McCutchan JA, Wolf JL, Ziegler EJ, Braude AI. Ineffectiveness of single-dose human antiserum to core glycolipid (*E. coli* J5) for prophylaxis of bacteremic, gram-negative infections in patients with prolonged neutropenia. **Schweiz Med Wschr 1983;14:40-45.**

McGiff JC, Itskovitz HD, Terragno A, Wong PY-K. Modulation and mediation of the action of the renal kallikrein-kinin system by prostaglandins. **Fec Proc 1976;35-175-180.**

McIntosh JK, Mule JJ, Jablons DM, Nordan RP, Rudikoff S, Lotze MT, Rosenberg SA. The kinetics of Interleukin-6 induction by the systemic administration of rhTNF-α in mice. **Ann NY Acad Sci 1989;557:572-575.**

McIntyre TM, Zimmerman GA, Satoh K, Prescott SM. Cultured endothelial cells synthesize both platelet activating factor and prostacyclin in response to histamine, bradykinin, and adenosine triphosphate. **J Clin Invest 1985;76:271-280.**

McManus LM, Hanahan DJ, Demopoulos CA, Pinckard RN. Pathobiology of the intravenous infusion of acetyl glyceryl ether phosphorylcholine (AGEPC), a synthetic platelet-activating factor (PAF), in the rabbit. **J Immunol 1980;124:2919-2924.**

McPhaden AR, Whaley K. The complement system in sepsis and trauma. **Br Med Bull 1985;41:281.**

Meager A, Leung H, Wooley J. Assay for tumour necrosis factor and related cytokines. **J Immunol Methods 1989;116:1-17.**

Meakins JL, Hohn DC, Simmons RL, Dunn DL, Hunt TK, Knigton DR. Host Defenses. In. **Surgical Infectious Diseases.** Howard RJ, Simmons RL, (editors). Appleton & Lange, 1988:1-13.

Meakins JL, Wicklund B, Forse RA, McLean PH. The surgical intensive care unit: Current concepts in infection. **Surg Clin North Am 1980;60:117-132.**

Mechanic RC, Frei E, Landy M, Smith WW. Quantitative studies of human leukocytic and febrile response to single and repeated doses of purified bacterial endotoxin. **J Clin Invest 1962;41:162-172.**

Mergenhagen SE, Gewurz H, Bladen HA, Nowotny A, Kasai N, Lüderitz O. Interactions of the complement system with endotoxins from a *Salmonella minnesota* mutant deficient in O-polysaccharide and heptose. **J Immunol 1968;100:227-229.**

Mergenhagen SE, Snyderman R, Gewurz H, Shin SH. Significance of complement to the mechanism of action of endotoxin. **Curr Top Microgiol Immunol 1969;5037-77.**

Merigan TC. Human ingerferon as a therapeutic agent. Current status. **N Eng J Med 1983;308:1530-1531.**

Metcalf DD, Lewis RA, Silbert JE, Rosenberg RD, Wasserman SI, Asuten KF. Isolation and characterization of heparin from human lung. **J Clin Invest 1979;4:1537-1543.**

Meuer S, Ecker U, Hadding U, Bitter-Wuermann D. Platelet-serotonin release by C3a and C5a: Two independent pathways of activation. **J Immunol 1981;126:1506-1509.**

Michie HR, Spriggs DR, Monogue KR, Sherman ML, Revhaug A, O'Dwyer ST, Arthur K, Dinarello CA, Cerami A, Wolff SM, Kufe DW, Wilmore DW. Tumor necrosis factor and endotoxin induce similar metabolic responses in human beings. **Surgery 1988a;104:280-286.**

Michie HR, Monogue KR, Spriggs DR, Revhaug A, O'Dwyer ST, Dinarello CA, Cerami A, Wolff SM, Kufe DW, Wilmore DW. Detection of circulating tumor necrosis factor after endotoxin admnistration. **N Eng J Med 1988b;318:1481-1486.**

Miller RL, Reichgott MJ, Melmon KL. Biochemical mechanisms of generation of bradykinin by endotoxin. **J Infect Dis 1973;128(Supplement):S144-S156.**

Milner KC. Patterns of tolerance to endotoxin. **J Infect Dis 1973;128(Suppl):237-245.**

Minami A, Fujimoto K, Ozaki Y, Nakamura S. Augmntation of host resistance to microbial infections by recombinant human interleukin-1α. **Infect Immun 1989;56:3116-3120.**

Mizel SB. Interleukin 1 and T cell activation. **Immunological Rev 1982;63:51-71.**

Mizel SB, Dayer JM, Krane SM, Mergenhagen SE. Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte activating factor (interleukin 1). **Proc Natl Acad Sci USA 1981;78:2474.**

Mizel SB, Kilian PL, Lewis JC, Paganelli KA, Chizzonite RA. The interleukin 1 receptor. Dynamics of interleukin 1 binding and internalization in T cells and fibroblasts. **J Immunol 1987;138:2906-2912.**

Moncada S, Mullane KM, Vane JR. Prostacyclin release by bradykinin *in vivo*. **Br J Pharmacol 1979;66:969-979.**

Moore RN, Larsen HS, Horohoo DW, Rouse BT. Endogenous regulation of macrophage proliferative expansion by colony stimulating factor-induced interferon. **Science 1984;223:178.**

