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Neutrophil-Endothelium Interactions in Patients with Systemic Inflammatory Response Syndrome

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ABSTRACT

Multiple Organ Dysfunction Syndrome (MODS) is associated with high mortality in patients admitted to the surgical intensive care unit (SICU). MODS begins with a systemic response described as Systemic Inflammatory Response Syndrome (SIRS). Studies on SIRS patients may provide an insight into the mechanisms by which SIRS progresses to MODS. In this thesis, the interactions between circulating polymorphonuclear neutrophils (PMNs) from patients with SIRS and endothelial cells (ECs) from human umbilical veins were measured in order to elucidate the mechanism for PMN adhesion and subsequent cytotoxicity of the ECs. PMNs from patients with SIRS were compared to PMNs from pre-operative surgical patients without SIRS and with healthy control subjects, *in vitro*. The results showed that PMNs adherence to ECs increased progressively from healthy controls to patients with SIRS. PMN-HUVE cytotoxicity, however, did not show this trend. PMNs from SIRS patients treated with lipopolysaccharide, unlike PMNs from patients without SIRS or healthy controls, showed no increase in PMN-EC adhesion. The results also showed that EC activation with TNF-α and Il-1β led to high levels of PMN-EC adhesion and cytotoxicity, whereas PMN treatment with lipopolysaccharide played a lesser role. Autologous plasma provided significant protection from PMN mediated EC damage. From this data I conclude that activation of the EC by cytokines associated with SIRS is far more important in promoting PMN-EC adhesion and subsequent cytotoxicity than PMN stimulation with lipopolysaccharide and that there are host factors in plasma that modulate this response.

ANALYSE

Le syndrome des déficiences polyviscérales (SDP) est associé à un taux élevé de mortalité, chez les patients admis à l'Unité de soins intensifs chirurgicaux (USIC). Le SDP débute avec une réponse systémique, laquelle est décrite comme étant le syndrome de réaction inflammatoire systémique (SRIS).

Il est possible que des études menées auprès de patients présentant le SRIS puissent permettre de comprendre les mécanismes par lesquels le SRIS évolue vers le SDP. Dans cette thèse, on a mesuré les interactions entre les leucocytes neutrophiles circulants (LNC) chez les patients présentant le SRIS et les cellules endothéliales (CE) provenant des veines ombilicales humaines, en vue d'élucider le mécanisme touchant l'adhésion des LNC ainsi que la cytotoxicité des CE. Des LNC provenant de patients présentant le SRIS ont été comparés, *in vitro*, à des LNC provenant de patients chirurgicaux en phase pré-opératoire qui ne présentent pas le SRIS et avec des sujets témoins en santé. Les résultats ont démontré que l'adhérence des LNC aux CE a augmenté de façon progressive entre les témoins en santé et les patients présentant le SRIS. Cependant, la cytotoxicité des LNC-HUVE n'a pas indiqué cette tendance. Les LNC provenant de patients présentant le SRIS et traités au moyen du complexe glucido-lipido-protéique, contrairement aux LNC provenant de patients ne présentant pas le SRIS ou de témoins en santé, n'ont démontré aucune augmentation en ce qui a trait à l'adhésion des LNC-CE.

Les résultats ont également démontré que l'activation des CE avec le FNT-α (facteur nécrosant des tumeurs) et l'Il-1β a mené à des niveaux élevés d'adhésion et de cytotoxicité des LNC-CE, tandis que le traitement des LNC au moyen du complexe glucido-lipido-protéique a joué un rôle moindre. Le plasma autologue a fourni une protection significative contre le dommage des CE imputable aux LNC.

A partir de cette donnée, j'en viens à la conclusion que l'activation des CE par cytokines, associée avec le SRIS, joue un rôle beaucoup plus important en ce qui a trait au fait de favoriser l'adhésion des LNC-CE et la cytotoxicité subséquente, que ne le fait la stimulation des LNC au moyen du complexe glucido-lipido-protéique et que, de plus, il y a des facteurs hôtes dans le plasma qui modulent cette réponse.

PREFACE

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TABLE OF CONTENTS

LIST OF ABREVIATIONS

BL	basal lamina
CAMs	cell adhesion molecules
ECs	endothelial cells
EDRF	endothelium-derived relaxing factor
FBS	fetal bovine serum
fMLP	N-formylmethionyl-leucyl-phenylalanine
EGF	epidermal growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	guanosine monophosphate
GP	glycoprotein
HAS	human serum albumin
HBSS	Hank's balanced salt solution
HEV	high endothelial venule
HUVE	human umbilical vein endothelium
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
LAD	leukocyte adhesion deficiency
LFA-1	lymphocyte function-associated antigen-1
IL-1β	interleukin-1β
IL-6	interleukine-6
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
MAb	monoclone antibody
MHCs	major histocompatibility antigens
MODS	multiple organ dysfunction syndrome
NO	nitric oxide

PAF	platelet-activating factor
PBS	phosphate-buffered saline solution
PMA	phorbol myristate acetate
PMNs	polymorphonuclear neutrophils
SICU	surgical intensive care unit
SIRS	systemic inflammatory response syndrome
SLe^x	sialyl Lewis X
TNF-α	tumor necroses factor-α
VLA-4	very late antigen 4

INTRODUCTION

General Perspective

The systemic inflammatory response syndrome (SIRS) has been used recently to describe the host systemic inflammatory process, independent of cause, associated with a large number of clinical conditions[1]. The remarkable similarity between the physiologic response in SIRS and multiple organ dysfunction syndrome (MODS) suggests that the mediators responsible for the expression of these clinical syndromes are similar or may be the same[2,3]. Transition from SIRS to clinically defined MODS does not occur in a clear-cut manner because these two entities may represent a continuum. Despite advances in critical care, the mortality of MODS has remained unchanged since the syndrome was characterized two decades ago[2,3], and there are no modalities that can actively reverse established organ failure at the present time[3]. The treatment of these patients consists of metabolic and hemodynamic support until the process reverses itself or death occurs. Therefore, the best management of the surgical patient at risk for MODS is prevention of the syndrome, especially at the systemic inflammatory response stage. A better understanding of the complex interactions of the various mechanisms involved in SIRS/MODS is necessary for development of appropriate therapies to combat the syndrome. Studies on SIRS patients may provide an insight into the mechanisms by which SIRS may progress to MODS, and may have implications in directing therapeutic strategies.

Infection remains the most common clinical event leading to MODS[3]. Polymorphonuclear neutrophils (PMNs) represent the first line of host defense against bacterial infections. Their complex biology involves the ability to sense chemotactic factors, adhere to vascular endothelial cells (ECs), migrate to areas of bacterial invasion of the host, and once there, phagocytose and kill the invading pathogens[4]. The process of exudation and pathogen specific cytotoxicity is subject to multiple biologic controls

which serve to maximize the defensive capacity of PMNs and at the same time limit cellular damage to the host. The EC barrier is susceptible to this damage. A means of influencing readiness for PMN bactericidal activity is cell priming, which is defined as functionally enhanced PMN activities after bacterial lipopolysaccharide (LPS) stimulation, including adherence, secretion of protein and lipid mediators, and N-formylmethionyl-leucyl-phenylalanine (fMLP)-stimulated superoxide release.[5] In addition to LPS, in-vitro studies have implicated tumor necrosis factor-α (TNF-α)[6], interleukins[7,8], arachidonic acid[9], platelet activating factor[10], colony stimulating factors[11], and the process of adhesion itself[4] as PMN priming agents.

In the microvasculature under flow conditions, PMNs roll along the EC surface of the post capillary venule[12]. The selectin class of adhesion molecules (L-, E-, P-selectins) and their ligands (e.g. Sialylated Le^x) are essential for this step[13]. The firm adhesion of PMNs to ECs, following the rolling step, requires both β2-integrin (CD11a/CD18, CD11b/CD18) expression on primed PMNs and intercellular adhesion molecule-1 (ICAM-1) expression on activated ECs[14]. At this point the PMN is at a critical crossroads for beneficial or detrimental reactions: migrating through EC junctions and reaching the pathogen invasion site; or prematurely releasing enzymes and superoxide, causing EC and basement membrane damage, followed by protein and fluid leaking into the interstitial space resulting in organ dysfunction.

ECs are distributed throughout the vasculature. An important finding is that the ECs are not identical throughout the vascular tree[15]. Such differences probably involve not only capillary EC but also veins and arteries[16]. The major biological properties of ECs include regulation of vascular growth, regulation of vessel tone, maintenance of selective vascular permeability, maintenance of hemostatic balance (platelet adhesion, coagulation, fibrinolysis), regulation of inflammatory and immune response, synthesis of stromal components, synthesis and secretion of peptides, and integration and transduction of blood-borne signals[17]. As mentioned above, an important part in inflammation is PMN adherence to EC. It is regulated not only by physical forces such as surface charges, but also by the presence of mediators in the medium (plasma) and specific receptors on the membranes of the involved cells. Cell adhesion molecules

(CAM) including (P- and E-) selectins and ICAM-1 are necessary for EC interaction with PMN, which are upregulated by the action of cytokines, most prominently IL-1β and TNF-α[17,18]. For firm EC-PMN adhesion, ICAM-1 is thought to be one of the most important CAM on EC[19]. Cytokines such as IL-1 and IL-8 are also produced by activated ECs in the inflammatory environment to regulate leukocyte adhesion and migration, or cause EC damage[20].

In summary, MODS is the most common cause of death in SICU. Because of the similarity between the physiologic responses of SIRS and MODS, studies on SIRS patients may provide an insight into the mechanisms by which SIRS may progress to MODS, and may have implications in directing therapeutic strategies. PMN-EC adhesion is the critical crossroads in inflammatory environment, with regulations of CAMs by mediators in milieu.

SIRS/MODS

Systemic Inflammatory Response Syndrome

The term *systemic inflammatory response syndrome* (SIRS) was coined[1] to imply a clinical response arising from a nonspecific insult and includes two or more of the following: (1) temperature greater than 38°C or less than 36°C, (2) heart rate greater than 90 beats per minute, (3) respiratory rate greater than 20 breaths per minute or a Pco_2 less than 32 mm Hg, or (4) white blood cell count greater than 12.0 x 10^9/L or less than 4.0 x 10^9/L or the presence of more than 0.10% immature neutrophils (bands). SIRS characterizes the clinical manifestations of hypermetabolism, often seen after a serious insult, and is proposed to describe this inflammatory process but independent of its cause. SIRS is seen in association with a large number of clinical conditions, such as burns, pancreatitis, ischemia, multitrauma, tissue injury, hemorrhagic shock, immune-mediated organ injury, and the exogenous administration of putative mediators of the inflammatory process (e.g. TNF), as well as an infectious insult[3]. It is likely that similar pathogenesis and pathophysiology underlie the various clinical entities that comprise

SIRS. When SIRS is the results of a confirmed infection, it is termed *sepsis*[1]. In this clinical circumstance, sepsis represents the systemic inflammatory response to infection. Rangel-Frausto et al[21] demonstrated in a prospective study that SIRS, sepsis, severe sepsis, and septic shock represent a hierarchical continuum of an inflammatory response to infection, in another words, the natural history of the inflammatory response to infection. A frequent complication of SIRS is the development of systemic organ dysfunction, including multiple organ dysfunction syndrome[3].

Multiple-Organ Dysfunction Syndrome

During 1950s and 60s, single organ failure, such as renal failure and respiratory failure, was the leading cause of death following major traumatic and surgical insults[22]. In the early 1970s, as the understanding, monitoring, and therapies of disease progressed, significant advances were made, and more patients survived previously lethal insults. With these advances, a new problem arose, namely, multiple organ failure, a progressive deterioration of organ function in patients who were critically ill or injured. Patients usually died of complications of their disease, rather than the disease itself. Multiple organ dysfunction syndrome, previously called multiple organ failure syndrome[3], was initially described as 'sequential system failure' by Tilney et al[23] in the mid 1970s. MODS is often the final complication of a critical illness, and a common pathway to death in the 20th century ICU[24]. A great deal of progress has been made during last 20 years, and new concepts have been developed to guide the battle against this syndrome. Recently, the term SIRS was used to describe the clinical manifestations of nonspecific systemic hypermetabolism and inflammation seen after a serious insult, and to replace 'sepsis syndrome' when infection is not confirmed[1]. It is based on the finding of numerous investigations over the last two decades that positive blood cultures and clinical infection are not necessary to initiate the MODS process[25]. The involved organs in MODS patient display similar patterns of tissue damage on autopsy and are often remote from the initial injury site or septic source, therefore theories concerning the pathophysiologic mechanism involved in MODS focus on common pathways and

interactions between the organ systems, rather than on isolated processes[3,25]. The search for a 'final common pathway' to the development of MODS has been the primary goal of recent research. Once thought to be related solely to cardiovascular instability and poor oxygen delivery, MODS is now recognized as a systemic syndrome mediated by numerous plasma enzyme cascades, cellular elements, and biochemical mediators commonly released and activated in inflammation and/or infection[25]. With accumulated clinical experience, availability of advanced instrumentation, and extensive research into the processes of MODS, different patterns of MODS have been described from etiology [26,27] and pathogenesis[2] points of view. Central to the understanding of MODS is that substances (mediators) and systems initially primed and activated to protect and defend the host actually cause severe tissue damage, shock, and death from MODS[1,2,3,24,25,26,27]. All the insults which cause SIRS can also produce MODS. Dysfunction can be subtle, including the complete failure of an organ (e.g. oliguric renal failure) or the chemical failure of an organ that may or may not result in clinical findings (e.g. an elevated serum creatinine level)[28].

Physiological and Metabolic Responses in SIRS/MODS

The major metabolic change that occurs in SIRS is an initial increase in oxygen consumption[29]. This must be met by an increase in oxygen supply or an ischemic (anaerobic) condition will result. Heart rate and cardiac output increases. Concomitantly, there is a fall in systemic vascular resistance caused by host mediators that can cause vasodilatation[30]. In the early stages of SIRS, the arterial-venous oxygen content difference is normal if oxygen delivery has been maintained by adequate cardiac output, hemoglobin, and arterial oxygen saturation[31]. As MODS begins (especially when sepsis is present), there is a further drop in systemic vascular resistance. It then seems to be a failure of cellular oxygen utilization, which is inappropriate because the oxygen requirement at the cellular level is increased[32]. There are two hypotheses to explain the causes of these changes. The first hypothesis points to the disorder in microcirculatory autoregulation[22]. Variable changes of peripheral vasodilatation within

different regional vascular beds cause maldistribution of flow and mismatch of oxygen delivery/consumption. This concept has some support in animal models but few in human studies[32,33,34]. The second hypothesis comes from the assumption that perfusion is adequate but the extraction failure of oxygen represents a cellular metabolic disturbance[35]. Metabolic reasons that may explain the phenomenon include interstitial edema or mitochondrial dysfunction[22,33,34]. Noticeable changes also occur in carbohydrate, protein, and fat hypermetabolism[29,35].

Pathogenesis of SIRS/MODS

A significant problem in our ability to treat SIRS/MODS is incomplete understanding of the biology and pathophysiology of the syndrome. Whatever stimulus, the cause of SIRS/MODS seems to be an uncontrolled systemic inflammatory response where the interactions and the effects of multiple mediator systems and their by-products are important. The ongoing stimulation of these systems, by the initiating cause or new insults, leads to SIRS and eventually MODS. There are several hypotheses put forth to explain SIRS/MODS on a cellular level, as well as 'multiple-hit' models to explain the behavior of a given patient with SIRS/MODS.

Macrophage/Cytokine Hypothesis

The macrophage/cytokine hypothesis postulates that an excessive or prolonged stimulation of macrophages and PMNs leads to the overproduction of cytokines [such as TNF-α, IL-1, IL-6, interferon-gamma (IFN-γ)] and other products which ultimately result in harmful local and systemic effects, SIRS/MODS.[2] Support for this theory is based on the findings that macrophage products such as TNF, IL-1 and IL-6 are present in the serum of patients with bacteraemia and endotoxemia and that the administration of these cytokines produce syndromes that are indistinguishable from bacterial sepsis[36,37]. Experimentally, the administration of monoclonal antibodies to LPS, TNF, IL-1, IL-6 and IFN-γ have decreased the mortality and the deleterious metabolic responses to infection in animal models[38]. However, initial clinical studies attempting to

block LPS as a proximal mediator in patients with Gram negative sepsis have not been successful[39]. One reason for the fact that therapy directed at individual inflammatory mediators or endotoxin have not been clinically effective may be that many of these substances have overlapping biologic activity and function synergistically to exert their effects[40]. In addition, since many of the cytokines exert their primary effects in a paracrine fashion, random plasma levels of the cytokines may not reflect accurately what is occurring at the tissue level thereby limiting our ability to identify the critical mediators of this syndrome.

Microcirculatory Hypothesis

The microcirculatory hypothesis is multifactorial and contains several overlapping components. The EC is central to the ischemia-reperfusion injury, followed by SIRS/MODS. Numerous studies have shown ECs being active in the regulation of blood flow, coagulation, and inflammatory response in tissue ischemia and injury[41,42,43]. Activated ECs express tissue factor, have increased capacity to find factor VIIa, and activate the extrinsic clotting pathway. They also express surface adhesion molecules such as E-selectin and ICAM-1, promote leukocyte adherence, and secrete leukocyte-activating factors such as IL-1, platelet-activating factor (PAF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-8[35]. Extensive or uncontrolled EC stimulation results in EC and tissue injury[38]. Another possible mechanism within the microcirculatory hypothesis is that tissue injury after ischemia-reperfusion is secondary to the generation of toxic oxygen metabolites during the reperfusion period[44,45]. The major sources of oxygen metabolites are through the tissue's xanthine oxidase pathway and oxygen radicals produced by leukocytes[44,45].

Gut Hypothesis

The gut hypothesis postulates that the gut acts as a reservoir of bacteria and endotoxin which can initiate and/or perpetuate the development of SIRS/MODS[46,47]. The phenomenon, known as translocation where bacteria, endotoxin, and gut-derived inflammatory products pass from the intestinal lumen to the regional lymph nodes and

the systemic circulation, is central to this hypothesis. The loss of intestinal barrier function as a result of infection, injury or hypoperfusion is the critical feature in bacteria translocation from the gut.

The 'Two-Hit' Model

This model proposes two or more sequential insults in the etiology of SIRS/MODS. The initial 'hit' primes the host's inflammatory response. The second and subsequent 'hits' activate this primed response[48,49]. This model satisfies the commonly observed clinical scenario of patients who survive their initial trauma or surgery and develop SIRS or MODS following a subsequent infection. MODS may also occur early in a patient's hospital course following severe infection, bacteraemia or trauma if the initial insult is of sufficient magnitude to both prime and activate the inflammatory response. The magnitude of the insult required to prime the system also appears to be several orders of magnitude less than that needed to activate macrophages and PMNs[50,51]. Consequently, a mild injury that would lead to no obvious clinical sequelae by itself may serve to prime the host such that subsequent insults become lethal.

Future Prospects for SIRS/MODS

The accumulated knowledge about SIRS/MODS gives great hope for the future, though no 'magic bullet' has yet been found. Control of various mediators and their effects will likely become more important and involve multiple agents (against multiple mediators) given at certain key times in the inflammatory response. Initial studies using monoclonal antibodies developed against single mediator, such as TNF-α and IL-1 did not demonstrate a major difference in control of SIRS[52,53]. Interference with PMN adhesion to the EC is another approach being pursued. Therefore, continued research with these approaches will be necessary before effective clinical therapy becomes available.

PMNs and PMN Adhesion Molecules

PMNs

Life Span of PMN

PMNs develop from undifferentiated precursors into cells capable of responding to and destroying a variety of microbial pathogens. The life of PMNs is spent in three environments in the normal adult human: marrow, blood, and tissues.

The *marrow* is the site of proliferation and terminal maturation of neutrophilic granulocytes (myeloblast to segmented neutrophil)[54,55,56]. Fifty five to sixty percent of the hematopoietic cells in the marrow are dedicated to the neutrophil lineage[54]. The earliest microscopically recognizable neutrophil precursor is the myeloblast. Proliferation takes place only during the first three stages of neutrophil maturation (*blast, promyelocyte,* and *myelocyte*) in seven days. After the myelocyte stage, these cells are no longer capable of mitosis and enter a large marrow storage pool (*metamyelocyte* and *band cell*). After five to seven days, they are released in a 'first in, first out' pattern into the blood, where they circulate for a few hours (a half life of about six hours) before entering tissues[57,58]. The normal human neutrophil production rate is $0.85\sim1.6 \times 10^9$ cells/kg per day[59]. The neutropoietic system has a high production volume, yet it is finely modulated in the steady state and has a great capacity to increase production in response to inflammatory stimuli. In the later situation, the myelocyte-to-blood transit time may be shortened (as short as 48 hours), division steps may be skipped, and the release into the blood may occur prematurely[60]. The humoral regulators involved in granulopoiesis, originally identified by their ability to stimulate colony formation from marrow progenitor cells, were named colony-stimulating factors (CSFs)[61]. With regard to neutrophil production, at least four human CSFs have been defined[62]. *GM-CSF* is a 22,000-M_r (Molecular Weight) glycoprotein that stimulates the production of

neutrophils, monocytes, and eosinophils; *G-CSF* has a M_r of 20,000 and stimulates only the production of neutrophils; *Interleukin-3* (IL-3) also has a M_r of 20,000 and acts relatively early in hemopoiesis, affecting multipotent stem cells; and *stem cell factor* (also known as *c-kit* ligand or *steel factor*), with a M_r of 28,000, acts in combination with IL-3 or GM-CSF to stimulate the proliferation of the earliest hemapoietic stem cells. Both G-CSF and GM-CSF also act directly on the PMN to enhance its function, and thus are important in regulating both the production and functional activity of neutrophils[62].

Circulation: Neutrophils leave the marrow storage compartment and enter the blood without significant reentry into the marrow. The total blood PMN pool is composed of the circulating pool and the marginated pool which is represented by PMNs adhering to the ECs of small vessels and account for approximately half of the total blood PMNs[63]. The behavior of PMNs in the blood appears to be controlled by two classes of membrane-bound adhesion proteins: *selectins* and *integrins*. PMNs circulate in the blood with a half-time of about six hours in a random manner[12,64]. Thus, PMNs newly released from the marrow are as likely to leave the blood as PMNs that have been circulating for several hours. Certain senescent PMNs, however, may be eliminated in a nonrandom fashion, perhaps by programmed cell death, or apoptosis[65,66]. Apoptosis is a physiologic phenomenon resulting in the death of mature cells. Apoptosis is characterized biochemically by internucliosomal DNA fragmentation and morphologically by nuclear and cytoplasmic condensation. One of the key features of programmed cell death in many tissues is the phagocytosis of apoptotic cells by macrophages. Ingestion of intact apoptotic granulocytes by macrophages may prevent the release of their toxic intracellular contents extracellularly, thereby promoting resolution of inflammation[66,67].

Tissue: Little is known of the fate of these cells in normal tissues. PMNs normally migrate into the lung, oral cavity, gastrointestinal tract, liver, and spleen[58,68]. They may be lost from mucosal surfaces or die in the tissues and be degraded by macrophages. The average life-span of the mature PMNs is thought to be very short, though an individual cell may survive for as long as two weeks. The PMN life-span is

further shortened if it takes in bacteria or other particles. PMNs may die in large numbers in areas of infection.

Activities of PMN

PMNs function to protect the host against infections. Their function is closely integrated with that of lymphocytes and macrophages, and cells that are also involved in the response to infection. PMN priming is thought to prepare PMN for optimal microbicidal function[5].

Priming of PMNs: Priming of PMNs is defined as the act of preparing the neutrophil for an enhanced response to subsequent stimulation[4]. Enhancement of fMLP induced superoxide anion (O_2^-) generation has been characteristically used to demonstrate PMN priming. The modulation of calcium-mediated signal transduction also appears to be a central feature[69]. Other functional changes of primed PMNs include increased adherence to endothelial cells with upregulation of adhesion molecules. In-vitro studies have implicated LPS[70], tumor necrosis factor-α[6], interleukins[7,8], arachidonic acid metabolites[9], platelet activating factor[10], colony stimulating factors[11], and the process of adhesion itself[4] as PMN priming agents. Although *in-vivo* interactions between these different immunomodulating agents are likely to be complex, their ultimate goal is thought to render PMNs optimally effective for microbicidal function at sites of infection[71].

Chemotaxis, Motility, Phagocytosis and Degranulation: Chemotactic factors, or chemotaxins, which are generated by the interaction of plasma proteins with antigens or pathogens, attract PMNs from the blood to sites of infection[72]. The diffusion of these factors creates a chemical gradient that directs the migration of PMNs, with the cells moving towards the source of the chemotactic factor(s). The PMN chemotactic factors exert their effects via binding to specific cell surface receptors. The way in which the extracellular binding event translates into intracellular signaling has been a subject of intense investigation. The formylated peptide fMLP (by-product of bacterial metabolism), complement factor C5a, leukotriene B_4 (LTB_4), platelet-activating factor

(PAF), and IL-8 are all recognized chemoattractants. The cDNA sequences of the PAF[73], fMLP[74], IL-8[75], LTB_4[76], and C5a[77] receptors predict tertiary structures that are remarkably similar to those of the known heterotrimeric GTP-binding protein (G protein)-coupled receptors. All the heterotrimeric G protein-coupled receptor families have guanine nucleotide binding sites, GTPase activity, and specificity for a given receptor system encoded in their α subunits[72]. The membrane lipids also flow[78], and enhanced cytosolic calcium is observed along the membrane margin[79] when PMNs move at a rate of up to 50 μm/min, similar to locomotion of amebas. The formation of a pseudopodium is essential for PMN locomotion and is also required for PMN ingestion[80]. PMNs ingest the opsonized microorganisms by surrounding them with moving pseudopodia that fuse to enclose the microbe within a vesicle called the *phagosome*[81]. The cytoplasmic granules of PMN fuse with the phagosome and discharge their contents into it, a process called *degranulation*[81]. PMNs consume molecular oxygen and enzymatically generate 'activated' metabolites such as superoxide and hydrogen peroxide which, together with material discharged into the phagosome from the granules, can kill ingested microbes[81]. Granule contents and oxygen metabolites may leak from PMN into extracellular fluid, where they can injure normal tissue. This leakage results from both direct secretion as well as from partially closed phagosomes[81].

PMN Granules

Azurophilic (Primary) Granules: In addition to myeloperoxidase, the azurophilic granule contains numerous lysosomal enzymes. Of the ten antimicrobial proteins of known sequence in human azurophil granules, two have unique primary structures (lysozyme and bactericidal permeability-increasing protein) while the remaining eight fall into two families of four members each: the defensins which account for thirty to fifty percent of granule protein on the one hand, and cathepsin G, elastase, proteinase 3, and azurocidin on the other[82]. The later four proteins can be termed serprocidins to denote that they are closely related to serine proteases with microbicidal activity[82].

Specific (Secondary) Granules: The specific or secondary granules, which by definition do not contain peroxidase, contain lactoferrin, lysozyme, B12-binding protein,

and other proteins[83]. These peroxidase-negative granules vary greatly in size, shape, electron-lucency, isopyknic density, and granule content. However, they can be loosely categorized by the distribution of two proteins, lactoferrin and gelatinase. Approximately sixteen percent of peroxidase-negative granules contains only lactoferrin, twenty four percent contains only gelatinase, and sixty percent contains both marker enzymes[83]. Thus, based on ultrastructure alone, three types of peroxidase-negative granules can be identified: peroxidase-negative granules containing gelatinase but no lactoferrin, peroxidase-negative granules containing lactoferrin but no gelatinase, and peroxidase-negative granules containing both lactoferrin and gelatinase[84]. This heterogeneity may be a result of overlapping synthesis and packaging of different granule proteins during granulopoiesis and is functionally significant since the gelatinase-containing granules are released from the cells induced by certain inflammatory mediators more readily than those containing lactoferrin. In this regard, the lactoferrin-containing granules also contain membrane proteins, Mac-1 and cytochrome b_{558}[84].

Secretory (Tertiary) Granules: Secretory vesicles have recently been discovered within neutrophils[83]. These vesicles are distinct from the azurophilic or specific granules, and they have been defined as intracellular organelles that contain tetranectin and latent alkaline phosphatase[83,84]. The later enzyme is located on the luminal side of the vesicle membrane and can therefore be identified in the presence of detergent as latent alkaline phosphatase. Further, these secretory vesicles contain plasma proteins, such as albumin, which are not synthesized by the cells but are endocytosed from plasma. These vesicles represent a specialized form of endocytic vesicle. Secretory vesicles are transported to the cell surface after the stimulus of formyl methionyl-leucyl phenylalanine or certain cytokines[83].

PMN Adhesion Molecules and Their Functions

Selectins and Their Functions

Selectins are polypeptides containing a sugar-binding site and oligosaccharides ending in a Lewis^x determinant[13]. Structural features common to the selectins are the presence of a NH_2-terminal C-type (Ca^{++}-dependent) lectin-like binding domain, an epidermal growth factor (EGF)-like region, a variable number of consensus repeats of sequences similar to those appearing in complement-regulatory proteins, a membrane-spanning region, and a short cytoplasmic region[13]. The genes for the selectin family are closely linked on chromosome 1 (q21-24)[13].

L-Selectin: L-selectin is a 38.5 kD protein also found on lymphocytes and monocytes, through which circulating PMNs attach reversibly to and roll along the ECs by interacting with its counterpart Lewis^x, and retain their spherical shapes[85]. The contribution of L-selectin to the adhesive interaction between leukocytes and endothelium becomes evident when the assay is performed under conditions of shear stress[86], demonstrating that L-selectin is involved in the initial attachment of leukocytes to endothelium. Exposure to inflammatory mediators causes L-selectin to be shed and PMN integrins to be upregulated[87]. The functional relevance of this is not fully defined but may relate to either or both of the following explanations. Shedding may be a protective mechanism such that activated leukocytes that re-enter the circulation cannot adhere to sections of normal ECs elsewhere in the circulation. Alternatively, shedding of L-selectin may be required to prevent steric hindrance that allows leukocyte integrins to interact with endothelial ICAM-1. L-selectin binds to SLe^x and Sle^a structures on ECs in a Ca^{++}-dependent manner[88]. L-selectin is also an important counter receptor for E-selectin and P-selectin in mediating adhesion of PMN[89]. The property of L-selectin involved is not its activity as a lectin, but rather as a glycoprotein that carries SLe^x-containing carbohydrates. Its surface distribution on the tips of microvilli is well positioned for initial contact of the PMN with selectins on the activated EC.

β_2 Integrins and Their Functions

Integrins are transmembrane cell surface proteins that bind to cytoskeletal proteins and communicate extracellular signals[14]. Each integrin consists of

noncovalently linked heterodimeric α and β chains. To date, eight known β chain subunits and twelve of fifteen reported α subunits have been cloned[14]. Integrins are arranged in subfamilies according to their β subunits and each α subunit may have from one to eight different subunits associated with it.

The β_2 integrin family consists of three heterodimeric integral membrane proteins including the lymphocyte function-associated antigen-1 (LFA-1) (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18)[14]. Each of these prototype integrin glycoproteins consists of noncovalently associated α and β subunits with $\alpha_1\beta_1$ stoichiometry. They share an identical β subunit (Mr = 95 kD) and are distinguished immunologically by α subunits. The molecular weights of α are 165 kD for Mac-1α, 177 kD for LFA-1α, and 150 kD for p150,95α. The CD18 gene is localized to band 21q22 of chromosome 21[90]. Heterogeneity of glycosylation among β_2 integrins has been reported, but the functional significance of these findings are unclear[91]. The three-dimensional structures of the αβ integrins and the individual contributions of α and β subunits in this subfamily are undefined. The α subunits of LFA-1, Mac-1, and p150,95 and their common β_2 subunit are synthesized as distinct precursors that are glycosylated cotranslationally with N-linked, high-mannose carbohydrate groups[92]. An association of α subunit and β subunit precursors, which occurs 1 to 2 hours after synthesis, is required for further conversion to complex-type, N-linked carbohydrates in the Golgi apparatus[93]. Matured αβ complexes are then transported to the cell surface or to intracellular secretory vesicles[92]. Because integrins within a given subfamily share a common β subunit and yet demonstrate distinct ligand-binding specificities, α subunits might impart binding specificity. Alternatively, α subunits may influence recognition by changing the conformation of β subunits to recognize specific ligands. LFA-1, Mac-1, and p150,95 have different and yet overlapping roles in adhesion, partly due to their characteristics of expression on leukocytes. Intercellular adhesion molecule-1 (ICAM-1) on the EC surface upregulated as the principal PMN integrin counterligand causes PMN-EC tight adhesion under low sheer force of blood flow[14]. As a results of the

interaction between the PMN integrins and adhesive molecules of EC, the PMNs tightly adhere to ECs and begin to migrate into the tissues.

LFA-1: LFA-1 is expressed on all immune cells, with the exception of some tissue macrophages[94]. LFA-1 is expressed at relatively constant levels on exposure to chemotactic stimuli[95]. The mechanisms may exist to actively suppress LFA-1 in low-avidity/affinity states on unstimulated leukocytes, and to elicit high-avidity binding states on cells, with Ca^{++} flux after exposure to inflammatory mediators[96]. Phorbol esters stimulate LFA-1-dependent homotypic aggregation of lymphocytes without altering the surface expression of LFA-1 (or its ligand ICAM-1) on these cells[97]. Three members of the immunoglobulin gene family, including ICAM-1, ICAM-2, and ICAM-3, are defined as functional ligands for LFA-1[98,99,100]. PMN adhesion to unstimulated EC is largely LFA-1 dependent and completely inhibited by ICAM-1 MAb[101]. Thus the relative role and importance of ICAM-2 in inflammatory reactions in vivo must be determined. The high level of expression of ICAM-3 on resting leukocytes of all lineages and its lack of expression on either resting or cytokine-activated ECs suggests that its pattern of expression is distinct from that of ICAM-1 and ICAM-2[98]. ICAM-3 may be the most important ligand for LFA-1 in the initiation of the immune response, because expression of ICAM-1 on resting lymphocytes is relatively low. LFA-1 participates in a range of adhesive interactions of immune cells, and is important in natural killing and antibody-dependent killing by granulocytes[102] and other immune cells.

Mac-1: In contrast with LFA-1, the distribution of Mac-1 is more limited and predominantly expressed on blood monocytes, macrophages, and granulocytes. Mac-1 recognizes the molecular ligand ICAM-1, whereas Mac-1, but not LFA-1, interacts with iC3b[14]. After biosynthesis, some Mac-1 heterodimers (but not LFA-1) are stored in secondary and tertiary granules in granulocytes[103]. The surface expression of Mac-1 increases several times over baseline level after cell activation by chemotactic factors, phorbol esters, and certain cytokines[104]. This occurs, in part, as a result of the translocation of granule-associated intracellular pools to the cell surface. This process of up-regulation occurs within minutes after agonist exposure and is not impeded by protein synthesis inhibitors, and appears to involve the fusion of granule and cell

membranes and to be accompanied by degranulation. Ligation of Mac-1 also induces the adhesion-dependent respiratory burst by PMNs stimulated with chemoattractants[105].

p150,95: Like Mac-1, p150,95 heterodimers are stored in secondary and tertiary granules in granulocytes after biosynthesis and upregulated with cell activation[103,104]. Like Mac-1, p150,95 also interacts with iC3b[14]. However, the functional importance of this determinant is less well defined.

Endothelium and Endothelial Cell Adhesion Molecules

Endothelium

Morphology

Vessel wall ECs constitute the internal surface of the whole vascular tree, as a roughly 1 to 2 kg organ consisting of about 10^{11} cells, with an aggregate surface area exceeding 100 sq. meters[106]. Veins, arteries, and capillaries show different histology, but in all of them ECs are constantly in contact with blood components. Morphologically, the EC is approximately 50 μm in diameter and 3μm in section, but its structure varies in the different vascular areas[19]. These differences have been studied specifically in the capillary area, in which two major groups of capillaries have been found: *continuous* and *fenestrated*[19]. Continuous capillaries (present in connective tissue, central nervous system, and skeletal muscle) share ECs thickened in the nucleus area with a thin cytoplasm covering the remainder of the internal vessel surface. In these continuous capillaries, cell-to-cell junctions are usually tight. Fenestrated capillaries (found in endocrine glands or in renal glomeruli) have an attenuated EC that appears fenestrated by pores of eighty to one hundred nm in diameter, with each EC adjoined to its neighbor by loose junctions (named gap junctions). A basement membrane underlies ECs throughout the vasculature. ECs show typical organelles as mitochondria or microtubules, as well as a great number of pinocytic vesicles[107,108]

which are involved in the transfer of material from blood to the vessel wall and *vice versa*. In addition, the EC contains a unique rod-shaped tubular organelle (or Weibel-Palade body) that is approximately 0.1 μm in diameter and three μm or more in length[109] that seems to derive from the Golgi apparatus[110]. These Weibel-Palade bodies are seen throughout the vascular ECs, especially in large veins, but are rare in capillaries, and their function seems to be closely related to hemostasis[13]. ECs have a low turnover rate in the resting state. Most ECs in mammals have a reproductive cycle that varies between several months to a few years[111]. The labeling of the cells by tritiated thymidine varies from 0.01 to 0.13%[112], with a more elevated turnover in the arterial area subjected to higher shear stress[113]. Tritiated thymidine EC labeling may be increased in a variety of blood vessel injuries and in the vasculature of neoplasms[114,115].

Function of the Endothelium

ECs are no longer considered to be a passive lining but rather a highly metabolically active tissue with a wide range of functions. An important finding is that the ECs are not identical throughout all the vascular tree[15]. There are different physiological functions of the ECs in different vascular territories, e.g. the ECs located in capillary, veins, and arteries, and also the ECs between species in the same territory[116]. EC heterogeneity involves in the important biological functions of the cells such as antigen expression, stromal composition (i.e., heterogeneity of collagens), proliferative rate, and response to injury[15,116]. Some of these differences between different ECs are ascribed to an adaptive pattern to different vascular microenvironments of potentially identical ECs rather than to innate cell characteristics of a wide range of EC populations[117]. The major biological properties of ECs include regulation of vascular growth, regulation of vessel tone, maintenance of selective vascular permeability, maintenance of hemostatic balance (platelet adhesion, coagulation, fibrinolysis), regulation of inflammatory reactions, regulation of the immune response, synthesis of stromal components, synthesis and secretion of peptides, and integration and transduction of blood-borne signals[117]. Here, I briefly review the following

functions: angiogenesis, coagulation, inflammation and immune response, synthesis of stromal components, and vascular tone regulation.

Angiogenesis: Angiogenesis is defined as the formation of new microvessels that extend from the existing vascular network[116]. A similar process to angiogenesis, in the embryo, is called vasogenesis (or vasculogenesis by some), leading to the formation of the entire cardiovascular system[116]. The maintenance of an intact layer of ECs is essential for the regulation of cell proliferation. Angiogenesis is a dynamic process that takes place in either physiological (for example, wound healing) or pathological situations (processes involving neoplasms, diabetic retinopathy, or chronic inflammatory diseases)[118]. Although EC turnover is usually low, ECs are capable of rapidly migrating and proliferating in response to angiogenic stimuli[119]. In response to an angiogenic stimulus, ECs in a 'mother' vessel separate from each other, leaving segments of basement membrane uncovered, which are rapidly subjected to enzymatic digestion. Through the formed gaps, ECs migrate. Other ECs divide. Migrating and proliferating cells form loops and then tubes. Basement membrane is secreted to cover the 'sprouts,' and the lumen of these tubes communicates with that of the mother vessel. This formation of sprouts continues until the necessary microvascular network is formed and accomplishes its function[118]. Angiogenesis is regulated by a wide set of agonists and antagonists[119,120,121,122,123,124], whereas regulation of vasogenesis and the molecular signals responsible for vasogenesis are unknown. Heterogeneity of ECs is evidenced by its different capacity to proliferate depending upon the organ and by its different responses to the same stimuli. Many of the referred angiogenic agonists are able to stimulate both proliferation and migration of the ECs[119].

Coagulation: Vascular ECs are also closely involved in the maintenance of hemostatic balance. They play an important role in modulating several aspects of platelet function, coagulation, and fibrinolysis[117]. They have both procoagulant and anticoagulant roles in relation to the soluble coagulation system[125,126]. Likewise, in relation to platelet aggregation, ECs have both agonist and antagonist effects. In normal blood flow, the luminal membrane of the ECs of intact vascular monolayers provides a physiologically antithrombotic surface, with thrombomodulin to enhance the ability of

thrombin activating protein C which is a substance that suppresses Factor Va and Factor VIIIa by proteolysis[125]. ECs synthesize protein S for optimal activation of protein C[127]. Heparin-like molecules on the EC membrane accelerate the inactivation of thrombin by plasma antithrombin III[124]. Perhaps through a similar mechanism, ECs may contribute to the inactivation of Factor IXa, Xa, and XIIa by antithrombin III[125]. Meanwhile, ECs possess several potentially interacting mechanisms with circulating cells and proteins. ECs synthesize and release factor V, and bind (from plasma) Factor V, Factor IXa, and Factor Xa[125]. And ECs enzymatically activate Factor XII[126]. These mechanisms will be triggered by any EC alteration or disruption, such as by endotoxin, thrombin, interleukin-1, TNF, leukocytes, or platelets stimulation, which result in increased tissue factor activity of ECs[116]. Platelets do not adhere to intact ECs. This property has been ascribed to endothelium-derived relaxing factor (EDRF) on the surface of ECs[117]. Nitric Oxide (NO) accounts for at least some of the EDRF biologic activity, in response to aggregating platelets, by directly acting on the vascular smooth muscle to produce relaxation through a cyclic-GMP-dependent pathway[128]. Even stimulated platelets do not adhere to normal ECs, which appears to be mediated by prostacyclin (PGI_2), through a cyclic-AMP-dependent mechanisms[129]. The actions of NO and PGI_2 are synergistic. ECs synthesize and secrete PGI_2 in response to the stimulus of many substances which include thrombin, histamine, bradykinin, lipoproteins, adenine nucleotides, cytokines (IL-1, TNF-α, basal lamina), trypsin, etc[116]. PGI_2 secretion is also stimulated by cellular events such as shear stress, immunologic injury, and activation of PMNs[117]. Damaged EC surface leads to the exposure of endothelial and subendothelial structures to which circulating platelets adhere, thus forming the primary platelet aggregate. The initial adhesion of platelets is mediated by subendothelial substances, plasma factors, and endothelial and platelet surface membrane receptors[116]. von Willebrand factor, which is synthesized and released by ECs, is one of the mediators for platelet adhesion to subendothelium[130]. The newly generated platelet monolayer is rapidly followed by further aggregation of platelets, thus stabilizing the platelet plug[131].