Mori K, Matsumoto K, Gans H. On the in vivo clearance and detoxification of endotoxin by lung and liver. **Ann Surg 1973;177:159-163.**

Morrison DC, Ryan JL. Endotoxins and disease mechanisms. **Annu Rev Med 1987;38:417-432.**

Morrison DC, Jacobs DM. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. **Immunochemistry 1976;13:813-818.**

Morrison DC, Ulevitch RJ. The effects of bacterial endotoxins on host mediation systems. A review. **Am J Pathol 1978;93:527-617.**

Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. **J Immunol Methods 1983, 65:55-63.**

Movat HZ, Cybulsky MI, Colditz IG, Chan W, Dinarello CA. Acute inflammation in gram-negative infection: Endotoxin, Inrterleukin-1, Tumor Necrosis Factor, and neutrophils. **Federation Proc 1987;46:97-104.**

Munford RS, Andersen JM, Dietschy JM. Sites of tissue binding and uptake in vivo of bacterial lipopolysaccharide-high density lipoprotein complexes. **J Clin Invest. 1981;68:1503-1513.**

Munford RS, Hall CL, Lipton JM, Dietschy JM. Biological activity, lipoprotein-binding behavior, and in vivo disposition of extracted and native forms of *Salmonella typhimurium* lipopolysaccharides. **J Clin Invest 1982;70:877-888.**

Murray HW. Macrophage activation in the acquired immunodeficiency syndrome. In: **Mechanisms of Host Resistance to Infectious Agents, Tumors, and Allografts,** edited by Steinman RM, North RJ. Rockefeller University Press, New York, 1986:321-333.

Nagy S, Nagy A, et al. Histamine level changes in the plasma and tissues in hemorrhagic shock. **Cric Shock 1986;18:227.**

Nakane AT, Minagawa T, Kazuyuki K. Endogenous tumor necrosis factor (cachectin) is essential to host resistance against Listeria monocytogenes infection. **Infect Immun 1988;56:2563.**

Natanson C, Eichenholtz PW, Danner RL, Eichacker PQ, Hoffman WD, Kuo GC, Banks SM, MacVittie TJ, Parrillo JE. Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. **J Exp Med 1989;169:823-832.**

Nathan C. Macrophage secretory products. **J Clin Invest 1987;79:319-326.**

Nathan CF, Kaplan G, Levis WR, Nusrat A, Witmer MD, Sherwin SA, Job CK, Horowitz CR, Steinman RM, Cohn ZA. Local and systemic effects of intradermal recombinant interferon-τ in patients with lepromatous leprosy. **N Eng J Med 1986;315:6-15.**

Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. **J Exp Med 1986;163:1363.**

Nedwin GE, Svedersky LP, Bringman TS, Palladino MA, Goedel DV. Effect of interleukin 2, interferon-τ, and mitogens on the production of tumor necrosis factors α and β. **J Immunol 1985;135:2492-2497.**

Neidwin GE, Svedersky LP, Bringhman TS, Palladino MA, Goeddel DV. Effect of interleukin 2, interferon-τ, and mitogens on the production of tumor necrosis factors α and β. **J Immunol 1985;135:2492-2497.**

Neilson IR, Neilson KA, Junis EJ, Rowe MI. Failure of tumor necrosis factor to produce hypotensive shock in the absence of endotoxin. **Surgery 1989;106:439-43.**

Neugebauer E, Lorenz W, Maroske D, Barthlen W, Ennis M. The role of mediators in septic/endotoxic shock. A meta-analysis evaluating the current status of histamine. **Theor Surg 1987;2:1-28.**

Nies AS, Melmon KL. Variation in endotoxin-induced kinin production and effect between the rabbit and the rhesus monkey. **Am J Physiol 1973;225:230-233.**

Nishijima M, Amano F, Akamatsu Y, Akagawa K, Tokunaga T, Raetz CRH. Macrophage activation by monosaccharide precursors of *Escherichia coli* lipid A. **Proc Natl Acad Sci USA 1985;82:282-286.**

Ogletree ML. Overview of physiological and pathophysiological effects of thromboxane A_2. **Federation Proc 1987;46:133-138.**

Okusawa S, Gelfand JA, Ikejima T, Connolly RJ, Dinarello CA. Interleukin 1 induces a shock-like state in rabbits. **J Clin Invest 1988;81:1162-1172.**

Old LJ. Tumor Necrosis Factor (TNF). **Science 1985;230:630-632.**

Olofsson P, Nylander G, Olsson P. Endotoxin: Routes of transport in experimental peritonitis. **Am J Surg 1986;151:443-447.**

Olson NC, Robinson NE, Scott JB. Effects of histamine on lung water and hemodynamics before and after β-blockade. **J Appl Physiol 1983;54:967-971.**

O'Malley WE, Achinstein B, Shear M. Action of bacterial polysaccharide on tumors. II Damage of sarcoma 37 by serum of mice trated with *Serratia marcescens* polysaccharide, and induced tolerance. **J Nat Cancer Inst 1962;29-1169.**

Onozaki K, Matsushima K, Aggarwal B, Oppenheim JJ. Human interleukin 1 is a cytocidal factor for several tumor cell lines. **J Immunology 1985;135:3962-3968.**

Oppenheim JJ, Stadler RP, Siraganian RP, Mage M, Matieson B. Lymphokines: theri role in lymphocyte responses. Properties of interleukin 1. **Federation Proc 1982;41:257-262.**