Inflammation and the Immune Response: ECs contain the antigens of the ABO system, and have major histocompatibility antigens (MHCs), Class I and II[117]. The

surface expression of the MHC can be induced by cytokines: IFN-γ, TNF-α, and TNF-β induce Class I (HLA-A and B); IFN-γ induces Class II (HLA-DR, DP, DQ)[117]. Resting ECs rarely express Class II (Ia) antigens, whereas ECs stimulated by activated lymphocytes or γ interferon consistently express Ia.[117] There is evidence that ECs can act as antigen-presenting cells. It is also apparent that under appropriate conditions ECs are capable of phagocytosis[120]. The MHCs and other endothelial antigens are not uniformly distributed throughout the vascular system of a given individual[132]. The ECs play the role of gate-keepers in regard to the deployment of phagocytic cells (PMNs and monocytes) from blood and the trafficking of lymphocytes. Thus, ECs control the efflux of granulocytes and monocytes guided by CAMs in the regions of inflammation and infection[133]. ICAM-1 of the Ig gene superfamily and P-, E-selectins of the selectin family are among the most important CAMs of ECs for regulation of PMN exudation. The adhesion of granulocytes, monocytes-macrophages, and lymphocytes to ECs is enhanced by the action of cytokines, most prominently IL-1, TNF-a, and IFN-γ[18]. These products not only increase leukocyte adhesion, but also stimulate the expression of molecules that serve as receptors for certain leukocytes on the surface of ECs. These cytokines and other circulating compounds such as thrombin and endotoxin induce the synthesis and/or release, by ECs, of various substances involved in the immune response[117]. Such substances include PGI_2, GM-CSF, platelet-activating factor, EC-derived growth factor (ECDGF, which supports the growth of other ECs), and IL-1[117].

Synthesis of Stromal Components: ECs secrete the constituents of their basal lamina (BL), mainly collagens IV and V and laminin (also some elastin, mucopolysaccharides, fibronectin, thrombospondin, etc.)[134,135]. The types of stromal constituents secreted by cultured ECs, either into the medium or into the extracellular matrix, vary with the animal and with the site from which such ECs have been obtained[135]. Stimulated ECs contain and release collagenases capable of digesting Collagen I, II, III, IV, and V[134]. Therefore, the endothelium is not only able to produce stromal elements, but also to remodel them (especially its own basal lamina).

Vascular Tone Regulation: The normal intact EC can be considered an important paracrine organ that secretes or metabolizes a host of vasorelaxant and vasoconstrictor

substances[136]. Among the vasorelaxants are endothelium-derived relaxing factor (EDRF)-nitric oxide (NO) and prostacyclin[129,137]. EDRF-NO is synthesized in ECs with the essential amino acid L-arginine by the action of NO synthase stimulated by various agents, including acetylcholine, substance P, bradykinin, and the calcium ionophore A23187.[138] EDRF-NO is also produced in response to stimulation by thrombin, adenine nucleotides, thromboxane A_2, peptidoleukotrienes, histamine, endothelin, and platelets aggregating[139]. EDRF-NO stimulates the enzyme guanylate cyclase, leading to accumulation of cyclic guanosine monophosphate (cGMP) in the vascular smooth muscle and resulting in vasorelaxation[130]. The flow-induced changes of shear stress are especially important in maintaining coronary artery tone[140]. PGI_2, a product of the arachidonic acid cascade, is also rapidly released from the EC after stimulation by thrombin, bradykinin, histamine, high-density lipoproteins, adenine nucleotides, leukotrienes, thromboxane A_2, calcium ionophore A23187, platelet-derived growth factor, tissue hypoxia, and hemodynamic stress[139]. The effects of PGI_2 are mediated by an increase in the generation of intracellular cyclic adenosine monophosphate[131]. ECs also produce vasoconstrictors such as thromboxane A_2[141], endothelin[142], and endothelium-derived hyperpolarizing factor[143] that hyperpolarizes the vascular smooth-muscle cell membrane. ECs also generate superoxide radicals, which have been considered a vasoconstrictor factor[144].

Endothelial Cell Adhesion Molecules

Adhesive interactions are essential for maintaining the structural and functional integrity of the vascular and immune system. CAMs of EC include integrins, the immunoglobulin (Ig) gene superfamily, selectins, leucine-rich motif-containing receptors, and cadherins[145].

Integrins: The firm attachment of ECs to the subendothelial extracellular matrix is mediated via CAMs[146]. Most of these matrix adhesive molecules are the ligands for integrin receptors expressed on ECs[147].

The immunoglobulin gene superfamily: The Ig gene superfamily consists of cell-surface proteins that are involved in antigen recognition (C1-type) and complement-binding or cellular adhesion (C2-type). Common features of C2-type proteins include a variable number of extracellular Ig-like domains with conserved cysteine sequences that form disulfide bonds to stabilize β-sheets of the tertiary structure[145]. Ig gene superfamily of ECs includes ICAM-1, ICAM-2, PECAM-1, and VCAM-1[145]. ICAM-1 and ICAM-2, in the interaction of leukocytes with vascular ECs are discussed later. VCAM-1, expressed on cytokine-stimulated ECs, serves as the membrane ligand or counter-receptor for integrin $\alpha_4\beta_1$ (VLA-4, or very late antigen 4) expressed on the membrane of lymphocytes and monocytes with distinct recognition mechanism[148]. PECAM-1 (CD31 or EndoCAM) is made of six Ig type C2 domains and is localized in EC intercellular junctions[149]. It is also expressed on platelets, monocytes, and granulocytes, but its ligand or counter-receptor is unknown[149].

Selectins: Selectin gene family represents the molecular recognition sites on the vascular EC for circulating lymphocytes, PMNs, and macrophages bearing the appropriate 'zip codes'[150]. P- and E-selectin are expressed on activated ECs for facilitating the attachment of PMNs to postcapillary venules, the main area for PMN migration to the zone of infection and inflammation in tissue (discussed later in detail).

Cadherins: Ca^{++}-dependent adhesive molecules named cadherins make up a family of transmembrane glycoproteins involved in the cell-cell adhesion of epithelial and ECs, also include liver cell adhesion molecule (L-CAM), E-cadherin (uvomorulin), A-CAM, N-cadherin, and P-cadherin[151].

Leucine-rich motif-containing receptors: Leucine-rich motif-containing receptors are a family member of CAMs. Among them, glycoprotein Ib (GP Ib) serves as ligand for von Willebrand factor in extracellular matrix and in plasma[152]. GP Ib is composed of two subunits, α and β, linked by a disulfide bond[152]. While GP Ib coupled with GP IX is mainly expressed in megakaryocytes and platelets, there is emerging evidence that cultured human ECs express GP Ib_α[153]. This expression is enhanced by TNF-α. The cloning and sequencing of GP Ib_α and GP Ib_β and their companion molecule GP IX revealed the following features: all three are non-integrins, and they share the leucine-

rich motif, a twenty-four-amino-acid sequence characteristic of leucine-rich glycoprotein presented in human plasma and in a diverse group of proteins[152].

ICAM-1 of Endothelium

Human ICAM-1 is a single-copy gene located on chromosome 19[154]. Molecular cloning showed that ICAM-1 has a core protein of 55 kD with five extracellular Ig-like domains[99]. Amino acid substitutions in the extracellular domains have indicated that the primary binding site for leukocyte CD11a/CD18 is located in the NH_2-terminal first domain of ICAM-1[155]. A second ligand-binding site for a leukocyte integrin (CD11b/CD18) is localized to the third Ig-like domain[156]. The cytoplasmic domain of ICAM-1 binds to the cytoskeleton, which may localize ICAM-1 within regions of the EC membrane to facilitate leukocyte adhesion and transmigration[157]. Induced ICAM-1 expression requires mRNA and protein synthesis and is observed at 4-6 hours, because there is apparently no storage form of this adhesion protein[158]. Its counter-receptors are integrins CD11a/CD18 and CD11b/CD18. It is also the receptor for over 90% of serotypes of human rhinoviruses causing the common cold and for malaria-infected erythrocytes[158]. ICAM-1 expression of EC peaks by 12 hours and persist for at least 72 hours after TNF-α stimulation[158]. ICAM-1 is transcriptionally regulated by cytokines (such as TNF-α, IL-1, TNF-β, IFN-γ), LPS, phorbol esters, or other mediators of inflammation[159]. Expression of ICAM-1 is regulated at multiple levels, with some agonists more selective (IFN-γ, for example) and others (e.g. IL-1, TNF-α, and LPS) less specific[159]. These multiple levels of regulation provide for precise modulation of the expression of ICAM-1 that is involved in recruitment of leukocytes to sites of inflammation or immune reaction.

ICAM-2 of Endothelium

ICAM-2 is another member of the Ig gene superfamily that is expressed on EC and is involved in leukocyte adherence. Human ICAM-2 is a single-copy gene located on chromosome 17[100]. Molecular cloning of ICAM-2 showed a core protein of 29 kD with six residues for possible N-linked glycosylation which, if fully used, would yield a

mature protein of 46 kD. ICAM-2 has only two extracellular Ig-like domains, with 34% homologous to the two NH_2-teminal Ig-like domains of ICAM-1[159]. The counter receptor of ICAM-2 is CD11a/CD18. ICAM-2 was found to be expressed constitutively on vascular EC both in vivo and in vitro, and was not subject to upregulation by cytokines (TNF-α, IL-1, or IFN-γ) or LPS[159]. However, surface expression of ICAM-2 has been reported to be increased on HEV (High Endothelial Venule) and small vessel EC in malignant versus nonmalignant lymph modes[160], suggesting that EC expression of ICAM-2 may be inducible under some circumstances.

E-Selectin of Endothelium

E-selectin (CD62E) is a 115-kD antigen, with the C-type lectin-like binding domain, the EGF-like domain, and six complement-regulatory protein regions[13]. Translation of E-selectin yields a core protein of 64 kD with 11 potential N-glycosylation sites. The 32-amino acid cytoplasmic domain contains tyrosine residues that have been suggested to mediate the internalization of other transmembrane proteins, and may account for the short half-life of E-selectin at the cell surface[161]. Mapping of E-selectin domains by MAbs has shown that the NH_2-terminal nine amino acids of the lectin domain and an epitope within the EGF-like region are important for ligand binding, and two positively charged amino acids (arginine 97 and lysine 113) of the lectin domain of human E-selectin are critical for ligand binding[162]. The expression of E-selectin on ECs requires the de novo synthesis of both mRNA and protein because cycloheximide and actinomycin D were shown to inhibit the generation of E-selectin[159]. *In vitro* the surface expression of E-selectin has been reported to peak 4 hours post stimulation with a return to basal levels of expression within 24 hours. However, studies *in vivo* have shown that E-selectin persists beyond 24 hours, suggesting that additional factors may determine the duration of E-selectin[159]. Like ICAM-1, E-selectin regulation is at multiple levels with various patterns of cytokines or inflammatory mediators leading to the differential induction and duration of E-selectin. The sialyl Lewis X (SLe^x) or closely related structures, including L-selectin and β_2 integrins[163],

which are heavily expressed on PMNs and monocytes and natural killer cells, are E-selectin ligands[164]. E-selectin also recognizes an isomer of SLe^x, sialyl Lewis A (Sle^a)[164]. SLe^a is expressed on some tumor cells but is not usually found on leukocytes, this interaction is more relevant to tumor metastases than to leukocyte trafficking[164].

P-Selectin of Endothelium

P-selectin is present on ECs and platelets[13]. In both cell types P-selectin is synthesized and stored in cytoplasmic granules: in platelets it is contained in α-granules, whereas in ECs it is found in Wiebel-Palade bodies. With appropriate activation P-selectin is mobilized to the external plasma membrane. The cloning of P-selectin showed an organization of domains common to selectins, but with nine consensus repeats of the complement-regulatory protein regions[159]. The core protein has a predicted molecular weight of 86 kD. There are 12 potential N-linked glycosylation sites which, if fully used, would yield a protein of 122 kD. Computer modeling and generation of site-specific mutants of the lectin domain of human P-selectin have indicated that two residues (tyrosine 48 and lysine 111) are critical for ligand binding[165]. SLe^x or other fucose-containing carbohydrate structures are critical for PMN binding to P-selectin, as well as to E-selectin, though P-selectin also binds to Sle^a [166]. However, candidate high-affinity glycoprotein ligands for P-selectin, distinct from L-selectin, have been described[159]. Binding of P-selectin to this glycoprotein was calcium-dependent, and was specifically inhibited by a blocking anti-P-selectin MAb[167]. With EC activation by thrombin, histamine, phorbol esters, calcium ionophores, or complement proteins, cytoplasmic storage granules fuse with the cell membrane and externalize their contents[159]. Initial reports showed that surface expression of P-selectin was rapid and transient, peaking by 10 minutes and returning to baseline within 20 to 30 minutes. Rapid loss of surface P-selectin was believed to be secondary to internalization of the protein. However, recent studies have shown that surface expression of P-selectin can last several hours. Stimulation of ECs by thrombin or oxygen radicals led to P-selectin-dependent PMN adhesion lasting 1.5 to 4 hours respectively[159]. P-selectin was demonstrated to mediate adhesion of PMNs to LPS-stimulated human EC that had been

pretreated for 4 hours[159]. Comparing to E-selectin binding, P-selectin binding happens quicker but weaker[168].

Polymorphonuclear Neutrophil-Endothelial Cell Interactions

The Process of PMN-EC Adhesion

PMN-EC adhesion is multiple-step process. Circulating PMNs are functionally quiescent but respond to exogenous stimuli, resulting in an increase in their adhesion (both to each other and other substrata), secretion of granule contents including tissue-destructive proteases, and activation of NADPH with the generation of toxic oxidative metabolites[169]. Initial contact with the vessel wall is in large part a random event, perhaps enhanced by local alteration in flow characteristics. The phenomenon of PMN rolling along endothelial cells is observed only under conditions of flow and is a prerequisite for higher-affinity interactions with the inflamed endothelium that take place under conditions of lower shear stress[85]. The frequency of leukocyte rolling on venular endothelium is much higher than that on arterioles[170]. There may be an intrinsic difference in the expression of endothelial adhesive components involved in leukocyte rolling, i.e., the selectins and their counter-structures on venular versus on arteriolar endothelium. An activated PMN enters postcapillary venules adjacent to inflammatory foci and develops low-avidity adhesive interactions with inflamed endothelia via specific classes of adhesion molecules that include the selectins. It has been postulated that low-affinity PMN rolling (facilitated by selectin-mediated PMN-EC cell binding) serves to slow the intravascular movement of PMNs over the inflamed endothelia. Such decreased movement of PMNs. would promote their exposure to soluble activating factors that stimulate PMN sticking mediated by β-integrin-ICAM-1 interactions that occur only during conditions of low shear stress. The high-affinity interactions (tight adhesion) are mediated by a separate class of adhesion molecules whose membrane density and/or functional affinity is up-modulated by locally high concentrations of

inflammatory stimuli. Specifically, in endothelial cells the intercellular adhesion molecule-1 (ICAM-1) is inducibly expressed. ICAM-1 serves as a recognition target for PMN β-integrin counter-receptors Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18) whose relative affinity for ICAM-1 is increased by PMN exposure to activating stimuli that include C5a, N-formulated bacterial peptides, IL-8, and leukotriene B_4 (LTB_4)[101]. High-affinity beta-integrin-dependent PMN-EC sticking promotes subsequent transendothelial migration through the basement membrane and into the extracellular matrix.

The Role of Cell Adhesion Molecules in PMN-EC Adhesion

In the early 1980s, the phenomena of leukocyte adhesion to EC were analyzed by applying the dynamic theory of physical principles[171]. The first protein involved in leukocyte adherence to EC, the murine lymphocyte homing receptor $gp^{90\text{-}Mel}$ (later termed L-selectin), was clearly identified immunologically by Gallatin *et al* in 1983[172]. Since then there has been an explosion of interest in this field, fueled by remarkable advances in the elucidation of the molecular basis of leukocyte adherence to EC and the potential for new therapies directed at these adhesion molecules. In the subsequent years nine EC and nine leukocyte surface proteins involved in this heterotypic adhesion have been molecularly cloned, and several other distinct leukocyte and EC molecules have been identified functionally or immunologically[159]. More efforts have been made to focus on expression, regulation, and function of the CAMs, and use of 'anti-adhesion' therapy in animal models of several diseases.[159]

Pretreatment of PMNs with anti-L-selectin MAbs or with agonists that cause activation-induced shedding of L-selectin decreases PMN adhesion to cytokine-stimulated endothelium[173]. The participation of P- and E-selectins (of ECs) are also involved in PMN rolling and adhesion with higher shear forces in vitro[85,168]. Administration of a blocking anti-P-selectin MAb was shown to decrease leukocyte rolling in vivo[174] and spontaneous rolling was reported to be virtually absent[175].

However, late PMN accumulation in inflamed peritoneum was not markedly reduced by treatment of normal animals with L-selectin Ig-chimeric protein or in P-selectin-deficient mice, whereas a MAb to E-selectin effectively inhibited PMN accumulation in the inflamed peritoneum and lungs of normal animals at 4 hours[176]. Following PMN rolling phase, β2 integrins (LFA-1 and Mac-1) activation or upregulation on PMNs are responsible for firm adherence of PMNs to ECs, resulting in greater affinity for ligands and/or to post-receptor events. Studies in vitro demonstrated that stimulated neutrophil adhesion to endothelium under static conditions was dependent on CD11/CD18[177], and observations by intra-vital microscopy showed that administration of CD18 MAbs prevented neutrophil sticking without affecting rolling[178]. PMNs from patients with Leukocyte Adhesion Deficiency type I (LAD type I, a rare autosomal recessive disorder involving CD18 deficiency) showed normal rolling, but were unable to stick and emigrate upon chemotactic stimulation; PMNs from patients with LAD type II (a PMN adherence defective presumably on the basis of absence of the sialyl-Lewis X antigen on the PMN surface) rolled poorly and failed to stick and emigrate under the shear forces provided by flow[179]. ICAM-1 upregulation on EC is the most important counterpart receptor of beta 2 integrins and responsible for the firm PMN adhesion, and is also responsible for later PMN transmigration[180,181].

OBJECTIVES

A popular hypothesis to explain the development of the systemic inflammatory response syndrome (SIRS) and subsequent multiple organ dysfunction involves PMN adherence and subsequent destruction of ECs of the microcirculation[2,45]. Previous studies showed that circulating PMNs from critically ill anergic patients demonstrated increased adherence to human umbilical vein endothelial cells (HUVE cells) and increased EC damage compared with healthy controls and preoperative reactive patient PMNs.[182] Numerous other investigators demonstrated that activated ECs express CAMs which play an important role in the regulation of inflammation following infection[117]. Since PMN-EC interactions occur in plasma, plasma may have important modulating effects in these interactions. Prior to designing therapeutic strategies (such as antibody blockade of adhesion molecules) to combat the "septic" sequelae of infections i.e. the progression of SIRS to MODS, the following questions must be answered:

1. *Does infection with a SIRS response, termed sepsis, result in increased PMN adhesion to endothelial cells and does the increased adhesion correlate with increased EC damage ?*

2. *What is the relative contribution of PMN activation vs. EC activation to the adhesion of PMN to ECs and subsequent EC cytotoxicity ?*

3. *Are there factors in plasma that modulate PMN-EC interactions ?*

The experiments described in this thesis were designed to answer these questions in order to bring basic research findings to the bedside.

MATERIALS AND METHODS

Subject Selection

Study participants consisted of patients admitted to the wards and the surgical intensive care unit of the department of Surgery, Royal Victoria Hospital. The experimental protocol was approved by the Royal Victoria Hospital Research Ethics Committee. Informed consent was obtained from all subjects. The non-infection patient group (n=27) consisted of patients admitted for major elective operations. Exclusion criteria were steroid administration, recent transfusion with blood or blood products, chemotherapy, or radiotherapy. Entry criteria for the SIRS patient group (n=37) were a documented life threatening infection plus two or more of the following: 1) fever > 38° C or hypothermia < 36°C; 2) WBC > $12.0x10^9$/L or < $4.0x10^9$/L, or the presence of > 10% immature neutrophils (bands); 3) tachypnea > 20 bpm on room air or requirement for mechanical ventilation; 4) tachycardia > 90 bpm; and 5) altered mental function. Exclusion criteria for this group included administration of > 5 units of blood or blood products within 48 hours of venipuncture, chemotherapy or radiotherapy, steroid administration, hemodialysis, liver failure (Child's C), or known or suspected HIV infection. Healthy controls (n=33) were recruited from the students and faculty of McGill University. None of them consumed medication or chemical substances during the study.

Reagents & Plastic Ware

Lipopolysaccharide (LPS, *Escherichia coli* 011:B4), tumor necrosis factor -α (TNF-α,), interleukine-1β (IL-1β), endothelial cell growth substance (ECGS), collagenase, trypsin-EDTA, PMA, fMLP and heparin were obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll-paque reagent and Dextran were purchased from Pharmacia Inc. (Piscataway, NJ). Chromium 51, as sodium chromate, was obtained

from New England Nuclear Co. (Boston, Mass). Iodine125-Human Serum Albumin was from ICN Biomedicals (Irvine, CA). Biocoat Cell Culture Inserts were obtained from Becton Dickinson (Bedford, MA). Hank's balanced salt solution (HBSS), gelatin, Dulbecco's phosphate-buffered saline solution (PBS), penicillin-streptomycin, RPMI-1640, and medium 199 in Earle's salts with glutamine, were bought from GIBCO (Grand Island, NY). Fetal bovine serum and HEPES buffer were obtained from Flow Laboratories (Mississauga, Ont.). Anti-human ICAM-1 was purchased from R&D Systems (Minneapolis, MN). Factor VIII-FITC was obtained from The Binding Site Co. (Birmingham, England). Forty eight well cell culture cluster was obtained from Costar Co. (Cambridge, MA). 25-mm plastic culture flasks were purchased from Fisher Scientific Co. (Montreal, Quebec).

Isolation of Peripheral Blood PMNs

Glassware was baked at 250°C for 2 hours to reduce LPS contamination. LPS free culture media and buffers, singly wrapped sterile pipettes, tubes, and pyrogen-free water were used. Venous blood was drawn in preservative-free heparinized tubes between 8:00 to 9:30 am and processed immediately. Except for the initial red blood cell sedimentation in 10% Dextran-70 performed at room temperature for 40 to 60 minutes[183], the rest of the isolation procedure was performed at 4°C. The erythrocyte-poor supernatant was centrifuged at 400xg for 5 minutes. The pellet was gently resuspended in 2 ml of PBS with free ionized calcium and magnesium, layered on 3 ml of Ficoll-paque, and centrifuged at 400xg for 20 minutes. The pellet was resuspended in PBS with free ionized calcium and magnesium. Erythrocytes that remained in the neutrophil pellet were hypotonically lysed by a 20-second exposure to endotoxin-free distilled water. Isotonicity was then restored by adding hypertonic saline (3.6%). The PMNs were washed twice, counted, resuspended in PBS with free ionized calcium and magnesium, and separated in tubes resting on ice. PMN purity was assessed by flow cytometry using forward-and side-scatter or microscopic visual examination.

Preparation of Autologous Plasma

A separate blood sample was drawn using heparinized tubes as above from the same individual and centrifuged immediately at 4°C at 400xg for 10 minutes. The plasma was aspirated carefully and kept at 4°C. Before use, the plasma was warmed up to 37°C.

Human Umbilical Endothelial Cell Culture

Human umbilical vein endothelial (HUVE) cells were obtained using the method of Jaffe[184]. Human umbilical cords were obtained from the Birthing Center of the Royal Victoria Hospital, Montreal, Quebec. HUVE cells were harvested by 0.1% collagenase perfusion of human umbilical veins and maintained in culture medium consisting of medium 199 in Earl's salts with glutamine 100 μg/ml, supplemented with 20% fetal bovine serum; EC growth substance 30 μg/ml; heparin 15 U/ml; penicillin G 100 U/ml; streptomycin 0.1 mg/ml, and HEPES 25 mM. The HUVE cells were cultured in 25-mm plastic culture flasks that had been previously coated with 0.3% of gelatin, in a humidified atmosphere of 5% carbon dioxide at 37°C until confluence.

Factor VIII-FITC stain[185] was used to verify the purity of HUVE cells: The HUVE cell monolayer was washed twice with PBS and 4% formaldehyde was added for 10 minutes at 4°C. This was washed twice with PBS, a 1:200 dilution of FITC labeled Factor VIII was added for 30 minutes at 37°C, and this was followed by a final wash with PBS before taking photographs under UV light. The purity of the HUVE cells was also verified with phase contrast microscopy.

Cultured cells were detached using Trypsin-EDTA, and plated in gelatin-coated 48 well plates (1 cm^2 each well), and incubated in culture medium until confluence. First and second passage of HUVE cells were used in the adhesion assays and only first passage cells were used in the HUVE cytotoxicity assays.

The integrity of the monolayer was verified by ^{125}I-human serum albumin (^{125}I-HSA) leakage. HUVE cells were cultured on 3 nm pore filters which were then inserted in 24 well plates with 0.5 ml of culture medium in the lower chamber and 0.1 ml culture medium in the upper chamber. The cells were then allowed to grow to confluence. The HUVE cell monolayer was washed twice with PBS, and 100 µl culture medium containing 30 µl ^{125}I-HSA (1.89 mCi/ml) was added to the upper chamber for 30 minutes at 37°C. Aliquots of the upper chamber and the lower chamber were taken for gamma counting. The ^{125}I-HSA leak rate was calculated as:

$$\textbf{Leak \%} = L / (U + L)$$

where U = CPM from upper chamber

L = CPM from lower chamber

To verify the PMN pipetting accuracy, PMNs were labeled as above and 25 aliquots of 250 µl were pipetted into 25 tubes for manual cell number counts and radioactivity determinations and the pipetting variability calculated.

HUVE Cell Monolayer Activation

HUVE cell monolayers in half of the 48 wells were treated with IL-1β 15 U/ml and TNF-α 25 ng/ml in 1% FBS-RPMI-1640 at 37°C for 3 hours in a humidified atmosphere of 5% carbon dioxide. To verify ICAM-1 expression, HUVE monolayers were washed with PBS twice after IL-1β and TNF-α activation. Then, FITC labeled anti-human ICAM-1 (1:100 dilution) was added for 10 minutes, washed and photographed under UV light.

Activation (Priming) and Labeling of PMNs

For the adhesion assays, all the PMNs were labeled with chromium-51 (^{51}Cr), 18.5×10^{7} Bq/ml at 37°C for 40 minutes with periodic gentle agitation. At the same time,

half of the PMNs were primed by adding LPS 100 ng/ml in separate tubes. After the incubation period, free ^{51}Cr and LPS were removed by two washes with PBS with free ionized calcium and magnesium. Cells in separate tubes were recounted and suspended in autologous plasma or in 1% fetal bovine serum (FBS)-RPMI-1640, at a final concentration of 2×10^6/ml, in order to generate the following four subgroups:

Unprimed PMN in 1% FBS-RPMI;

Unprimed PMN in autologous plasma;

Primed PMN in 1% FBS-RPMI;

Primed PMN in autologous plasma.

Effect of Activating (Priming) PMNs or HUVE Cells on PMN-EC Adhesion

Immediately prior to the assay, HUVE monolayers were washed twice with RPMI to remove the culture medium, IL-1β and TNF-α, and unattached HUVE cells. Radiolabeled PMNs in 1% FBS-RPMI, previously LPS treated or not, were added to different rows of HUVE wells that were treated or not with IL-1β and TNF-α at a density of 5×10^5 PMNs per well in six-plicate as shown in table 1. Six 250 µl samples of PMNs from the above groups were sent for gamma count to obtain the total PMN cpm. After 30 minutes, the supernatant from each well consisting of medium and non-adherent PMNs was carefully aspirated. The monolayers were washed twice with RPMI. The washed monolayers consisting of HUVE cells and adhered PMNs were solubilized with 1N NaOH. The radioactivity in the lysate was measured with a gamma counter. The PMN adherence in each well was calculated as follows:

Table 1. Experimental setup for testing the *effect of activating the PMNs or the HUVE cells on PMN-EC Adhesion in RPMI*

Unprimed PMN		Primed PMN	
HUVE-	HUVE+	HUVE-	HUVE+
1	1	1	1
2	2	2	2
3	3	3	3
4	4	4	4
5	5	5	5
6	6	6	6

PMN Adherence % = {^{51}Cr cpm in monolayer /Total ^{51}Cr cpm} ×100

As a check on this method, the numbers of PMNs in the supernatant fluid were determined using a hemocytometer in 8 of 24 randomly determined wells at each experiment and PMN adherence rate also calculated as:

PMN adherence % = [($5x10^5$ PMNs - $n_{\text{supernatant PMNs}}$)/ $5x10^5$ PMNs] x 100

Activating (Priming) PMNs or HUVE Cells on PMN-EC Adhesion - Effect of Plasma

The same experimental procedure as above was used except that the RPMI was replaced with plasma.

Determination of the Proportion of PMNs that Adhered to ECs During Repeated PMN-EC Adhesive Contacts

For the 'first pass' PMN-HUVE interaction, primed or unprimed PMNs, $2x10^6$/ml in 250 µl of 1% FBS-RPMI were added to each HUVE cell monolayer (stimulated or unstimulated), for 30 minutes at 37°C, in a 5% CO_2 atmosphere following an experimental setup of six-plicate as above:

PMN unprimed and HUVE monolayer unstimulated;
PMN primed and HUVE monolayer unstimulated;
PMN unprimed and HUVE monolayer stimulated;
PMN primed and HUVE monolayer stimulated.

Six 250 µl samples of PMNs from the above groups were counted to obtain the total PMN cpm. After 30 minutes of the 'first pass' interaction period, HUVE monolayers were washed with RPMI-1640 twice. The wash solution of each subgroup was pooled

together and centrifuged; PMNs were counted, adjusted to a concentration of $2x10^6$/ml in 1% FBS-RPMI, and added to the corresponding HUVE monolayers (fresh stimulated or unstimulated) for 30 minutes at 37°C, in a 5% CO_2 atmosphere, to measure the 'second pass' PMN-HUVE interaction. Three 250 µl samples of PMNs from the above groups, were counted to obtain the total PMN cpm. Both the HUVE monolayers adhered with fresh PMNs (the first pass)or washout PMNs (the second pass) were treated with 1N NaOH and radioactivity determined as before and adherence calculated.

HUVE Cell Monolayer Labeling with ^{51}Cr

For the PMN-HUVE cytotoxicity assays, the HUVE monolayer was labeled with ^{51}Cr (instead of PMN labeling) as follows. Fifteen to eighteen hours before the experiment, HUVE cell monolayers were washed once with HBSS. A solution of 0.5 mCi ^{51}Cr in 30 ml HUVE cell culture medium was prepared and 0.3 ml of the solution was added to each well (for a total of 96 wells). Cell labeling was stopped by washing the HUVE monolayers three hours before the cytotoxicity assay.

HUVE Cytotoxicity Assay

After the HUVE monolayers were washed twice with medium RPMI, PMNs 0.5 million in 0.2 ml RPMI or PMN 0.5 million in 0.2 ml autologous plasma were added to wells (12 subgroups, with triplicates of each determinant). For the first two rows, only culture medium 0.2 ml for each well was added to determine the spontaneous ^{51}Cr release from labeled HUVE cells during the PMN-HUVE interaction period, providing duplicates of each determinant. After 30 minutes of PMN-EC interaction at 37°C in 5% CO_2, 0.05ml of PBS alone, or with PMA (final concentration of 5µg/ml), or fMLP (final concentration of 1 nM) in PBS were added to appropriate wells, for a 4 hr interaction period with gentle agitation (60 rpm) to prevent non adherent PMNs from settling onto the monolayer by gravity alone (and thus having the potential to interact with ECs by a non-adherent mechanism). At the end of the experiment, the supernatant was taken out

carefully, and wells washed twice with RPMI. Then, 1N NaOH was added to each well. All the supernatant and washout solution was collected as it contained ^{51}Cr released from HUVE cells reflecting the killed HUVE cells. The NaOH solution represented surviving HUVE cells that retained their ^{51}Cr label. HUVE cytotoxicity by PMNs was calculated as follows:

$$\textbf{\% HUVE Cytotoxicity} = \frac{\text{cpm of supernatant}}{\text{total cpm}} - \text{\% corrected spontaneous release}$$

where the spontaneous release % = cpm of supernatant (no PMNs) / total cpm;
and the corrected spontaneous release % = (1-PMN adhesion %)* x spontaneous release %.

The application of this correction factor changes the uncorrected data by an average +2% in HUVE- groups and by an average +15% in HUVE+ groups.

*It was noted at the beginning of the above experiments that the cpm of ^{51}Cr detected in the supernatants of HUVE wells that contained no PMNs (spontaneous release) was greater than the ^{51}Cr cpm from HUVE wells that contained PMNs (HUVE cytotoxicity). This was interpreted to mean that adhered PMNs that did not kill the HUVE cells that they adhered to, partially inhibited ^{51}Cr release from such HUVE cells during the 4 hour interaction. It was thought necessary to introduce a correction factor to account for ^{51}Cr release differences caused by PMN adhesion without cytotoxicity. Therefore, the PMN adhesion rates from the corresponding adhesion assay for each subject group (table 2) were used for this correction factor.

Table 2. *The mean PMN adhesion to HUVE cells from each subject group used as a correction factor in the PMN HUVE cytotoxicity assay.*

		In RPMI	In Plasma
Control	HUVE unstimulated	4.2%	5.6%
	HUVE activated	34.0%	30.3%
Preoperative group	HUVE unstimulated	5.4%	6.0%
	HUVE activated	38.5%	33.3%
SIRS group	HUVE unstimulated	8.8%	12.4%
	HUVE activated	44.4%	36.4%

In these cytotoxicity experiments four basic experimental variations were studied with each subject group:

PMN in Medium and unstimulated HUVE monolayers

PMN in autologous plasma and unstimulated HUVE monolayers

PMN in Medium and stimulated HUVE monolayers

PMN in autologous plasma and stimulated HUVE monolayers

Effect of Time on HUVE Cytotoxicity

The time course of HUVE cytotoxicity was done using the same protocol as above with 3, 5, and 8 hours of PMN-HUVE interaction periods. The effect of a second signal in the form of fMLP 1 nM was also tested using 3, 5, and 8 hours of PMN-EC interaction periods.

Comparison of Spontaneous Release of ^{51}Cr from HUVE to Different Media

These experiments were done to look for different spontaneous ^{51}Cr release (without PMNs' cytotoxicity effect) from HUVE cells bathed in culture medium, autologous plasma, or RPMI.

The cell monolayers were washed twice with RPMI. Culture medium, autologous plasma, or RPMI, was added 0.2 ml each well to cover the labeled HUVE cells in duplicate. After a 30 minute incubation, PBS 50μl was added to each well and incubated for 2, 4, 6, and 8 hours periods respectively at 37°C with gentle agitation. The supernatants were taken out carefully. The cell monolayers were washed twice with RPMI. The wash-out was added to the supernatant for gamma count. 1N NaOH then was added to each well. The dissolved solution was taken out for gamma count. The ^{51}Cr release rate was calculated as:

$$^{51}Cr \text{ Release } \% = U / (U + L)$$

where U = CPM of supernatant

L = CPM of NaOH solution with dissolved cell monolayer.

Statistical Analysis

Data are expressed as mean ± SD for all measurements. Analysis of variance (ANOVA) was used to assess for statistical significance across more than two patient data groups. Between group statistical differences were examined using Bonferonni corrected t-tests. Significance was set at $p < 0.05$.

RESULTS

Technical Factors

Photograph 1 shows the typical 'cobble stone' appearance of a HUVE monolayer examined with phase contrast microscopy depicting the typical endothelial cell appearance. Photograph 2 shows a similar monolayer stained for Factor VIII to demonstrate cell purity and specificity. Photograph 3 depicts a HUVE monolayer after activation with TNF-α and IL-1β stained with FITC labeled anti-ICAM-1 demonstrating significant expression of ICAM-1 by HUVE cells with more then 99% of cells expressing high density of ICAM-1.

The integrity of the HUVE cell monolayers was verified with the ^{125}I-HSA leak test which showed an everage albumin leak rate of 0.72% $\pm$ 0.21% (table 3). In contrast empty filters, i.e. without a HUVE cell monolayer, had a leak rate of 21%.

Table 4 shows the variability of pipetting PMN aliquots and the ability to distribute PMNs reliably into the appropriate experiment such as the HUVE wells for the adherence and cytotoxicity assay.

Two healthy control subjects (one man and one woman, age 40 and 30 years old) and 2 patients with SIRS, age range 46 and 33 years old, whose demographic details are shown in table 11, were used to measure spontaneous ^{51}Cr release. Spontaneous ^{51}Cr release from labeled HUVE cells in RPMI medium was much higher compared to that from HUVE cells in culture medium or in human plasma. Human plasma derived from healthy control subjects had the same stabilizing effect on the HUVE monolayer as endothelial cell culture medium (Chart 27 and 28). Plasma derived from patients with SIRS also stabilized the HUVE monolayer to the same extent as plasma from healthy controls in both unstimulated (chart 29) and stimulated (chart 30) HUVE cell monolayers.

PMNs Adherence to HUVE Cells in Health and Disease

Ten volunteers, four women and six men, age range 21 to 65 years old, served as healthy control subjects. None consumed medication or chemical substances during the study. Ten preoperative patients, six men and four women, age range 38 to 79, served as the non-infected subjects without SIRS. Their demographic details and diagnosis are shown in Table 5. Fourteen patients from the SICU, eight men and six women, age range 23 to 82 formed the SIRS patient group. Their demographic details and diagnosis upon admission to the SICU are shown in Table 6. Chart 1 shows the data, with a progressive increase in the adherence of unstimulated PMNs to unstimulated HUVE cells from healthy subjects to those with SIRS. The PMN adherence of patients with SIRS, 8.8 $\pm$ 3.2 %, is significantly higher than that of subjects without SIRS, 5.4 $\pm$ 2.4%, or healthy subjects, 4.2 $\pm$ 1.4%. The results are similar to previous data with anergic patients reported from this laboratory[184].

HUVE Cell Cytotoxicity in Health and Disease

Eleven volunteers, eight men and three women, age range 24 to 48 years old served as the healthy control group. Twelve preoperative patients, seven men and five women, age range 24 to 78 years old, with a mean 49, served as the non-infected control subjects without SIRS. Their demographic details and diagnosis are shown in table 7. Sixteen patients from the SICU, eight men and eight women, age range 28 to 88, with a mean 65, served as the SIRS patient group. Their demographic details and diagnosis are shown in table 8. Chart 2 shows the data of unstimulated PMN cytotoxicity to unstimulated HUVE cells. Unlike the significant increase in the adherence of PMNs obtained from patients with SIRS to HUVE cells, these PMNs did not show an increase in HUVE cell cytotoxicity compared to healthy control subjects without SIRS.

Interestingly, pre-operative patients without infection or SIRS demonstrated a significant increase in HUVE cell cytotoxicity.

Effect of PMN Activation (Priming)

To test the effects of PMN activation with lipopolysaccharide on HUVE adherence and cytotoxicity, PMNs from the same groups used in the adherence and cytotoxicity experiments shows above, were pre-treated with LPS 100 ng/ml for 40 minutes prior to use in the standard adherence and cytotoxicity assays. The adherence data are shown in chart 3. LPS stimulation was able to increase the adherence of healthy subject and pre-operative patient PMNs to the level of PMN adherence observed with PMNs from patients with SIRS. PMNs obtained from patients with SIRS could not be further stimulated to increase their adherence to HUVE cells above that level observed with unstimulated PMNs from these patients. The cytotoxicity data are shown in chart 4. Of interest, pre-treatment of PMNs with LPS did not alter their capacity to kill HUVE cells in a plasma free system in any of the groups studied.

Effect of HUVE Cell Activation

To test the effects of HUVE cell activation with TNF-α and IL-1β for 3 hours on PMN adherence and cytotoxicity, PMNs from the same groups used in the adherence and cytotoxicity experiments shows above, were also used in the following adherence and cytotoxicity assays. The adherence data are shown in chart 5. HUVE cell activation resulting in ICAM-1 expression markedly increased the adherence of PMNs to HUVE cells in all groups. The significantly increased adherence of PMNs from patients with SIRS was maintained in both the basal and activated HUVE cell monolayers. The increase in PMN adherence to activated HUVE cells resulted in increased cytotoxicity in all groups, but again, the increased adherence observed with PMNs from patients with

SIRS did not result in increased cytotoxicity in this group compared to its two control groups (chart 6). The slightly increased cytotoxicity of PMNs from pre-operative patients seen with unstimulated HUVE cells was maintained in stimulated HUVE cells (chart 6). Even with maximally activated HUVE cells, PMN pre-treatment with LPS 100 ng/ml for 40 minutes showd the same augmentation of PMN adherence in control and pre-operative patients, but not in patients with SIRS (chart 7). HUVE cytotoxicity was not enhanced significantly by PMN pre-treatment with LPS even with maximally stimulated HUVE cells (chart 8).

Chart 9 clearly demonstrates the importance of HUVE cell activation (ICAM-1) expression over PMN stimulation (integrin expression) on the adherence of PMNs to HUVE cells. When Healthy control PMNs were used, HUVE activation results in over 1000% increase in adherence compared to PMN stimulation, whereas no such aumentation was observed with HUVE cytotoxicity.

Effect of "First Pass" PMN Adhesion vs. Subsequent PMN Adhesion to HUVE Cells

Chart 10 shows the data of an experiment designed to determine whether the PMN adherence measured in these experiments was due to a PMN subpopulation in the circulation with an avidity for HUVE cells. As can be seen in this chart, the adherence pattern of healthy control PMNs derived from the non-adhered population in the supernatent of the experiment used to generate the data shown in chart 9, is similar to those of chart 9, indicating that PMNs not adhered to HUVE cells during the first pass, were capable of similar adhesion as first pass PMNs. No PMN subpopulations were detected by these experiments.

Effect of a "Second Hit" on PMN Cytotoxicity of HUVE Cells

Adhered PMNs onto HUVE cells may receive additional stimuli that dictate their behaviour. Chart 11 shows the data of experiments designed to test whether a "second

hit" through a membrane acting agonist, fMLP 1 nM, would increase the unstimulated and stimulated HUVE cell cytotoxicity by PMNs obtained from the 3 groups. No such augmentation of HUVE cell cytotoxicity induced by fMLP was observed among three study groups. Similarly PMA 5 μg/ml, an agonist that bypasses membrane signalling, did not reveal an augmented HUVE cell cytotoxicity by PMNs from patients with SIRS (chart 12), as well as PMNs from control and pre-operative patients.

Effect of Plasma on PMN Adherence and Cytotoxicity of HUVE Cells

PMN-EC interactions occur in the presense of plasma *in vivo*, yet most scientists study these interactions in plasma free systems. As can be seen from chart 13, plasma had no effect on the progressive increase in PMN adherence to HUVE cells seen in the three study groups. In contrast, chart 14 shows that plasma eliminated the HUVE cytotoxicity seen with RPMI as the medium in all three study groups. Comparing PMN-HUVE interactions in plasma to in RPMI, stimulating the PMNs (chart 15) or the HUVE cells (chart 16) or combinations of the two (chart 17) had no extra effect on PMN adherence to HUVE cells in plasma and in particular, the adherence was not eliminated as was seen with HUVE cytotoxicity. Only with HUVE cell stimulation (chart 18) could we observe any appreciable HUVE cytotoxicity and this was not higher in patients with SIRS compared to pre-operative patients without SIRS or to healthy controls. The similar pattern of cytotoxicity was seen when fLMP (chart 19) or PMA (chart 20) was jointed to the PMN-HUVE interaction in all three study groups.

The protective effect of plasma was better appreciated when HUVE cytotoxicity was followed out to 8 hrs. Five volunteers, one man and four women, age range 35 to 48 years old served as the healthy control group. Five preoperative patients, two men and three women, age range 23 to 63 years old, with a mean 48, whose demographic details and diagnosis are shown in table 9, and 5 patients with SIRS, age range 53 to 83 years old, with a mean 67, whose demographic details are shown in table 10, were used to test for the effect of plasma on PMN adhesion and subsequent cytotoxicity of HUVE

cells in a series of time checking points. As shown in chart 21 using PMNs and plasma from healthy controls with unstimulated and stimulated HUVE cells, plasma provides nearly complete protection of HUVE cells from PMN cytotoxicity up to 8 hours whereas RPMI resulted in as much as 68% HUVE cytotoxicity after 8 hours. A "second hit" in the form of fMLP 1 nM produced minimal extra cytotoxicity even with stimulated HUVE cells overlaid with plasma (chart 22). Similar results were obtained with plasma from pre-operative patients without SIRS (charts 23 and 24), as well as plasma obtained from patients with SIRS (charts 25 and 26). Plasma from patients with SIRS was able to protect HUVE cells against PMN induced cytotoxicity even after HUVE cell activation or "second hit" stimulation of the adhered PMNs with fMLP 1 nM.