Otterness IG, Golden HW, Brissette WH, Seymour PA, Daumy GO. Effects of continuously administered murine Interleukin-1a: Tolerance development and granuloma formation. **Infect Immun 1989;57:2742-2750.**

Pabst MJ, Johnston RB. Bacterial lipopolysaccharide: a mediator of inflammation. In: **Handbook of Inflammation, Volume 6: Mediators of the Inflammatory Process.** Henson PM, Murphy RC editors. Elsevier, New York, **1989:361-393.**

Pace JL, Russell SW, Torres BA, Johnson HM, Gray PW. Recombinant mouse τ-interferon induces the priming step in macrophage activation for tumor cell killing. **J Immunol 1983;130:2011.**

Palladino MA, Patton JS, Figar IS, Shalby MR. Possible relationships between *in vivo* anti-tumour activity and toxicity of tumour necrosis factor-α. In: **Tumour necrosis factor and related cytotoxins.** Ciba Foundation Symposium 131, Bock G, Marsh J, editors. Wiley & Sons, Chichester, **1987a:21-38.**

Palladino MA, Shalaby MR, Kramer SM, Ferraiolo BL, Baughman RA, Deleo AB, Crase D, Marafino B, Aggarwal BB, Figari IS, Liggitt D, Patton JS. Characterization of the antitumor activities of human Tumor Necrosis Factor-α and the comparison with other cytokines: Induction of tumor-specific immunity. **J Immunol 1987b;138:4023-4032.**

Parant M. Effect of LPS on non-specific resistance to bacterial infections. In, **Benefical effects of endotoxins.** Alois Nowotny, Editor. Plenum Press, NY. **1983;179-196.**

Parker MM, Shelhgamer JH, Bacharach SL, Green MV, Natanson C, Frederick TM, Damske BA, Parrillo JE. Profound but reversible myocardial depression in patients with septic shock. **Ann Intern Med 1984;100:483-490.**

Parker MM, Parrillo JE. Septic shock: Hemodynamics and pathogenesis. **JAMA 1983;250:3324.**

Parratt JR. Neurohuumoral agents and their release in shock. In. **Handbook of shock and trauma, Vol. 1: Basic science.** Altura BM, Lefer AM, Schumer W. (editors), Raven, New York, **1983:311-336.**

Parrillo JE, Septic shock: Clinical manifestations, pathogenesis, hemodynamics, and management in a critical care unit. In: Parrillo JE, Ayres SM (eds.) **Mayor issues in critical care medicine.** Baltimore: Williams & Wilkins, **1984:111-125.**

Paul NL, Ruddle NH. Lymphotoxin. **Ann Rev Immunol 1988;6:407-438.**

Peters WP, Johnson MW, Friedman PA, Mitch WE. Pressor effect of naloxone in septic shock. **Lancet 1981;1:529.**

Philip R, Epstein LB. Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. **Nature 1986;323:86-89.**

Pilaro AM, Larskin DL. Accumulation of activated mononuclear phagocytes in the liver following Lipopolysaccharide treatment of rats. **J Leuk Biol 1986;40:29-41.**

Pinckard RN, Ludwig JC, McManus LM. Platelet-activating factors. In. **Inflammation: Basic Principles and Clinical Correlates.** Edited by Gallin JI, Goldstein IM and Snyderman R. Raven Press, Ltd. New York, **1988:139-167.**

Pinckard RN, McManus LM, Demopoulos CA, Halonen M, Clark PO, Shaw JO, Knieker WT, and Hanahan DJ. Molecular pathobiology of acetyl glyceryl ether phosphorylcholine (AGEPC). Evidence for the structural identity with platelet activating factor (PAF). **J Reticuloendothelial Soc 1980;28:95s-103s.**

Plotnikoff NP, Murgo AJ, Miller GC, Corder CN, Faith RE. Enkephalins: immunomodulators. **Federation Proc 1985;44:118-122.**

Polák L, Turk JL. Suppression of the haemorrhagic component of the Shwartzman reaction by anti-complement serum. **Nature 1969;223:738-739.**

Polk HC, Shields CL. Remote organ failure: A valid sign of occult intraabdominal infection. **Surgery 1977;81:310.**

Polk HC, Fry DE. Infection and fever in the surgical patient. In. **Complications in Surgery and their management.** Hardy JD (editor) WB Saunders Co. Philadelphia, **1981:1-19.**

Proctor RA, Will JA, Burhop KE, Raetz CRH. Protection of mice against lethal endotoxemia by a Lipid A precursor. **Infect Immun 1986;52:905-907.**

Raetz CRH, Purcell, S. Takayama K. Molecular requirements for B-lymphocyte activation by *Escherichia coli* lipopolysaccharide. **Proc Natl Acad Sci USA 1983;80:4624-4628.**

Raines EW, Dower SK, Ross R. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. **Science 1989;243:393-396.**

Ramadori G, Van Damme J, Rieder H, zum Büschenfelde KHM. Interleukin 6, the third mediator of acute-phase reaction, modulates hepatic protein synthesis in human and maouse. Comparison with interleukin 1 β and tumor necrosis factor-α. **Eur J Immunol 1988;18:1259-1264.**

Ramsay G, Newman PM, McCartney AC, Ledingham IMcA. Endotoxemia in multiple organ failure due to sepsis. **Prog Clin Biol Res 1988;272:237-246.**