DISCUSSION

PMNs are armed with an impressive arsenal of bactericidal agents and play a vital role in the host defense against invading pathogens. However, these same agents can produce extensive cellular damage to host tissues within an inflammatory enviroment. PMN migration through venules, usually post capillary venules, is a critical stage in carrying out beneficial or detrimental function in the inflammatory process. This includes PMN rolling, adherence to, and finally passing through the vascular endothelial cell layer. PMN-EC adhesion represents specific arrest of neutrophils in venules leading to rapid accumulation PMNs during local inflammatory responses. CAMs are required on both PMNs and ECs for this firm adhesion and are modulated by multiple inflammatory factors.

The techniques required for the experiments in this thesis were carefully developed and documented. The ability to isolate and subculture HUVE cells[184] was shown by the typical 'cobble stone' appearance of the endothelial monolayer. Strict control of the harvest procedure avoided the most common contamination by smooth muscle cells. This was further demonstrated by a Factor VIII stain which is a specific marker of endohtelial cells[185]. Most authors agree that ICAM-1 is expected soon after endothelial cell activation with a peak at 4-6 hr[158]. In this study, we used a 3 hr incubation with TNF-α and IL-1β which induced ICAM-1 in more than 99% of endothelial cells. Kukielka and colleagues[186] demonstrated that ICAM-1 mRNA and ICAM-1 expression could be detected within one hour of reperfusion in a canine myocardial ischemia/reperfusion model.

Most authors use the modified ^{125}I-HSA leak test as introduced by Drake et al[187] to verify the integrity of the HUVE cell monolayers. Less than 2% leak rate is acceptable and we certainly maintained this standard. A potential source of error from the variability of pipetting PMN aliquots, especially in the cytotoxicity assays, was also addressed, as this was kept to a SD <10%.

An interesting observation was made during optimization of the cytotoxicity assay. We found that the different media used produced differences in spontaneous ^{51}Cr release from the labeled HUVE cells. Plasma alone, both from healthy controls and from patients with SIRS, appears to stabilize HUVE cells in culture, resulting in lower spontaneous ^{51}Cr release compared to RPMI in unstimulated and stimulated HUVE cells.

We found a progressive increases of PMN adhesion to unstimulated HUVE cell monolayers when PMNs were obtained from healthy donnors, patients without SIRS and patients with SIRS. This increased PMN adherence observed with progressive disease was not accompanied by a comparable increase in HUVE cell cytotoxicity. This is unlike our previous observation with anergic surgical patients in the intensive care unit where anergic ICU patients demonstrated increased PMN adherence to HUVE cells and a significantly higher HUVE cytotoxicity[182]. Other works in this Laboratory have demonstrated that circulating PMNs from SICU patients have enhanced adhesion receptor expression and decreased cell delivery to inflammatory foci[188,189,190,191]. Sahin et al recently reported that serum from patients with Behcet's disease[192] increased the adhesion of control PMNs to HUVE cells *in vitro* but they did not measure the adherence of PMNs from Behcet's disease patients. Fein et al[193] studied PMN adherence to bovine endothelial cells in 13 patients with sepsis secondary to bacterial infections, 14 patients without infection, and 12 patients with myocardial infarction and found no differences in baseline PMN adhesion in the 3 groups. PMA stimulation of PMNs produced lower adhesion in patients with sepsis. We found no other clinical reports in the literature to compare with our findings.

LPS stimulation of PMNs increased the adherence of PMNs from healthy subjects and patients without SIRS to the level of adhesion of PMNs from patients with SIRS. PMNs from patients with SIRS could not be further stimulated to increase their adherence to HUVE cells. This suggests that PMNs from SIRS patients are maximally stimulated for HUVE adherence. CD11a (LFA-1) is reported to be expressed at relatively constant levels and is largely responsible for unprimed PMN adherence to

unstimulated EC[95,101]. Anti-CD11b Mabs had almost completely inhibited adhesion of primed PMNs to unstimulated ECs[101,194], whereas MAbs directed at CD11a had no obvious inhibitory effect in static adhesion assays, which demonstrated that upregulation of CD11b (Mac-1) is largely responsible for enhanced PMN-EC adhesion after PMN priming.

Unlike our previous findings with calcium flux[69], we found no evidence for PMN subpopulations that preferentially adhere to HUVE cells. We found that the "first pass" adherence pattern of PMNs (unstimulated and stimulated with LPS) from healthy controls was similar to a "second pass" experiment using PMNs derived from the non-adhered population in the supernatant of the first pass adherence step.

Based on these previous findings, it is possible that CD11b upregulation, in the environment of quiescent ECs, is time related (a small percent of PMNs at a time), where does this fit in metabolic processing of functional maturation of PMNs, and is responsive to inflammatory factors' stimulation.

PMN activation with LPS had no obvious effect on HUVE cell cytotoxicity in any of the studied groups. HUVE cell cytotoxicity was not significantly enhanced by PMN pretreatment with LPS even with maximally stimulated HUVE cells. The results are different from the reports that Mac-1 dependent adhesion to ECs enhanced secretion of H_2O_2 by chemotactic factors or cytokine activated PMNs[195196].

A dramatic increase in PMN adherence to HUVE cells occurred following HUVE cell monolayer activation with TNF-α and IL-1β with a concomitant increase in HUVE cell cytotoxicity.

Thomas et al reported that treatment of HUVE cells with LPS produced much higher PMN adherence than treatment of PMNs with LPS[194]. It is accepted so far by most that the Ig gene superfamily adhesion molecules of ECs, specifically ICAM-1, are involved in this PMN-EC adhesion[158,159]. There is no storage of ICAM-1 in quiescent ECs and several hours is required for ICAM-1 upregulation (mRNA and protein synthesis). Integrin expressions on PMNs are also required for firm PMN-EC adhesion (including LFA-1 and Mac-1)[14,101]. The result suggests that integrin upregulation after PMN-EC contact to meet upregulated ICAM-1 expression is so efficient that PMN

pretreatment (priming) is not necessary. Physiologically, it reflects the specific PMN function of massive accumulation as to quick response in an acute inflammation/infection environment which is labeled as EC activation. It also suggests that the maximum PMN-EC adhesion level is depended upon the maximum expression level of CAMs (most possibly ICAM-1) on ECs. The multiple level regulations of the ICAM-1 expression with different stimulators render PMNs at relevant multiple level adhesion to ECs, with limited modulation by PMN priming.

When the evidences were that PMN priming is not key factor for adhesion and primed PMNs have less capability of exudation, it seems that PMN priming is the host defense at alarming stage and PMNs at the function state ready to fight in the circulation against pathogens deeply invaded into system circulation, a situation often seen in life-threatening infection (for example, bacteremia and sepsis). However, primed PMNs in circulation do more damage than unprimed PMNs, especially to endothelial lining and underneath membranes in inflammatory environment. And many factors induced in non-infectious environment changes can also prime PMNs, which makes the side-effect of PMNs more complicated.

This PMN-HUVE adhesion study was under static condition, bypassed the 'rolling' stage of PMNs under a shear force condition *in vivo* which is considered necessary for stopping PMNs and initiating adhesion. It is possible that with (three hours of) TNF-α and IL-1β stimulation, E selectins are expressed on the HUVE surface and play a role in PMN-HUVE interaction[13]. It has been reported that MAbs directed against E-selectin on cytokine-stimulated EC monolayers inhibit PMN adhesion[197] and migration[180], whereas MAbs against L-selectin showed little migration inhibition[197,198].

Dukielka[186] and Barnett[199] reported that ICAM-1 expression promotes the neutrophil mediated cytotoxicity.

We found no evidence for the "multiple hit" hypothesis which states that primed PMNs by infection etc. respond more "aggressively" following a second hit by agonist stimuli. Membrane acting stimuli such as fMLP or stimuli that bypass membrane signaling mechanisms such as PMA failed to significantly alter cytotoxicity of HUVE cells in all study groups. This is contrary to the findings of Nathan[196] and Bratt[200]. One

reason may be that in this study, fMLP or PMA were added to the PMN-EC interaction system after PMNs were adherent to HUVE cells, and with this environment, single inflammatory factors may have very weak influence to PMN-EC interaction.

To mimic *in vivo* PMN-EC interactions, plasma was introduced into the PMN-EC interaction system. Plasma did not change the PMN adhesion pattern seen with RPMI medium in the three study groups. We were surprised to find that plasma nearly eliminated the PMN HUVE cytotoxicity seen with RPMI medium in the system in all study groups. Plasma exerted this protective effect even after HUVE cell activation with TNF-α and IL-1β. The data suggest that plasma does not interfere with PMN adhesion to endothelium but prevents cytotoxicity of endothelial cells *in vivo*; the plasma effect lasts up to 8 hours; and the key step of PMN adhesion to endothelial cells prior to PMN exudation can proceed without damaging the endothelial cell. *In vivo* PMN adherence to ECs may not result in EC cytotoxicity, contrary to most current views of sepsis and the pathophysiology of multiple organ dysfunction.

Krsek-Staples and Webster[201] reported that ceruloplasmin, a plasma antioxidant, increases in concentration during inflammation and protects both endothelial cells and neutrophil and endothelial cell proteins from oxidative injury by iron oxidation in xanthine oxidase/hypoxanthin reactions. Ma and Lefer[202] showed that low dose taprostene, a stable analogue of prostacyclin, combined with human superoxide dismutase inhibited neutrophil adherence and activation and to inactivate superoxide radicals, resulted in reduced cellular injury. Others reported that -platelet-activating factor (PAF)[203] which may induce PMN integrin expression and oxygen free radical production, and lactoferrin[204] which is released by activated PMNs and causes P-selectin-mediated adhesion and microvascular injury or dysfunction. These materials exist in plasma in a pathophysiologic environment to influence PMN-EC interaction in a negative way. S-nitroso-N-acetylpenicillamin (SNAP)[205] which is thought to maintain systemic blood pressure and preserve vascular endothelial integrity, and Sialyl Lewis^x-containing oligosaccharide[206] were tested to attenuate plasma based factors inducing harmful PMN-EC interaction.

We know little about the mechanism of plasma protection in PMN-EC interactions which, to our knowledge, has not been reported in literature. Its physiologic importance is obvious. In SIRS patients, high concentrations of inflammatory mediators and bacterial byproducts in plasma both prime PMNs systematically and activate ECs in situ. Any primed and adhered PMN has the potential to damage ECs, without a control mechanism to counterbalance the detrimental potential of PMNs. It suggests that normal circulation (in which powerful protecting materials exist) to inflammatory microenvironment is necessary to keep the plasma stabilizing mechanism in effect when high level of PMN adhesion occurs.

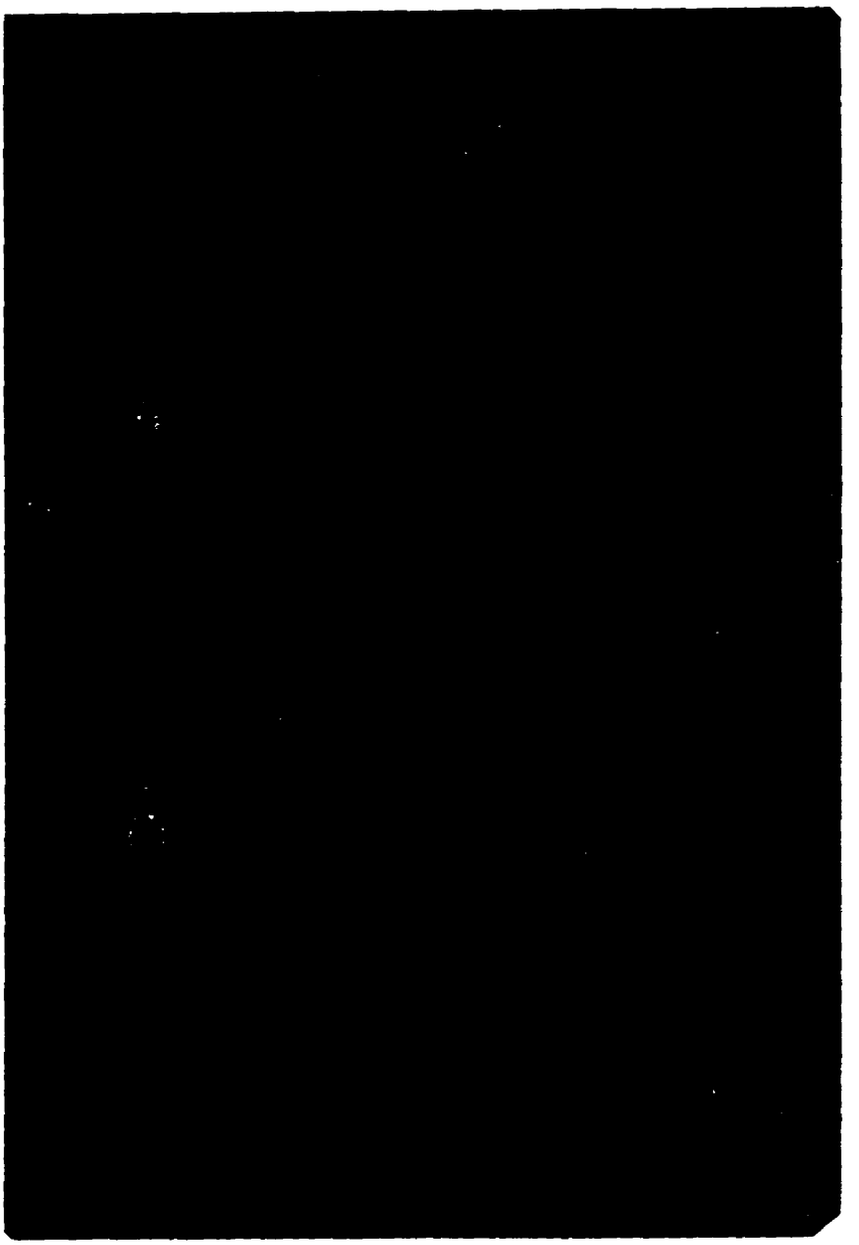
We know more positive feedback mediators and their mechanisms than negative feedback ones during systemic inflammatory response. Plasma functioning to prevent massive PMNs cytotoxicity to ECs is one example. From practical standpoint, more work is worthwhile to identify the unknown factor(s) and its protection mechanism.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

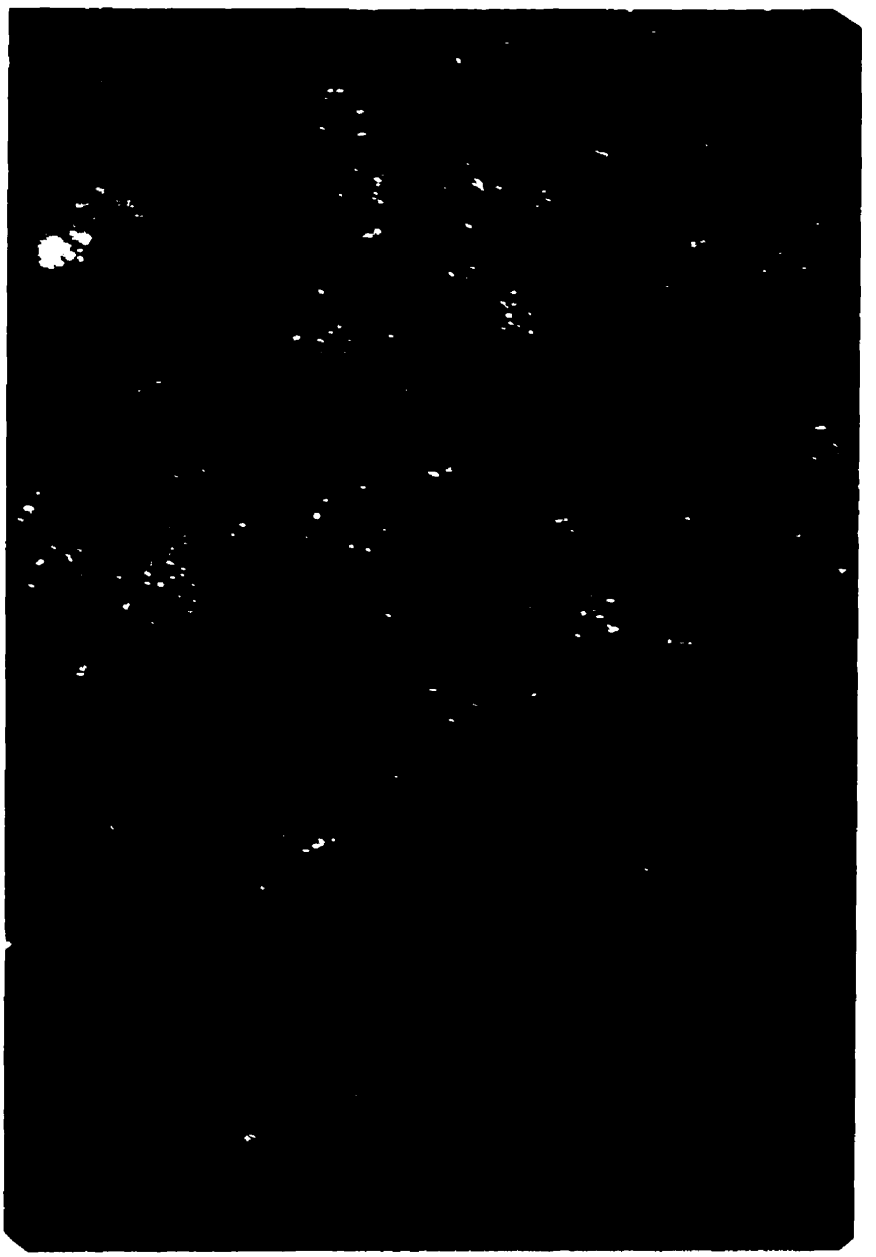
1. PMNs show progressively increased HUVE adherence as disease progresses from healthy controls to patients in the ICU with SIRS.
2. This increased PMN HUVE adherence is not associated with increased baseline HUVE cytotoxicity.
3. PMN pretreatment with LPS causes a slight but significant increase in PMN adhesion to HUVE cells, without an increases HUVE cell cytotoxicity.
4. HUVE cell activation with TNF-α and IL-1β produces a far greater increase in PMN adherence to HUVE cells than PMN treatment with LPS.
5. Treatment of PMNs from SIRS patients with LPS can not enhance their adhesion to HUVE cells, implying that PMNs from SIRS patients are maximally primed.
6. Plasma stabilizes HUVE cells and reduces spontaneous ^{51}Cr release compared to RPMI medium.
7. Plasma stabilizes adhered PMNs from destroying HUVE cells.

PHOTOGRAPHS AND TABLES

Photograph 1: *The appearance of the Human Umbilical Vein Endothelial cell monolayer under phase contrast microscopy (100 x).*



Photograph 2: *Human Umbilical Vein Monolayers stained with FITC -Factor VIII (1:200 dilution, 100x)*



Photograph 3: *FITC labaled anti-ICAM-1 staining (1:100) of TNF-α and IL-1β activated HUVE cell monolayer (100x).*

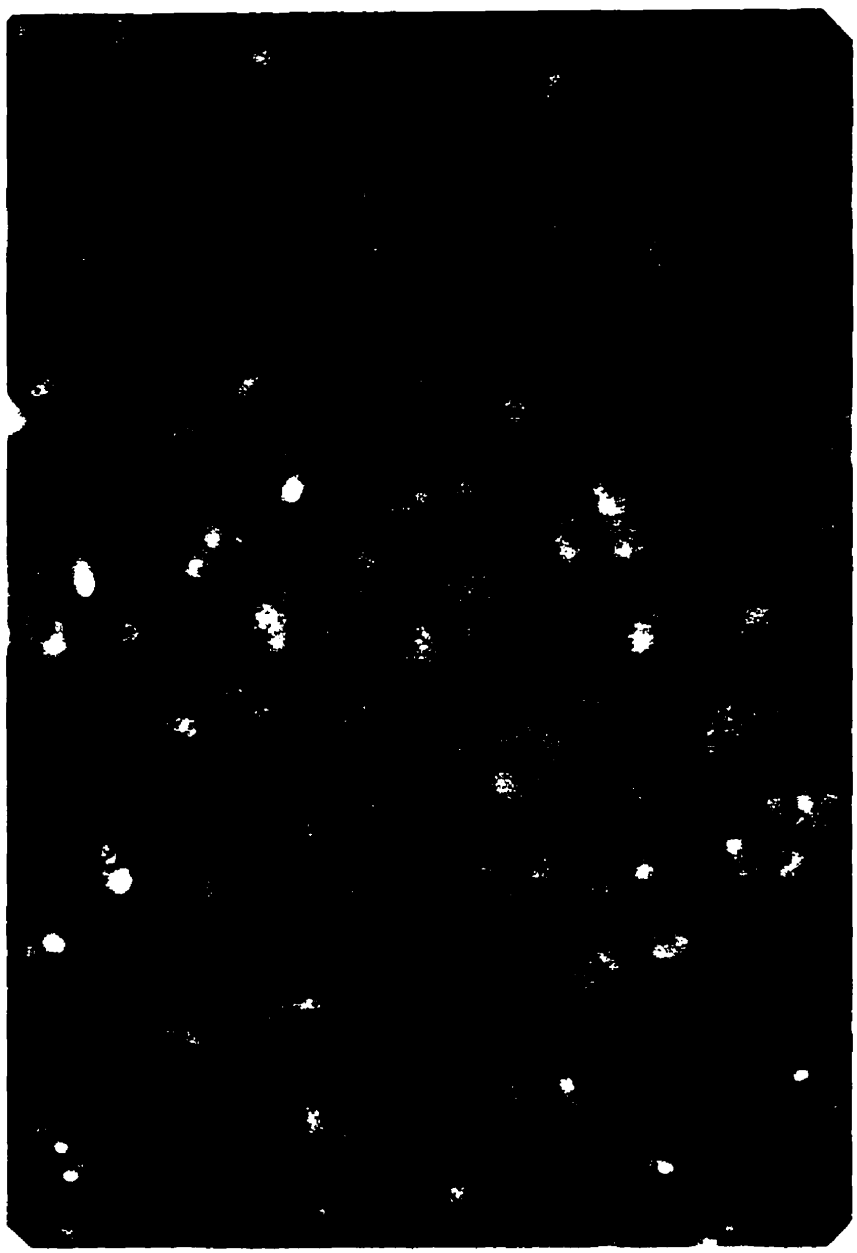


Table 3: *HUVE cells were cultured on 3 nm pore filters in transwell chambers untill confluence. The filters were washed twice with PBS, and placed in the transwell with 0.5 ml of culture medium in the bottom. An aliquot of 100 µl of ^{125}I-HSA in culture medium was added to the upper compartment of each chamber and the chambers were incubated for 30 minutes at 37°C in 5% CO_2. Aliquots form the upper and lower compartments were taken and counted, and the leak rate calculated.*

Filter No.	U-CPM	L-CPM	Leak %
1.	1081112	6088	0.56
2.	912858	5114	0.56
3.	1088064	5931	0.54
4.	1126997	5858	0.52
5.	1082372	5749	0.53
6.	1074861	6935	0.64
7.	1092919	6742	0.61
8.	1156643	6772	0.58
9.	876577	6724	0.76
10.	939727	7510	0.79
11.	1177517	6433	0.54
12.	1073260	8237	0.76
13.	1005920	7062	0.70
14.	1124637	8037	0.71
15.	1115430	7613	0.68
16.	1098762	6584	0.60
17.	1135314	8879	0.78
18.	1030615	14881	1.42
19.	906256	8273	0.90
20.	1139777	12542	1.09
21.	1156588	9946	0.85
22.	1290432	9358	0.72
mean	**1076665**	**7785**	**0.72**
SD	**98838**	**2299**	**0.21**

Table 4: *The variability in the pippeting of PMN aliquots. After labeling, PMNs were washed and concentrated to $2x10^6$/ml. Then, 250 µl was distributed into each tube for cell and radioactivity counts.*

No.	Cell Count	CPM Count
1.	167	35877
2.	98	35441
3.	193	37238
4.	166	35880
5.	123	34143
6.	113	34894
7.	127	34976
8.	100	36053
9.	127	35495
10.	113	35142
11.	152	35164
12.	141	35286
13.	152	31367
14.	142	36049
15.	158	35345
16.	157	36111
17.	160	35581
18.	156	35561
19.	197	36042
20.	191	35628
21.	195	36234
22.	188	38002
23.	122	34976
24.	128	36171
25.	131	35759
mean	**148**	**35568**
SD	**30**	**1144**

Table 5: *The 10 preoperative patients with no infections or SIRS that were used in the PMN-EC Adhesion Assay.*

Case	Gender	Age	Diagnosis
1	F	49	Gallstones
2	M	39	Post gastractomy syndrome
3	F	38	Colon Cancer
4	M	74	Biliary stricture
5	M	55	Choledocholithiasis
6	F	79	Left Breast Mass (CA)
7	M	79	Left Inguinal Hernia
8	M	56	Right Inguinal Hernia
9	M	68	Left Inguinal Hernia
10	F	49	Varicose Veins
mean		59	

Table 6: *The 14 patients with SIRS from the Surgical Intensive Care Unit that were used in the PMN-EC Adhesion Assay.*

Case	Gender	Age	Diagnosis	APACHE II
1	M	23	Sepsis/pneumonia	24
2	M	70	Fournier's gangrene	18
3	F	82	Pneumonia	37
4	F	50	Pneumonia	36
5	F	66	Pneumonia	12
6	M	65	Bacteremia/cholangitis	13
7	M	68	Necrotizing fasciitis	9
8	F	67	Pneumonia	18
9	F	82	Intraabdominal abscess	9
10	M	68	Pancreatic abscess	9
11	F	69	Generalized Sepsis	18
12	M	71	Pneumonia	22
13	M	66	Aortic graft infection	20
14	M	59	ARDS/pneumonia	9
mean		65		18.1

APACHE II: acute physiology and chronic health evaluation II;

ARDS: adult respiratory distress syndrome.

Table 7: *The 12 preoperative patients with no infection or SIRS that were used for the PMN-HUVE Cytotoxicity Assay.*

Case	Gender	Age	Diagnosis
1	F	24	Septoplasty
2	M	35	SeptoRhinoplasty
3	M	29	Corneal Graft
4	M	72	Cataract Surgery
5	F	78	Cataract Surgery
6	F	48	Carpal Tunnel
7	M	41	Duputran's Contracture
8	F	46	Cataract Surgery
9	F	71	Cataract Surgery
10	M	30	Inclusion Cyst
11	M	38	Femoral Artery Occlusion
12	M	70	Femoral Artery Occlusion
Mean		49	

Table 8: *The 16 patients with Systemic Inflammatory Response Syndrome from the SICU who were used in the PMN-HUVE Cytotoxicity Assay.*

Case	Gender	Age	Diagnosis	APACHE II
1	M	83	Pneumonia	10
2	M	67	Pneumonia	26
3	M	68	Bacteremia	7
4	M	63	Sepsis/Pneumonia	11
5	F	81	Pneumonia	28
6	F	65	Cholangitis	24
7	F	39	Abdominal wall cellulitis	31
8	M	77	Acute Pancreatitis	19
9	M	48	Pneumonia	6
10	M	87	Pneumonia	13
11	F	71	Perforated Colon	28
12	M	69	Mediastinitis	22
13	F	60	Perforated gastric ulcer	22
14	F	88	Pneumonia	12
15	F	28	Septic shock	39
16	F	38	Sepsis/Pneumonia	15
mean		65		19.6

Table 9: *The 5 preoperative patients with non-infectious diseases that were used in the Time Course of the PMN-EC Cytotoxicity Assay.*

Case	Gender	Age	Diagnosis
1	F	63	Gallstones
2	M	48	Gallstones
3	M	23	Urethroplasty
4	F	62	Gallstones
5	F	43	Tonsillectomy

Table 10: *The 5 patients with with Systemic Inflammatory Response Syndrome from the SICU who were used in the Time Course of PMN-EC Cytotoxicity Assay.*

Case	Gender	Age	Diagnosis	APACHE II
1	M	74	Urosepsis	20
2	M	66	Pneumonia	24
3	F	83	Peritonitis	14
4	F	53	Systemic Sepsis	23
5	M	59	Septic shock	30
mean		67		18.2

Table 11: *The 2 patients with Systemic Inflammatory Response Syndrome from the SICU who were used in the test of Spontaneous Release of ^{51}Cr from labeled HUVE cells in different media.*

Case	Gender	Age	Diagnosis	APACHE II
1	F	46	Intra-Abdominal Infection	20
2	F	33	Intra-Abdominal Infection	24
mean		40		22

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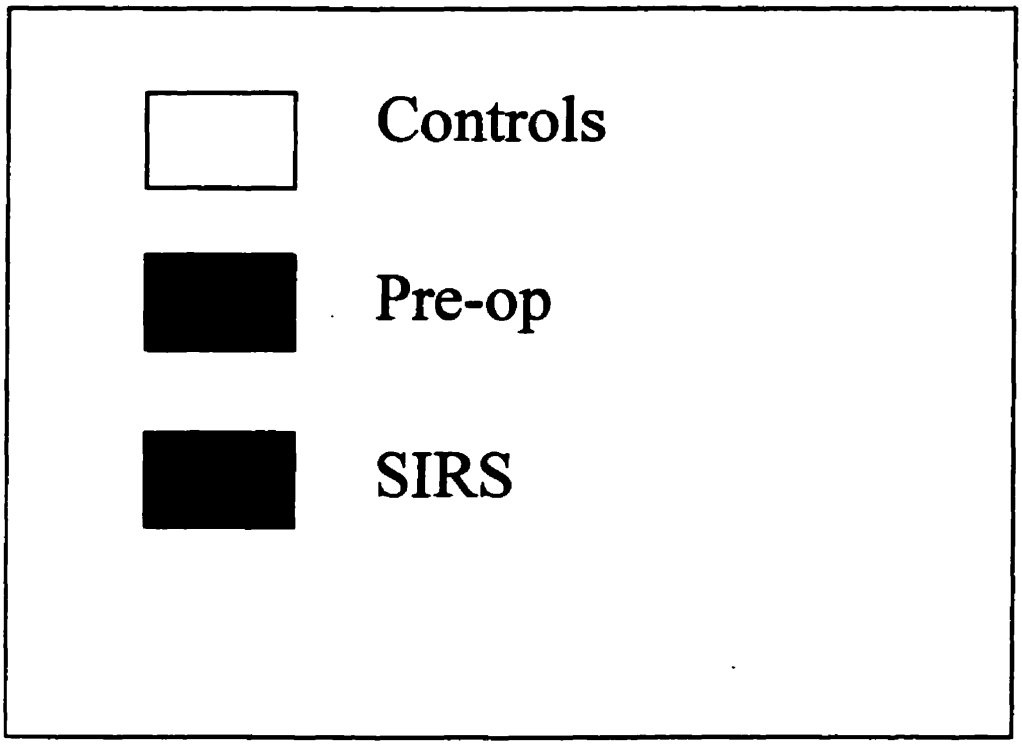


Please note that the above convention is maintained for all charts that follow

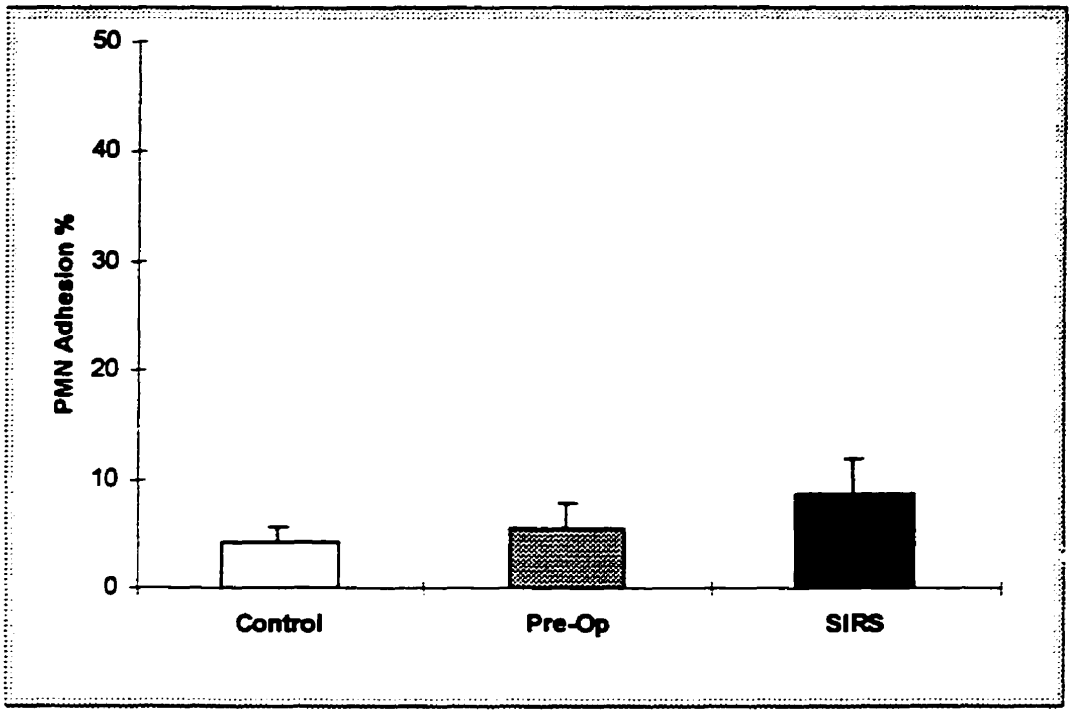


Chart 1. Baseline PMN-HUVE adhesion: no PMN activation, no HUVE activation, 30 minutes interaction, in medium RPMI.

Group	**PMN adhesion %**	*p* value
Control	4.2% ± 1.4%	Control vs. Pre-Op - NS
Pre-Op	5.4% ± 2.4%	Pre-Op vs. SIRS - < 0.01
SIRS	8.8% ± 3.2%	SIRS vs. Control: - < 0.05
ANOVA	p < 0.01	

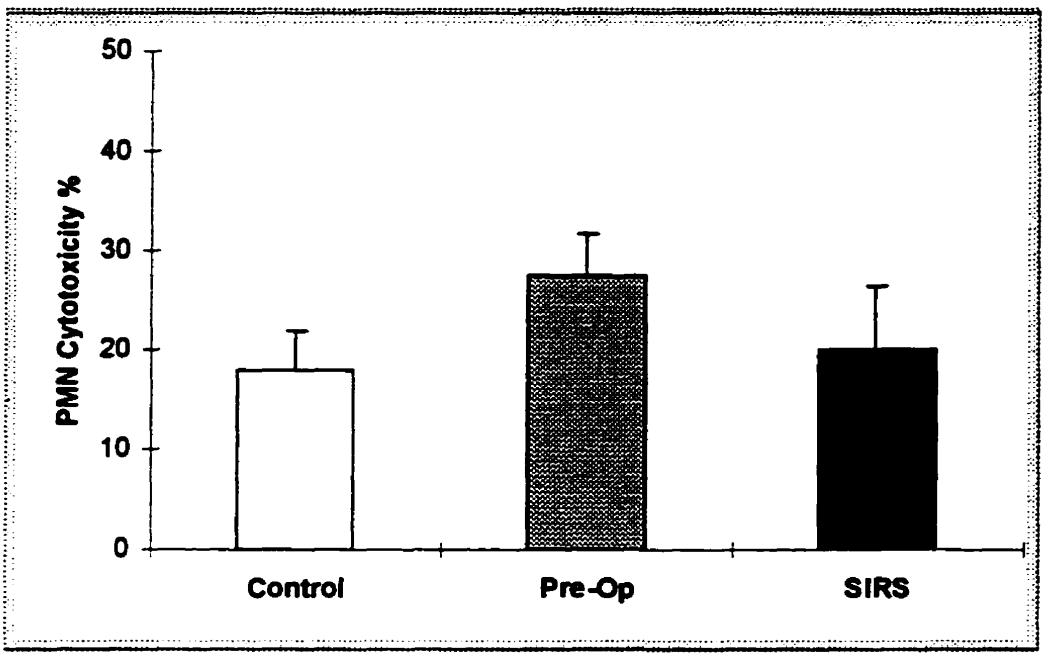


Chart 2. *Baseline PMN-HUVE cytotoxicity: no PMN activation, no HUVE activation, 4 hours interaction, in RPMI.*

Group	PMN Cytotoxicity %	*p* value
Control	18% ± 3.9%	Control vs. Pre-Op - < 0.01
Pre-Op	27.4% ± 4.3%	Control vs. SIRS - NS
SIRS	20.1% ± 6.4%	SIRS vs. Pre-Op - < 0.05
ANOVA	p< 0.01	

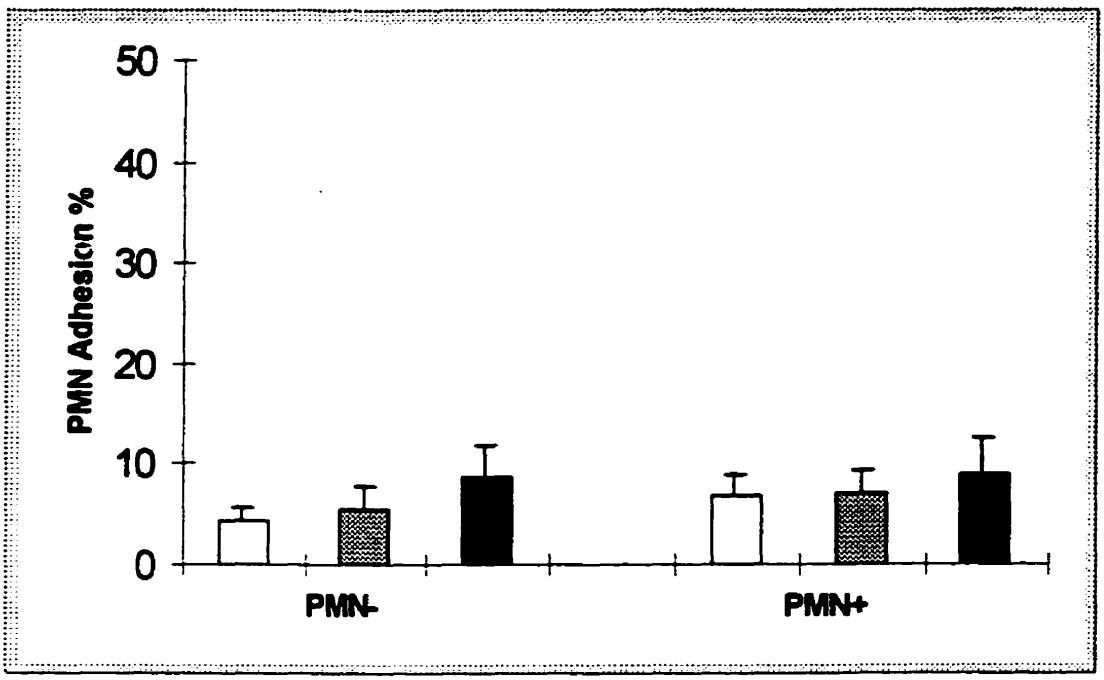


Chart 3. *The effect of PMN activation (priming) with LPS 100 ng/ml for 40 minutes on PMN adherence to unstimulated HUVE cells, in RPMI.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
Control	4.2% ± 1.4%	6.9% ± 2.1%	<0.05
Pre-Op	5.4% ± 2.4%	7.0% ± 2.4%	<0.05
SIRS	8.8% ± 3.2%	8.9% ± 3.8%	NS
ANOVA	p<0.01	NS	

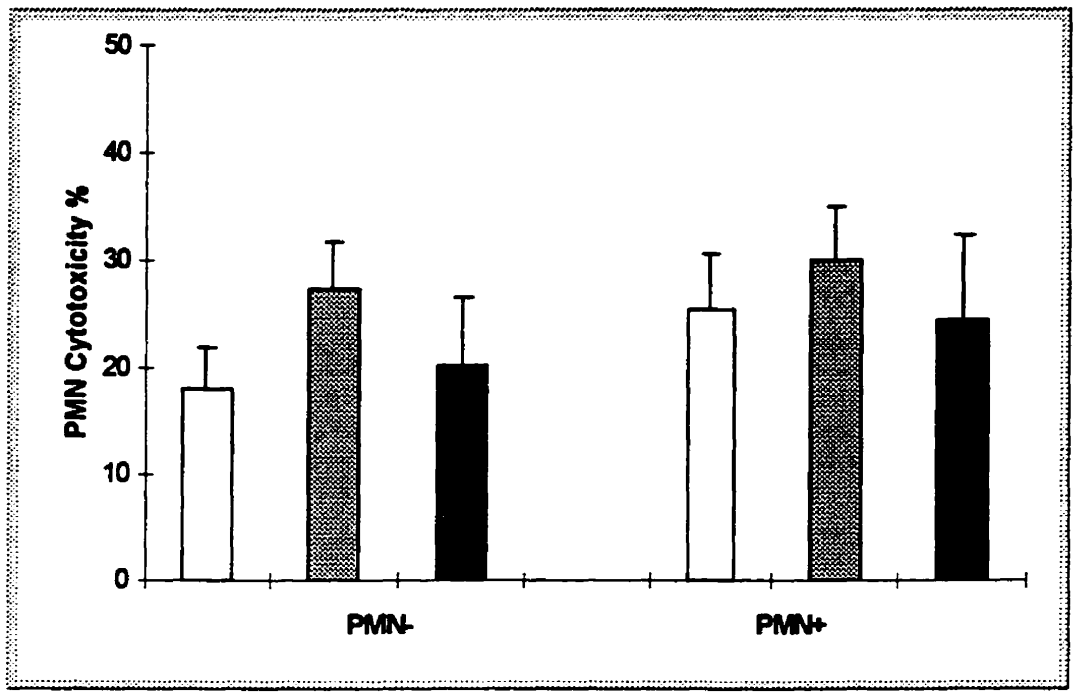


Chart 4. *The effect of PMN activation (priming) with LPS 100 ng/ml for 40 minutes on PMN cytotoxicity of unstimulated HUVE cells, in RPMI.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
Control	18.0% ± 3.9%	25.4% ± 5.1%	NS
Pre-Op	27.4% ± 4.3%	30.0% ± 5.0%	NS
SIRS	20.1% ± 6.4%	24.4% ± 8.0%	NS
ANOVA	p<0.01	NS	