Reichman BL, Handzel ZT, Segal S, Weinstein Y Levin S. Participation of a histamine-Sepharose-adherent subpopulation of human mononuclear cells in the production of leukocyte migration inhibition factor (LIF) in healthy children. **Clin Exp Immunol 1979;37:562-566.**

Regal JF, Pickering RJ. C5a-induced trachealcontraction: Effect of an SRS-A antagonist and inhibitors of arachidonate metabolism. **J Immunol 1981;126:313-316.**

Revel M, Zilberstein A, Chen L, Gothelf Y, Barash I, Novick D, Rubinstein M, Michalevicz R. Biological activities of recombinant human IFN-β2/IL-6 (*E. coli*). **Ann NY Acad Sci 1989;557:144-155.**

Revhaug A, Michie HR, Manson J McK, Watters JM, Dinarello CA, Wolff SM, Wilmone DW. Inhibition of Cyclo-oxygenase attenuates the metabolic response to endotoxin in humans. **Arch Surg 1988;123:162-170.**

Rhodes J, Ivanyi J, Cozens P. Antigen presentation by human monocytes: Effects of modifying MHC class II antigen expression and IL-1 production by using recombinant interferons and corticosteroids. **Eur J Immunol 1986;16:370.**

Ribi E, Cantrell JL, Takayama K, Qureshi N, et al. Lipid A and Immunotherapy. **Rev Inf Dis 1984;6:567-572.**

Rietschel ETh, Schade U, Jensen M, Wollenweber HW, Lüderitz O, Greisman SG. Bacterial endotoxins: Chemical structure, biological activity and role in septicemia. **Scand J Infect Dis [Suppl]. 1982;31:8-21.**

Rietchel ETh, Wollenweber HW, Russa R, Brade H, Sahringer U. Concepts of the chemical structure of Lipid A. **Rev Infect Dis 1984;6:432-438.**

Rocklin RE. Histamine-induced suppressor factor (HSF): effect on migration inhibitory factor (MIF) production and proliferation. **J Immunol 1977;118:1734-1738.**

Rogers TS, Halushka PV, Wise WC, Cook JA. Differential alteration of lipoxygenase and cyclooxygenase metabolism by rat peritoneal macrophages induced by endotoxin tolerance. **Prostaglandins 1986;31:639-650.**

Rosenstreich DL, Vogel SN, Jacques AR, Wahl LM, Oppenheim JJ. Macrophage sensitivity to endotoxin: Genetic control by a single codominant gene. **J Immunol 1978;121:1664-1670.**

Roscher AA, Mangiello VC, Jelsema CL, Moss J. Autoregulation of bradykinin receptors and bradykinin-induced prostacyclin formation in human fibroblasts. **J Clin Invest 1984;74:552-558.**

Rothstein JL, Schreiber H. Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. **Proc Natl Acad Sci USA 1988a;85:607-611.**

Rothstein JL, Schreiber H. Relationship of tumour necrosis factor and endotoxin to macrophage cytotoxicity, haemorrhagic necrosis and lethal shock. In: **Tumour necrosis factor and related cytotoxins.** Ciba Foundation Symposium 131. Bock G, Marsh J, editors. Wiley & Sons, Chichester, **1987:124-139.**

Rothstein JL, Lint TF, Schreiber H. Tumor necrosis factor/cachectin induction of hemorrhagic necrosis in normal tissue requires the fifth component of complement (C5). **J Exp Med 1988b;168:2007-2021.**

Rouzer CA, Cerami A. Hypertriglyceridemia associated with *Trypanosoma brucei* infection in rabbits: role of devective triglyceride removal. **Mol Biochem Parasitol 1980;2:31-38.**

Rubin BY, Anderson SL, Sullivan SA, Williamson BD, Carswell EA, Old LJ. Purification and characterization of a human tumor necrosis factor from the LuKII cell line **Proc Natl Acad Sci USA 1985;82:6637.**

Ruddle NH, Waksman BH. Cytotoxic effect of lymphocyte-antigen interaction in delayed hypersensitivity. **Science 1967;157:1060-1062.**

Sacco-Gibson NA, Filkins JP. Macrophages, monokines, and the metabolic pathophysiology of septic shock. **Prog Clin Biol Res 1989;286:203-218.**

Said SI, Mutt V. Polypeptide with broad biological activity isolated from small intestine. **Science 1970;169:1217.**

Sánchez-Cantú L, Rode HN, Christou NV. Endotoxin tolerance is associated with reduced secretion of Tumor Necrosis Factor. **Arch Surg 1989;124:1432-1436.**

Sánchez-Cantú L, MacPhee MJ, Christou NV, Rode HN. Lack of correlation between TNF levels and LPS lethal effect. (Abstract) **7th International Congress of Immunology, Berlin, 1989:111-19.**

Samuelsson B. Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. **Science 1983:220:568-575.**

Satomi N, Haranaka K Kunii O. Research on the production site of tumour necrosis factor (TNF). **Jpn J Exp Med 1981;51:317-322.**

Saunders RN, Handley DA. Platelet-activating factor antagonists. **Annu Rev Pharmacol Toxicol 1987;27:237-255.**

Sayers TJ, Macher I, Chung J, Kugler E. The production of tumor necrosis factor by mouse boner marrow-derived macrophages in response to bacterial lipopolysaccharide and chemically synthesized monosaccharide precursors. **J Immunol 1987;138:2935-2940.**

Schoeffel U, Lausen M, Ruf G, von Specht BU, Freudenberg N. The overwhelming inflammatory response and the role of endotoxin in early sepsis. **Prog Clin Biol Res 1989;308:371-376.**

Schutze S, Scheurich P, Schluter C, Ucer U, Pfizenmaier K, Kronke M. Tumor necrosis factor-induced changes of gene expression in U937 cells. Differentiation-dependent plasticity of the responsive state. **J Immunol 1988;140;3000-3005.**

Scuderi P, Sterling KE, Lam KS, Finley PR, Ryan KJ, Ray CG, Petersen E, Slymen DJ, Salmon SE. Raised serum levels of tumour necrosis factor in parasitic infections. **Lancet 1986;2:1364-1365.**

Segreti J. Nosocomial Infections and Secondary Infections in Sepsis. **Critical Care Clinics 1989;5:177-189.**

Senterfitt VC, Shands JW. Salmonellosis in mice infected with Mycobacterium tuberculosis BCG I. Role of endotoxin in infection. **J Bact 1968;96:287.**

Seow WK, Thong YH, Ferrante A. Macrophage-neutrophil interactions: contrasting effects of the monokines interleukin-1 and tumor necrosis factor (cachectin) on human neutrophil adherence. **Immunology 1987;62:357-361.**

Shanahan JI, Denburg JA, Fox J, Bienenstock J, Befus D. Mast cell heterogeneity: effects of neuroenteric peptides on histamine release. **J Immunol 1985;135:1331-1337.**

Sharp B, Tsukayama DT, Gekker G, Keane WF, Peterson PK. β-endorphin stimulates human polymorphonuclear leukocyte superoxide production via a stereospecific opiate receptor. **J Pharmacol Exp Ther 1987;242:579.**

Shavit Y, Lewis JW, Terman GW, Gale RP, Liebeskind JC. Opioid peptides mediate the suppressive effect of stress on natural killer cytotoxicity. **Science 1984;223:188.**

Silberstein DS, David JR. Tumor necrosis factor enhances eosinophil toxicity to Schistosoma mansoni larvae. **Proc Natl Acad Sci USA 1986;83:1055.**

Simon RH, Ward PA. Adult Respiratory Distress Syndrome. In: **Inflammation: Basic Principles and Clinical Correlates.** Edited by Gallin JI, Goldstein IM, and Snyderman R. Raven Press, Ltd, New York, **1988:815-827.**

Simpson SQ, Casey LC. Role of Tumor Necrosis Factor in Sepsis and Acute Lung Injury. **Critical Care Clinics 1989;5:27-47.**

Sironi M, Breviario F, Proserpio P, Biondi A, Vecchi A, van Damme J, Dejana E, Mantovani A. IL-1 stimulates IL-6 production in endothelial cells. **J Immunol 1989;142:549-553.**

Slordal L, Warren D, Moore MAS. Effect of recombinant murine tumor necrosis factor on hemopoietic reconstitiution in sublethally irradiated mice. **J Immunol 1989;142:833-835.**

Smith EF, Lefer AM, Smith JB. Influence of thromboxane inhibition on the severity of myocardial ischemia in cats. **Can J Physiol Pharmacol 1980;58:294-300.**

Smith EM, Harbour MD, Blalock JE. Lymphocyte production of endorphins and endorphin mediated immunoregulatory activity. **J Immunol 1985;136:779s.**

Smith RJ, Bowman BJ, Iden SS. Stimulation of the human neutrophil superoxide anion-generation system with 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine. **Biochem Pharmacol 1984;33:973-978.**

Socher SH, Friedman A, Martínez D. Recombinant human tumor necrosis factor induces acute reduction in food intake and body weight in mice. **J Exp Med 1988;167:1957-1962.**

Socrates' Defense (Apology). In: **Plato The Collected Dialogues.** Hamilton E, Cairns H. editors. Bollingen Series LXXI, Princeton, **1961:7-8.**

Solomkin JS, Cotta LA, Satoh PS, Hurst JM, Nelson RD. Complement activation and clearance in acutye illness and injury: Evidence for C5a as a cell-directed mediator of the adult respiratory distress syndrome in man. **Surgery 1985;97:668-678.**

Spink WW, Vick J. A labile serum factor in experimental endotoxin shock: Cross-transfusion studies in dogs. **J Exp Med 1961;114:501-508.**

Spink WW, Davis RB, Potter R, Chartrand S. The initial stage of canine endotoxin shock as an expression of anaphylactic shock: Studies on complement titers and plasma histamine concentration. **J Clin Invest 1964:43:696-704.**

Spitzer JJ, Bagby GJ, Meszaros K, Lang CH. Altered control of carbohydrate metabolism in endotoxemia. **Prog Clin Biol Res 1989;286:145-165.**

Stahl TJ, Cerra FB. Hemodynaimc and Metabolic Responses. In. **Surgical Infectious Diseases.** Howard RJ, Simmons RL, (editors). Appleton & Lange, Norwalk, Con. **1988:209-232.**

Staub NC, Schultz EL, Koike K. Albertine KH. Effect of neutrophil migration induced by leukotriene B_4 on protein permeability in sheep lung. **Federation Proc, 1985;44:30-35.**