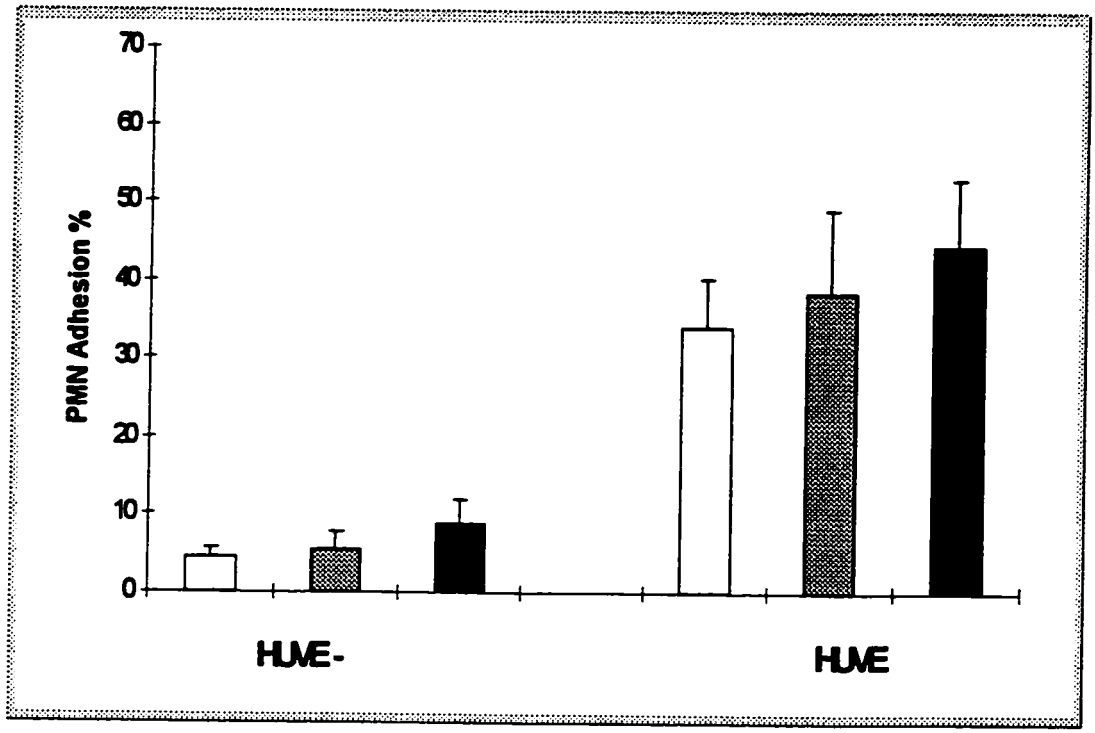


Chart 5. *The effect of HUVE cell activation with TNF-α and IL-1β for 3 hours on unprimed PMN adherence to HUVE cells, in RPMI.*

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	4.3% ± 1.4%	34.0% ± 6.2%	<0.001
Pre-Op	5.4% ± 2.4%	38.5% ± 10.5%	<0.001
SIRS	8.8% ± 3.2%	44.4% ± 8.5%	<0.001
ANOVA	p<0.001	p<0.05	

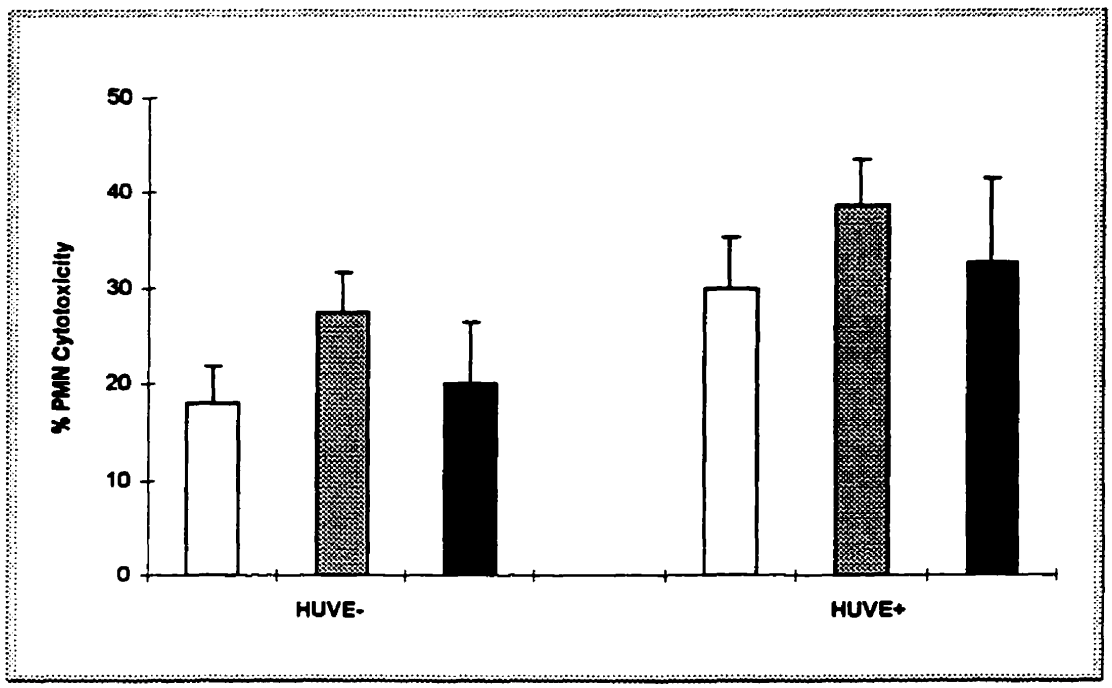


Chart 6. *The effect of HUVE cell activation with TNF-α and IL-1β on unprimed PMN cytotoxicity to HUVE cells, in RPMI.*

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	18.0% ± 3.9%	30.0% ± 5.3%	<0.01
Pre-Op	27.4% ± 4.3%	38.8% ± 4.8%	<0.01
SIRS	20.1% ± 6.4%	32.6% ± 9.0%	<0.01
ANOVA	p<0.01	p<0.05	

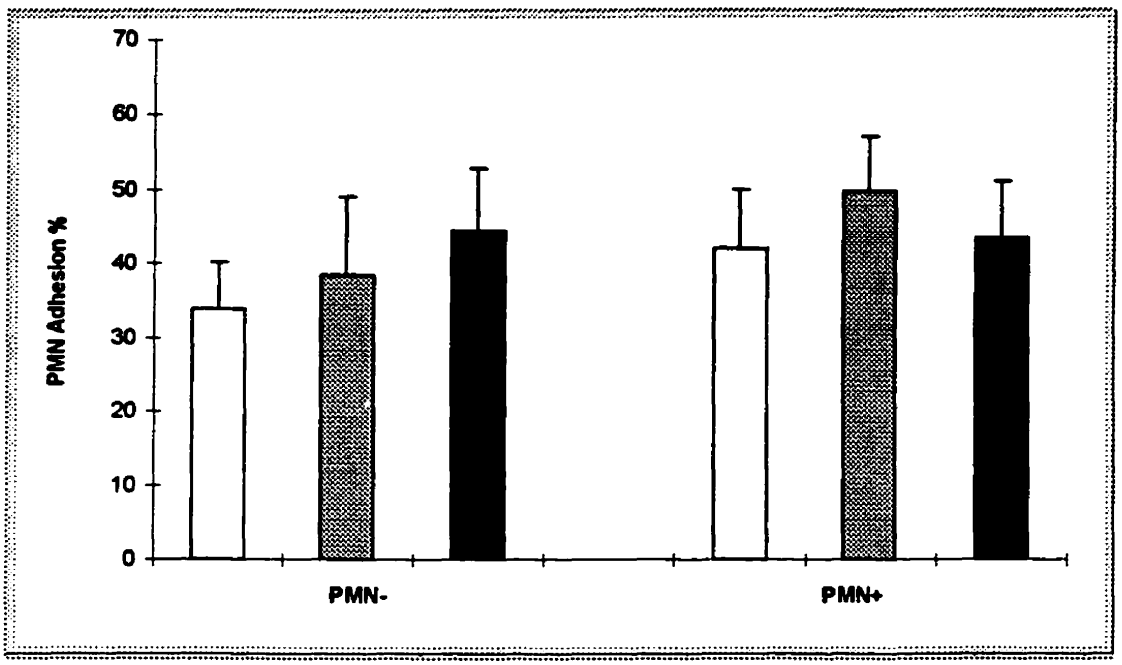


Chart 7 *The effect of PMN activation (priming) with LPS 100 ng/ml for 40 min on PMN adherence to stimulated HUVE cells, in RPMI.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
Control	34.0% ± 6.2%	42.1% ± 7.8%	<0.05
Pre-Op	38.5% ± 10.5%	49.7% ± 7.3%	<0.05
SIRS	44.4% ± 8.5%	43.4% ± 7.6%	NS
ANOVA	p<0.05	NS	

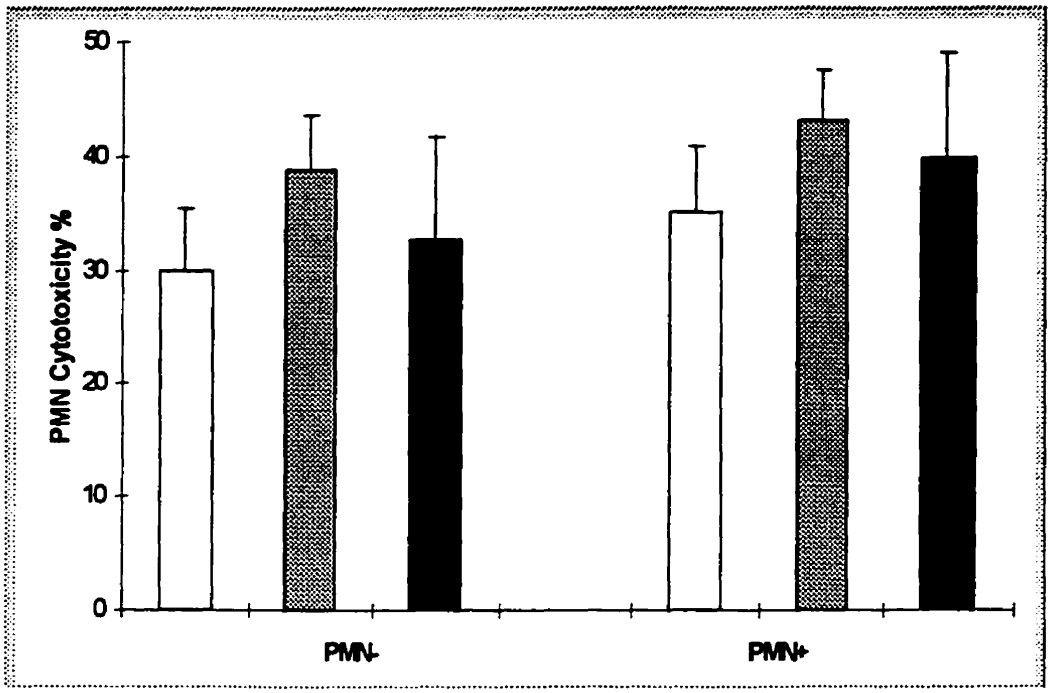


Chart 8 *The effect of PMN activation (priming) with LPS 100 ng/ml for 40 minutes on PMN cytotoxicity of stimulated HUVE cells, in RPMI.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
Control	30.0% ± 5.3%	35.1% ± 5.8%	NS
Pre-Op	38.8% ± 4.8%	43.1% ± 4.2%	NS
SIRS	32.6% ± 9.0%	39.8% ± 9.1%	NS
ANOVA	$p<0.05$	NS	

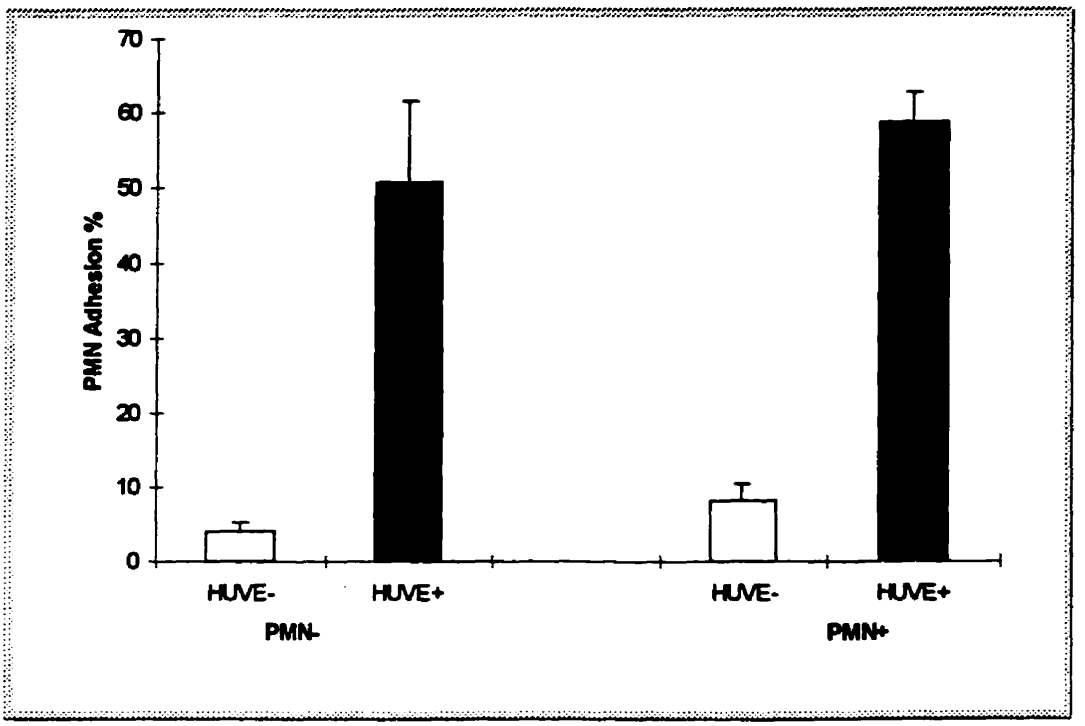


Chart 9 *The effect of PMN priming with LPS for 40 min vs. the effect of HUVE stimulation with TNF-α and IL-1β for 3 hours on PMN adherence to HUVE cells.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
HUVE-	4.1% ± 1.2%	8.2% ± 2.2%	<0.05
HUVE+	50.6% ± 11.0%	58.6% ± 4.1%	NS
p value HUVE- vs. HUVE+	<0.001	<0.005	

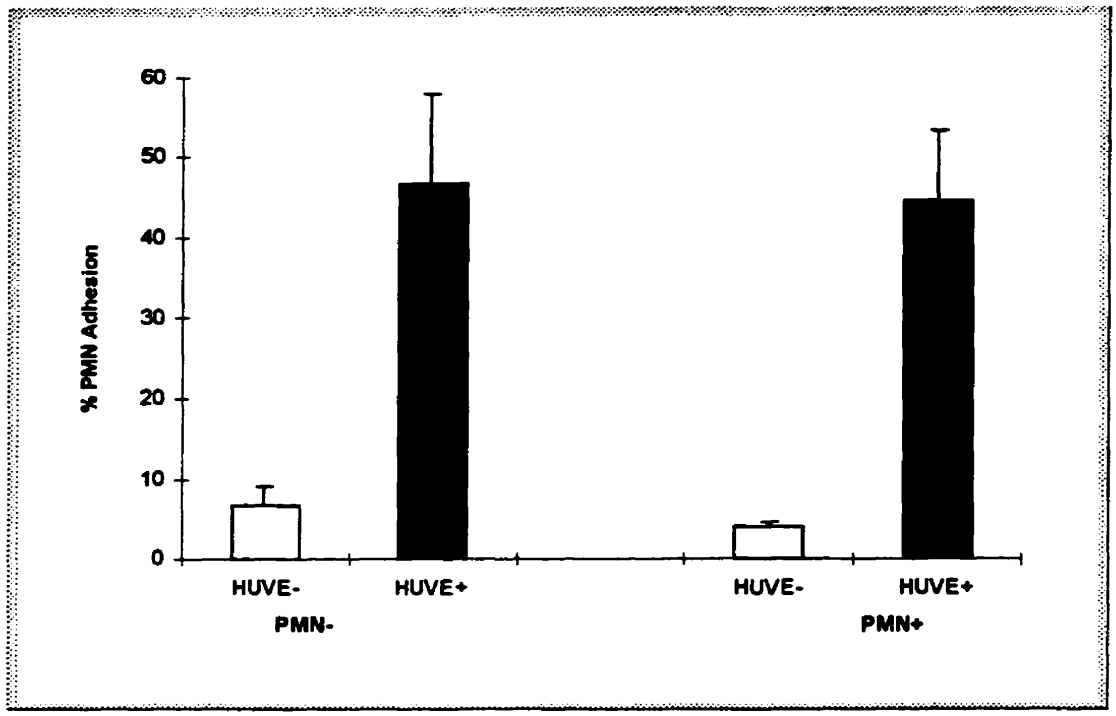


Chart 10 *The proportion of PMNs (which derive from the supernatants of the first PMN-HUVE interaction shown in Chart 9) that adhere to HUVE cells at the second adhesion step.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
HUVE-	6.8% ± 2.3%	3.9% ± 0.7%	NS
HUVE+	46.6% ± 11.5%	44.5% ± 8.7%	NS
p value HUVE- vs. HUVE+	<0.01	<0.001	

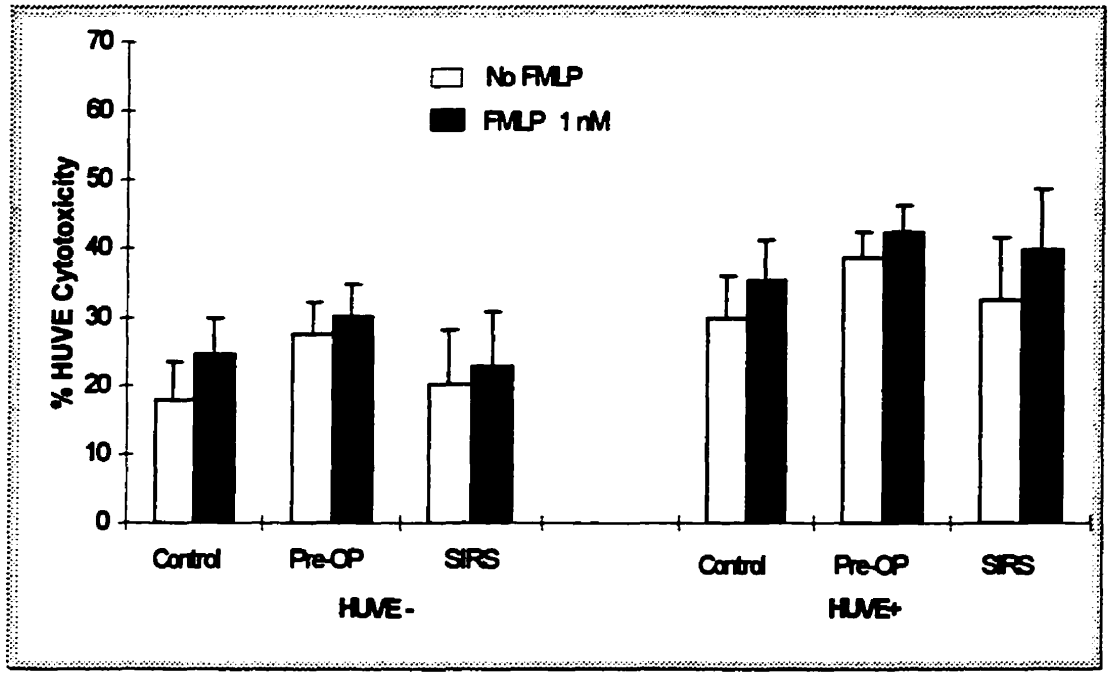


Chart 11 *The effect of a PMN stimulating factor in the form of fMLP 1 nM, on unstimulated PMN cytotoxicity to unstimulated and stimulated HUVE cells, in RPMI.*

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	24.6% ± 5.3%	35.3% ± 6.0%	<0.001
Pre-Op	30.1% ± 4.8%	42.6% ± 3.7%	<0.001
SIRS	22.8% ± 8.0%	39.7% ± 8.9%	<0.001
ANOVA	p<0.05	p<0.05	

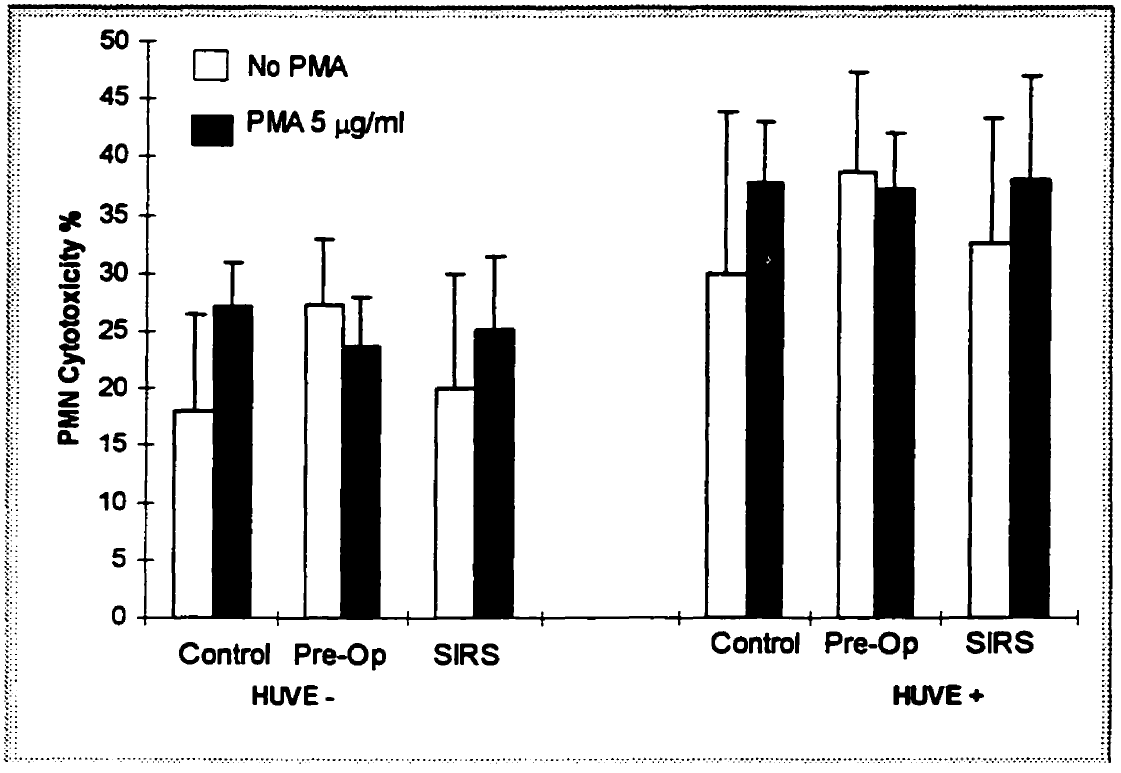


Chart 12 *The effect of a PMN stimulating factor in the form of PMA 5 ug/ml, on unstimulated PMN cytotoxicity to unstimulated and stimulated HUVE cells, in RPMI.*