Steffen M, Ottman OG, Moore MAS. Simulataneous production of tumor necrosis factor-α and lymphotoxin by normal T cells after induction with IL-2 and anti-T3. **J Immunol 1988;140:2621.**

Stenson WF, Parker CW. Prostaglandins, macrophages, and Immunity: Opinion. **J Immunol 1980;125:1-5.**

Strunk RC, Cole FS, Perlmutter DH, Colten HR. γ-Interferon increases expression of class III complement genes C2 and factor B in human monocytes and in murine fibroblasts. **J Biol Chem 1985;260:15280-15285.**

Stumacker RJ, Kovnat MJ, McCabe WR. Limitations of the usefulness of the *limulus* assay for endotoxin. **N Eng J Med 1973;283:1261-1264.**

Sugarmann BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Shepard HM. Recombinant human Tumor Necrosis Factor-α: Effects on proliferation of normal and transformed cells in vitro. **Science 1985;230:943-945.**

Sultzer BM. Genetic control of leucocyte responses to endotoxin. **Nature 1968;219:1253-1254.**

Sun X, Hsueh W. Bowel necrosis induced by Tumor Necrosis Factor in rats is mediated by Platelet-activating Factor. **J Clin Invest 1988;81:1328-1331.**

Sung SSJ, Bjorndahl JM, Wang CY, Kao HT, Fu SM. Production of tumor necrosis factor/cachectin by human T cell lines and peripheral blood T lymphocytes stimulated by phorbol myristate acetate and anti-CD3 antibody. **J Exp Med 1988;167:937.**

Sung SSJ, Jung LKL, Walters JA, Chen W, Wang CY, Fu SM. Production of tumor necrosis factor/cachectin by human B cell lines and tonsillar B cells. **J Exp Med 1988;168:1539-1551.**

Suter E, Ullman GE, Hoffman RG. Sensitivity of mice to endotoxin after vaccination with BCG. **Proc Soc Exp Biol Med 1958;99:167-169.**

Suzuki M, Mikami T, Matsumoto T, Suzuki S. Gelation of Limulus lysate by synthetic dextran derivatives. **Microbiol Immunol 1977;21:419.**

Takayama K, Qureshi N, Raetz CRH, Ribi EE, Peterson J, Cantrell JL, Pearson FC, Wiggins J, Johnson AG. Influence of fine structure of lipid A on *Limulus* amebocyte lysate clotting and toxic activities. **Infect Immun 1984;45:350-355.**

Takayama K, Qureshi N, Ribi E, Cantrell JL. Separation and characterization of toxic and nontoxic forms of Lipid A. **Rev Infect Dis 1984;6:439-443.**

Takayama K, Ribi E, Cantrell JL. Isolation of a nontoxic lipid A fraction containing tumor regression activity. **Cancer Res 1981;41:2654-2657.**

Tamm I. IL-6. Current research and new questions. **Ann NY Acad Sci 1989;557:478-489.**

Tamladge JE, Phillips H, Schneider M, Rowe T, Pennington R, Bowersox O, Lenz B. Immunomodulatory properties of recombinant murine and human tumor necrosis factor. **Cancer Res 1988;48:544-550.**

Tate WJ, Douglas H, Braude AI, Wells WW. Protection against lethality of E. coli endotoxin with "O" antiserum. **Ann NY Acad Sci 1966;133:746-762.**

Taylor GW, Morris HR. Lipoxygenase pathways. **Br Med Bull 1983;39-219.**

Teng NNH, Kaplan HS, Hebert JM, Moore C, Douglas H, Wunderlich A, Braude AI. Protection against gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. **Proc Natl Acad Sci USA. 1985;1790-1794.**

Thomas L. **The lives of a cell:** Notes of a biology watcher. New York. The Viking Press. **1974:p78.**

Thurman GB, Braude IA, Gray PW, Oldham RK, Stevenson HC. MIF-like activity of natural and recombinant human interferon-γ and their neutralization by monoclonal antibody. **J Immunol 1985;134:305-309.**

Tiengo M. Naloxone in irreversible shock. **Lancet 1980;1:690.**

Till GO, Johnson KJ, Kunkel R, Ward PA. Intravascular activation of complement and acute lung injury. Dependency on neutrophils and toxic oxygen metabolites. **J Clin Invest 1982;69:1126-1135.**

Till GO, Ward PA. Systemic complement activation and acute lung injury. **Federation Proc 1986;45:13-18.**

Tinley NL, Bailey GL, Morgan AP. Sequential system failure after rupture of abdominal aortic aneurysms: An unsolved problem in postoperative care. **Ann Surg 1973;178:117-122.**

Titus RG, Sherry B, Cerami A. Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis. **J Exp Med 1989;170:2097-2104.**

Tonnesen MG, Smedly LA, Henson PM. Neutrophil-endothelial cell interactions: Modulation of neutrophil adhesiveness induced by complement fragments C5a and C5a des Arg and formyl-methionyl-leucyl-phenylalanine. **J Clin Invest 1984;74:1581-1592.**

Tracey KJ, Beutler B, Lowry SF, Merrywether J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ, Zentyella A, Albert JD, Shires GT, Cerami A. Shock and tissue injury induced by recombinant human cachectin. **Science 1986;234:470-474.**

Tracey KJ, Lowry SF, Fahey TJ, Albert JD, Fong Y, Hesse D, Beutler B, Manogue KR, Calvano S, Wei H, Cerami A, Shires GT. Cachectin/Tumor Necrosis Factor induces lethal shock and stress hormone responses in the dog. **Surg Gynecol Obstet 1987;164:415-422.**

Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. **Nature 1987;330:662-664.**

Tracey KJ, Wei H, Manogue KR, Fong Y, Hesse DG, Nguyen HT, Kuo GC, Beutler B, Cotran RS, Cerami A, Lowry SF. Cachectin/Tumor Necrosis Factor induces cachexia, anemia, and inflammation. **J Exp Med 1988;167:1211-1227.**

Ulevitch RJ, Johnston AR. The modification of biophysical and endotoxic properties of bacterial lipopolysaccharides by serum. **J Clin Invest 1978;62:1313-1324.**

Urbaschek R, Urbaschek B. Tumor necrosis factor and interleukin 1 as mediators of endotoxin-induced beneficial effects. **Rev Infect Dis 1987;9:S607-S615.**

Vargaftig BB, Lefort J, Chignard M, Benveniste J. Platelet-activating factor produces bronchoconstriction unrelated to the formation of prostaglandin derivatives. **Eur J Pharmacol 1980;65:185-192.**

Vaughan GM, Becker RA. Cortisol and corticotrophin in burned patients. **J Trauma 1982;22:263.**

Vilcek J, Henriksen-DeStefano D, Palombella D, Tsujimoto M. In: **The biology of the Interferon System,** edited by Steward WE, Schellekens H. Elsevier, New York, **1985:249.**

Vilcek J, Klion A, Henriksen-DeStefano D, Zemtsov A, Davidson DM, Davidson M, Friedman-Kien AE, Le J. Defective γ-interferon production in peripheral blood leukocytes of patients with acute tuberculosis. **J Clin Immunol 1986;6:146-151.**

Vlassara H, Brownlee M, Monogue KR, Dinarello CA, Pasagian A. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. **Science 1988;240:1546-1548.**

Vogel SN, Douches SD, Kaufman EN, Neta R. Induction of colony stimulating factor in vivo by recombinant interleukin 1α and recombinant tumor necrosis factor α. **J Immunol 1987;138:2143-2148.**

Vogel SN, Kaufman EN, Tate MD, Neta R. Recombinant Interleukin-1 alfa and recombinant tumor necrosis factor alfa synergize in vivo to induce early endotoxin tolerance and associated hematopoietic changes. **Infect Immun 1988;56:2650-2657.**

Vogel SN, Moore RN, Sipe JD, Rosenstreich DL. BCG-induiced enhancement of endotoxin sensitivity in C3H/HeJ mice. I. In vivo studies. **J Immunol 1980;124:2004-2009.**

Voller A, Bidwell DE, Bartlett A. The Enzime Linked Immunosorbent Assay (ELISA). In. **A guide to abstracts of microplate applications.** Flonline Publications, Guernsey, **1979.**

Vukajlovich SW, Morrison DC. Activation of murine spleen cells by lipid A: Negative modulation of lipid A mitogenic activity by O-antigen polysaccharide. **J Immunol 1985;135:2546-2550.**

Waage A. Production and clearance of tumor necrosis factor in rats exposed to endotoxin and dexamethasone. **Clin Immunol Immunopathol 1987;45:348-355.**

Waage A, Brandizaeg P, Halstensen A, Kierulf P, Espevik T. The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between Interleukin 6, Interleukin 1, and fatal outcome. **J Exp Med 1989;169:333-338.**

Waage A, Espevik T, Lamvik J. Detection of tumour necrosis factor-like cytotoxicity in serum from patients with septicaemia but not from untreated cancer patients. **Scand J Immunol 1986;24:739-743.**

Waage A, Halstensen A, Espevik T. Association between tumour necorisis factor in serum and fatal outcome in patients with meningococcal disease. **Lancet 1987;1:355-357.**

Waage A, Halstensen A, Shalaby R, Brandtzaeg P, Kierulf P, Espevik T. Local production of Tumor necrosis factor α, Interleukin 1, and Interleukin 6 in meningococcal meningitis. Relation to the inflammatory response. **J Exp Med 1989a;170:1859-1867.**

Walker RI. Possibilities for use of endotoxin in compromised subjects. In, **Benefical effects of endotoxins.** Alois Nowotny, Editor. Plenum Press, NY. **1983;197-212.**

Wallach D, Holtmann H, Engelmann H, Nophar Y. Sensitization and desensitization to lethal effects of tumor necrosis factor and IL-1. **J Immunol 1987;140:2994-2999.**

Walsh SW. Preeclampsia: an imbalance in placental prostacyclin and thromboxane production. **Am J Obstet Gynecol 1985;152:335-340.**

Warren RS, Starnes HF, Gabrilove JL, Oettgen HF, Brennan MF. The acute metabolic effects of tumor necrosis factor administration in humans. **Arch Surg 1987;122:1396-1400.**

Watson J, Kelly K, Largen M, Taylor BA. The genetic mapping of a defective LPS response gene in C3H/HeJ mice. **J Immunol 1978;120:422-424.**

Waymack JP, Miskell P, Gonce SJ, Alexander JW. Immunomodulators in the treatment of peritonitis in burned and malnourished animals. **Surgery 1984;96:308-314.**

Waynforth HB. Methods of obtaining body fluids. In, **Experimental and Surgical technique in the Rat.** Academic Press, London, **1980:62-85.**

Weinberg JB, Hobbs MM, Misukonis MA. Phenotypic characterization of γ interferon-induced human monocyte polykaryons. **Blood 1985;66:1241-1246.**

Weinberg JR, Wright DJM, Guz A. Interleukin-1 and tumour necrosis factor cause hypotension in the conscious rabbit. **Clin Science 1988;75:251-255.**

Weinberg PF, Matthay MA, Webster RO, Roskos KV, Goldstein IM, Murray JF. Biologically active products of complement and acute lung injury in patients with the sepsis syndrome. **Am Rev Resp Dis 1984;130:791-796.**

Weissman C, Weber H. The interferon-activated genes. **Prog Nucleic Acid Res 1986;33:251.**

Westphal O, Lüderitz O, Bister F. Uber die Extraktion von Bakterien mit Phenol-Wasser. **Z Naturforsch 1952;7B:148-155.**

Westphal O. Bacterial endotoxins. The Second Carl Prausnitz Memorial Lecture. **Int Arch Allergy Appl Immunol 1975;49:1-43.**

Weinstein WM, Onderdonk AB, Bartlett JG, Gorbach SL. Experimental intra-abdominal abscesses in rats: Development of an experimental model. **Infect Immun 1974;10:1250-1255.**

White MV, Kaliner MA. Histamine. In: Inflammation: **Basic Principles and Clinical Correlates.** Edited by Gallin JI, Goldstein IM, Snyderman R. Raven Press, New York, **1988:169-193.**

Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock. A review of laboratory models and a proposal. **J Surg Res 1980;29:189-201.**

Wiggins RC, Giclas PC, Henson PM. Chemotactic activity generated from the fifth component of complement by plasma kallikrein of the rabbit. **J Exp Med 1981;153:1391-1404.**

Wildfeuer A, Heymer B, Schleifer KH, Haferkamp O. Investigations on the specificity of the Limulus test for the detection of endotoxin. **Appl Microbiol 1974;28:867.**

Williams Z, Hertogs CF, Pluznik DH. Use of mice tolerant to Lipopolysaccharide to demonstrate requirement of cooperation between macrophages and lymphocytes to generate lipopolysaccharide-induced colony-stimulating factor *in vivo*. **Infect Immun 1983;41:1-5.**

Williamson BD, Carswell EA, Rubin BY, Prendergast JS, Old LJ. Human tumor necrosis factor produced by human B-cell lines: Synergistic cytotoxic interaction with human interferon. **Proc Natl Acad Sci USA 1983;80:5397-5401.**

Winn R, Harlan J, Nadir B, Harker L, Hildebrandt J. Thromboxane A2 mediates lung vasoconstriction byt not permeability after endotoxin. **J Clin Invest 1983;72:911-918.**

Wirth JJ, Kierszenbaum F. Recombinant tumor necrosis factor enhances macrophage destruction of Trypanosoma cruzi in the presence of bacterial endotoxin. **J Immunol 1988;141:286.**

Wolff SM. Biological effects of bacterial endotoxins in man. **J Infect Dis 1973;128(Suppl):s259-s263.**

Wybran J. Enkephalins and endorphins as modifiers of the immune system: present and future. **Federation Proc 1985;44:92-94.**

Yasaka T, Boxer LA, Baehner RL. Monocyte aggregation and superoxide anion release in response to FMLP and PAF. **J Immunol 1982;128:1939-1944.**

Yip YK, Vilcek J. Molecular weight of human γ interferon is similar to that of other human interferons. **Science 1982;215:411-413.**

Young LS, Stevens P, Ingram J. Functional role of antibody against "core" glycolipid of enterobacteriaceae. **J Clin Invest 1975;56:850-861.**

Yourcenar M. **The Abyss.** Farrar, Straus and Giroux, New York **1976.**

Yurt RW, Pruitt BA Jr. Base-line and postthermal injury plasma histamine in rats. **J Appl Physiol 1986;60:1782-1788.**

Zaldivar NM, Scher I. Endotoxin lethality and tolerance in mice: Analysis with the B-lymphocyte-defective CBA/N strain. **Infect Immun 1979;24:127-131.**

Ziegler EJ, Douglas H, Braude AI. Human antiserum for prevention of the local Shwartzman reaction and death from bacterial lipopolysaccharides. **J Clin Invest 1973;52:3236-3238.**

Ziegler EJ, Douglas H, Sherman JE, Davis CE, Braude AI. Treatment of *E. coli* and *Klebsiella* bactermia in agranulocytic animals with antiserum to a UDP-Gal epimerase-deficient mutant. **J Immunol 1973a;111:433-438.**

Ziegler EJ, McCutchan JA, Douglas H, Braude AI. Prevention of lethal pseudomonas bactermia with epimerase-deficient *E. coli* antiserum. **Trans Assoc Am Physicians 1975;88:101-108.**

Ziegler EJ, McCutchan JA, Fierre J, Glauser MP, Sadoff JC, Douglas H, Braude AI. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant Escherichia coli. **N Eng J Med 1982, 307:1225-1230.**

Zuckerman SH, Bendele AM. Regulation of serum Tumor Necrosis Factor in Glucocorticoid-sensitive and -resistant rodent endotoxin shock models. **Infect Immun 1989;57:3009-3013.**