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	27.1% ± 8.5%	37.7% ± 13.8%	<0.01
Pre-Op	23.7% ± 5.5%	37.2% ± 8.5%	<0.01
SIRS	25.1% ± 9.9%	38.0% ± 10.7%	<0.01
ANOVA	NS	NS	

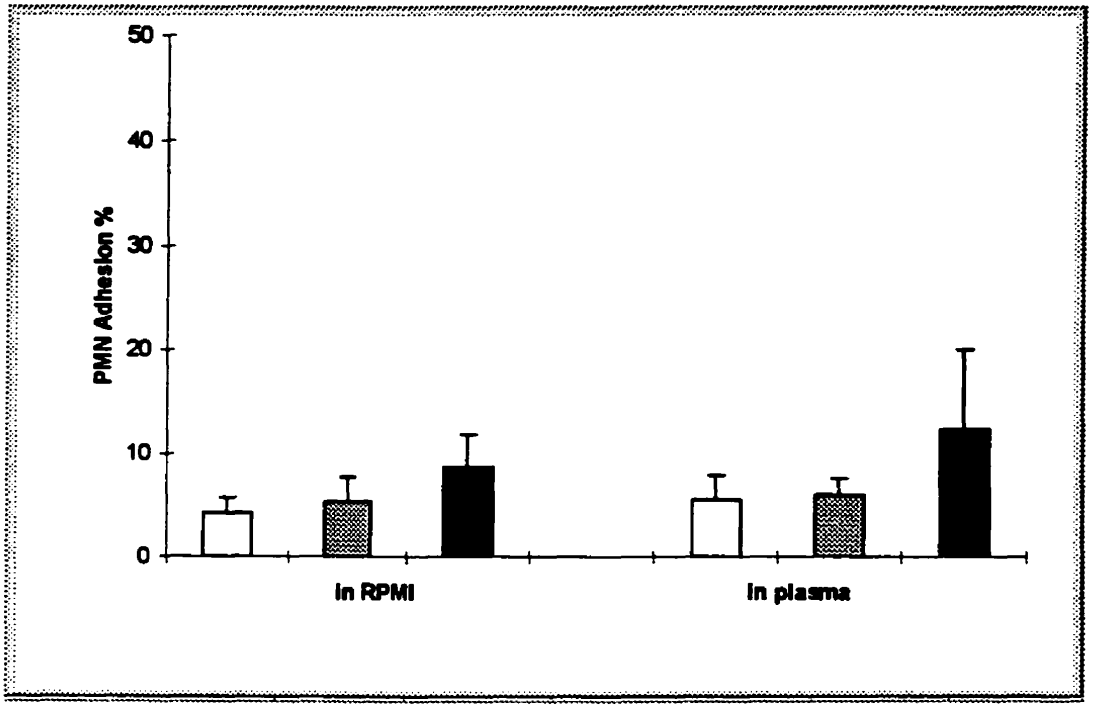


Chart 13 *The effect of plasma on the adhesion of unstimulated PMN to unstimulated HUVE cells.*

	In RPMI	**In plasma**	*p* value RPMI vs. plasma
Control	4.3% ± 1.4%	5.6% ± 2.4%	NS
Pre-Op	5.4% ± 2.4%	6.0% ± 1.6%	NS
SIRS	8.8% ± 3.2%	12.4% ± 7.7%	NS
ANOVA	p<0.01	p<0.01	

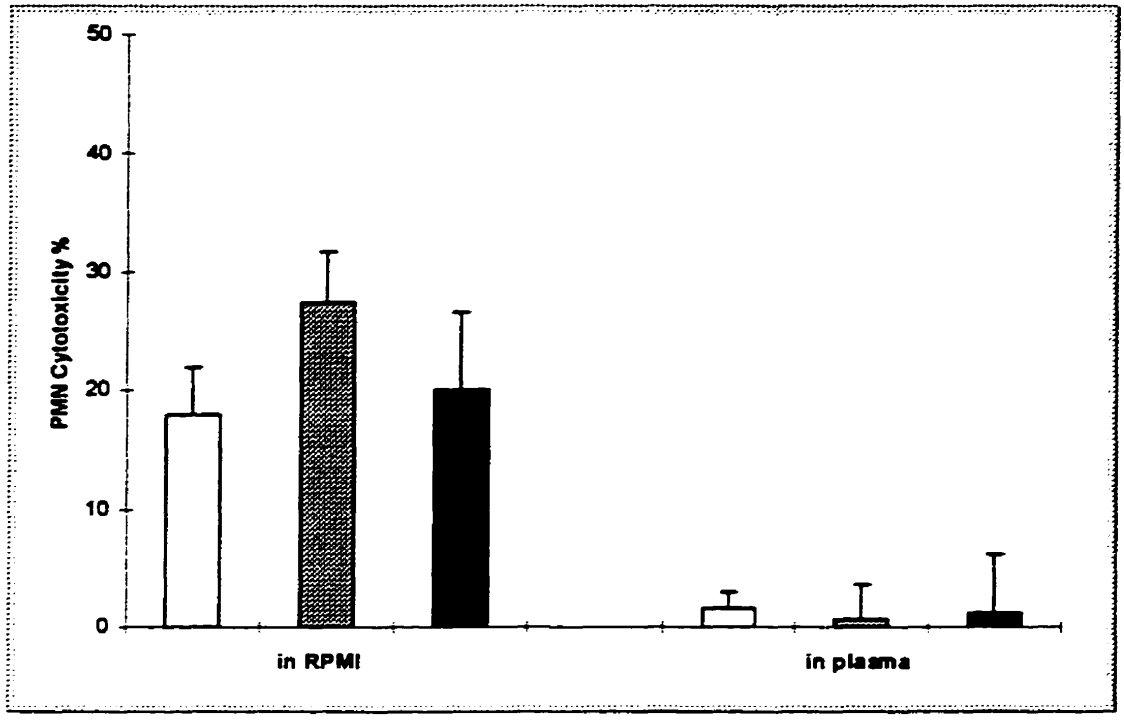


Chart 14 *The effect of plasma on the cytotoxicity of unstimulated PMN to unstimulated HUVE cells.*

	In RPMI	**In plasma**	*p* value RPMI vs. plasma
Control	18.0% ± 3.9%	1.6% ± 1.4%	<0.001
Pre-Op	27.4% ± 4.3%	0.7% ± 2.9%	<0.001
SIRS	20.1% ± 6.4%	1.2% ± 5.0%	<0.001
ANOVA	p<0.01	NS	

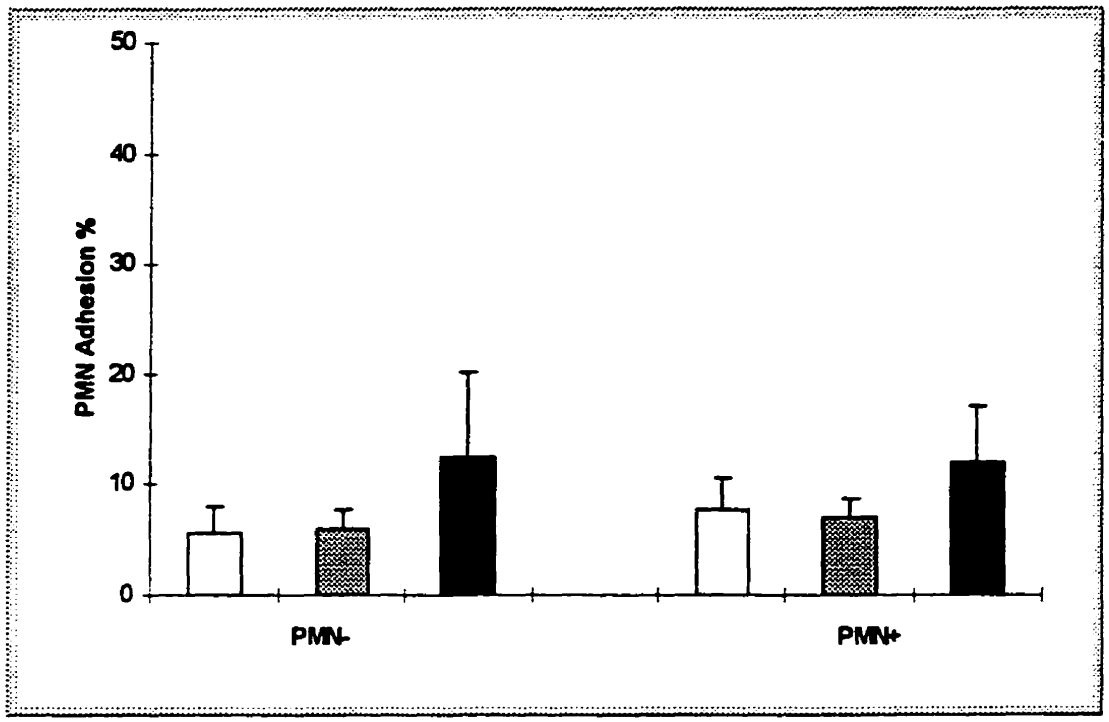


Chart 15 *The effect of plasma on the adherence of unstimulated and stimulated PMN (priming with LPS 100 ng/ml for 40 minutes) on to unstimulated HUVE cells.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
Control	5.6% ± 2.4%	7.7% ± 2.9%	<0.05
Pre-Op	6.0% ± 1.6%	7.0% ± 1.7%	<0.05
SIRS	12.4% ± 7.7%	12.0% ± 5.2%	NS
ANOVA	p<0.01	p<0.01	

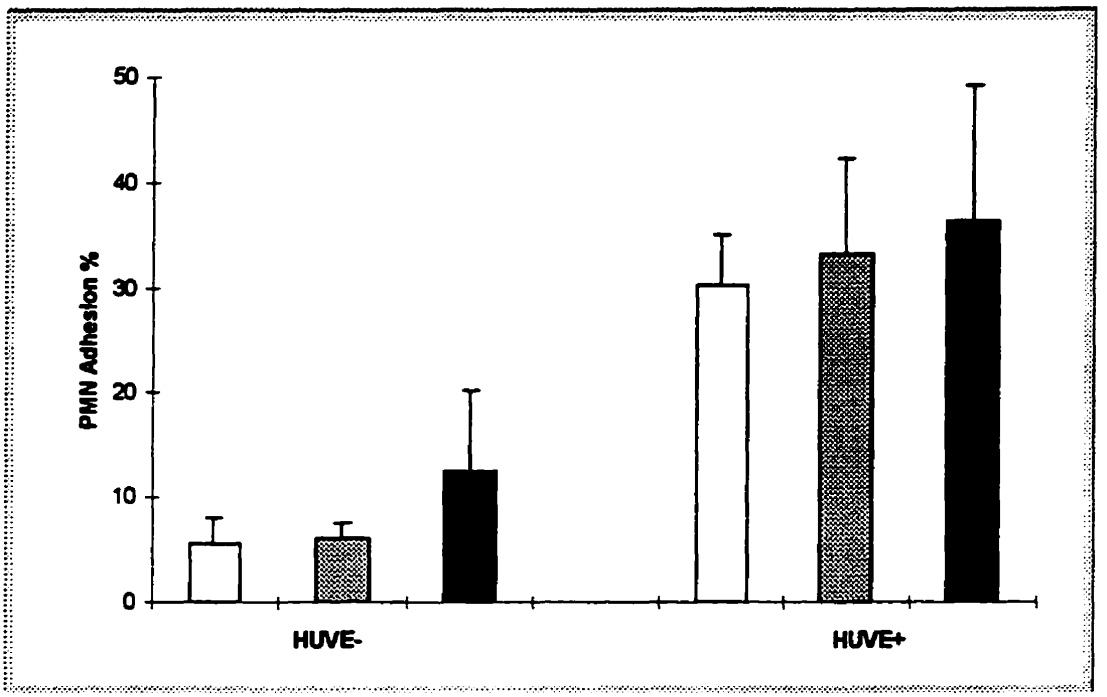


Chart 16 *The effect of plasma on stimulated PMN adherence to unstimulated and stimulated (TNF-α and IL-1β for 3 hours) HUVE cells.*

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	5.6% ± 2.4%	30.3% ± 4.8%	<0.001
Pre-Op	6.0% ± 1.6%	33.3% ± 9.0%	<0.001
SIRS	12.4% ± 7.7%	36.4% ± 12.8%	<0.001
ANOVA	p<0.01	NS	

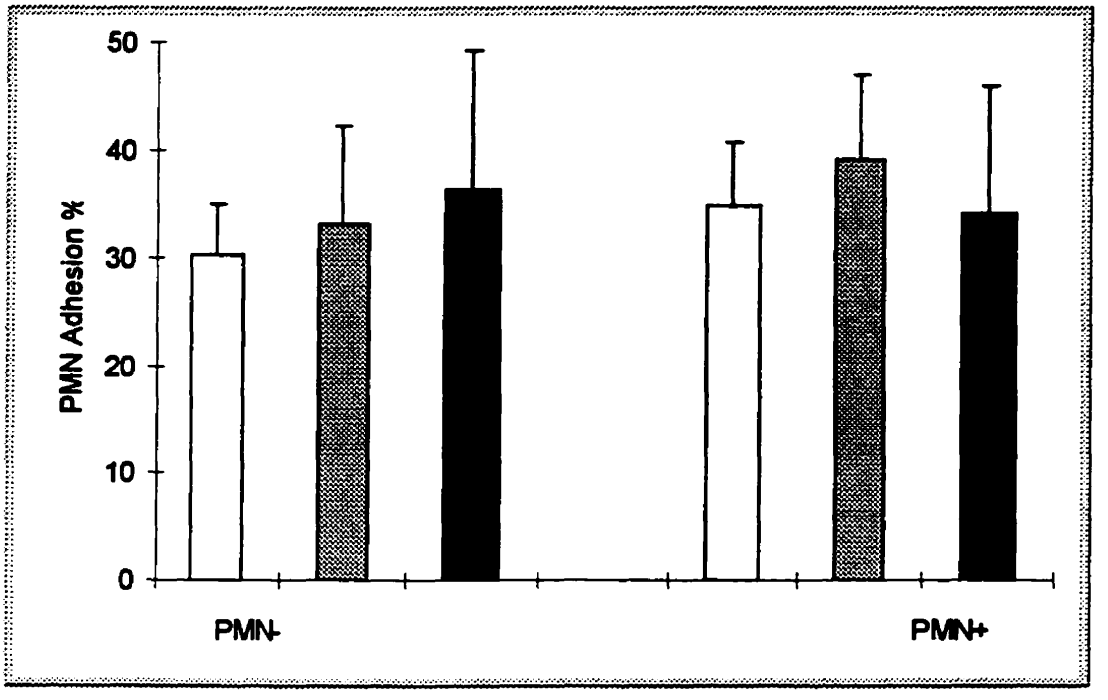


Chart 17 *The effect of plasma on the adhesion of unstimulated and stimulated PMN (priming with LPS 100 ng/ml for 40 min) to TNF-α and IL-1β stimulated HUVE cells.*

	PMN unprimed	**PMN primed**	*p* value PMN- vs. PMN+
Control	30.3% ± 4.8%	34.9% ± 5.9%	<0.05
Pre-Op	33.3% ± 9.0%	39.1% ± 7.9%	<0.05
SIRS	36.4% ± 12.8%	34.2% ± 11.7%	NS
ANOVA	NS	NS	

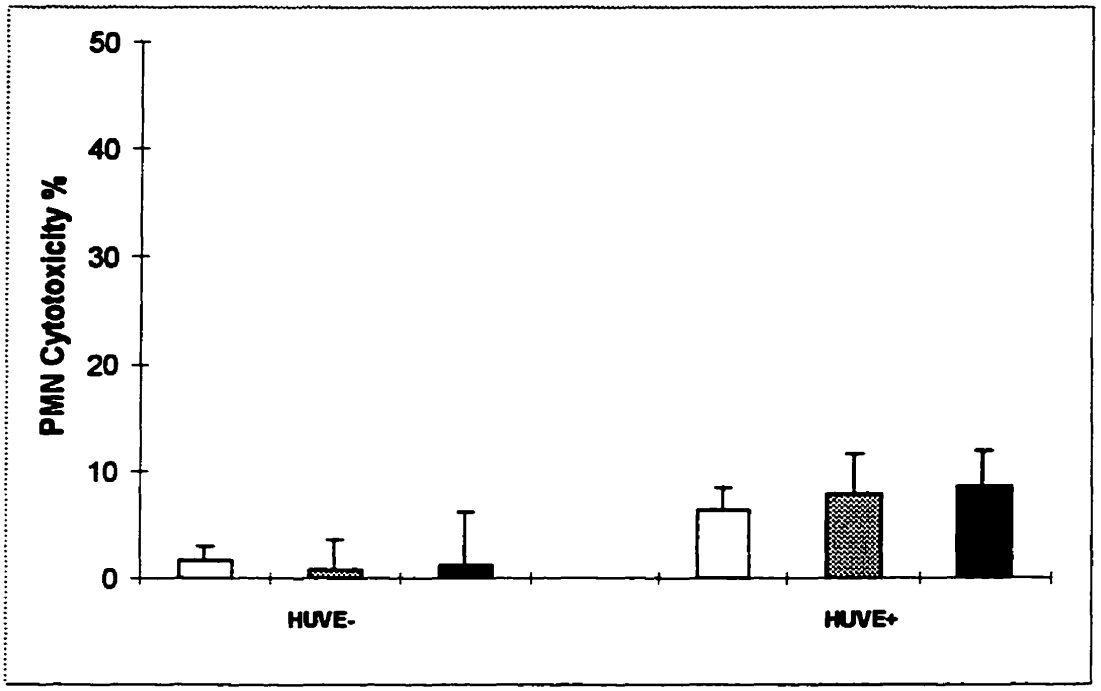


Chart 18 *The effect of plasma on the cytotoxicity of unstimulated PMNs towards unstimulated or stimulated (TNF-α and IL-1β for 3 hours) HUVE cells.*

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	1.6% ± 1.4%	6.3% ± 2.1%	<0.001
Pre-Op	0.7% ± 2.9%	7.9% ± 3.8%	<0.001
SIRS	1.2% ± 5.0%	8.6% ± 3.4%	<0.001
ANOVA	NS	NS	

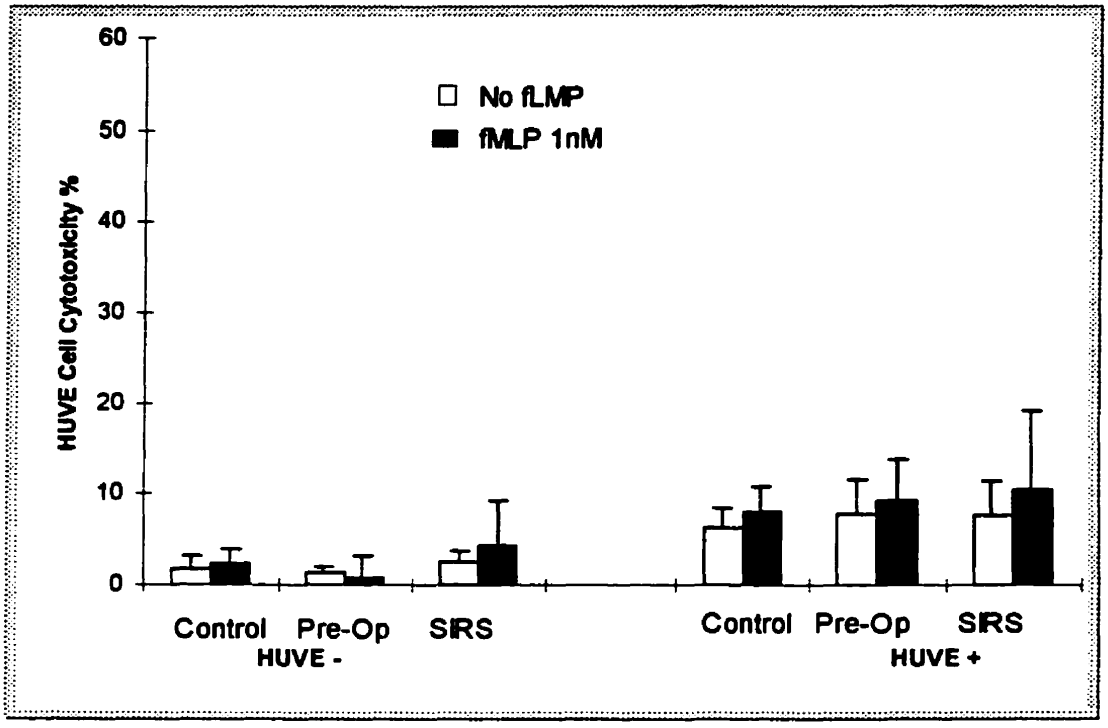


Chart 19 The effect of plasma on the cytotoxicity of unstimulated PMNs towards unstimulated or stimulated (TNF-α and IL-1β for 3 hours) HUVE cells, with the PMN stimulating factor in the form of fLMP 1nM.

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	2.3% ± 1.6%	8.0% ± 2.9%	<0.001
Pre-Op	0.8% ± 2.4%	9.2% ± 4.6%	<0.01
SIRS	4.4% ± 4.9%	10.4% ± 8.7%	<0.05
ANOVA	<0.05	NS	

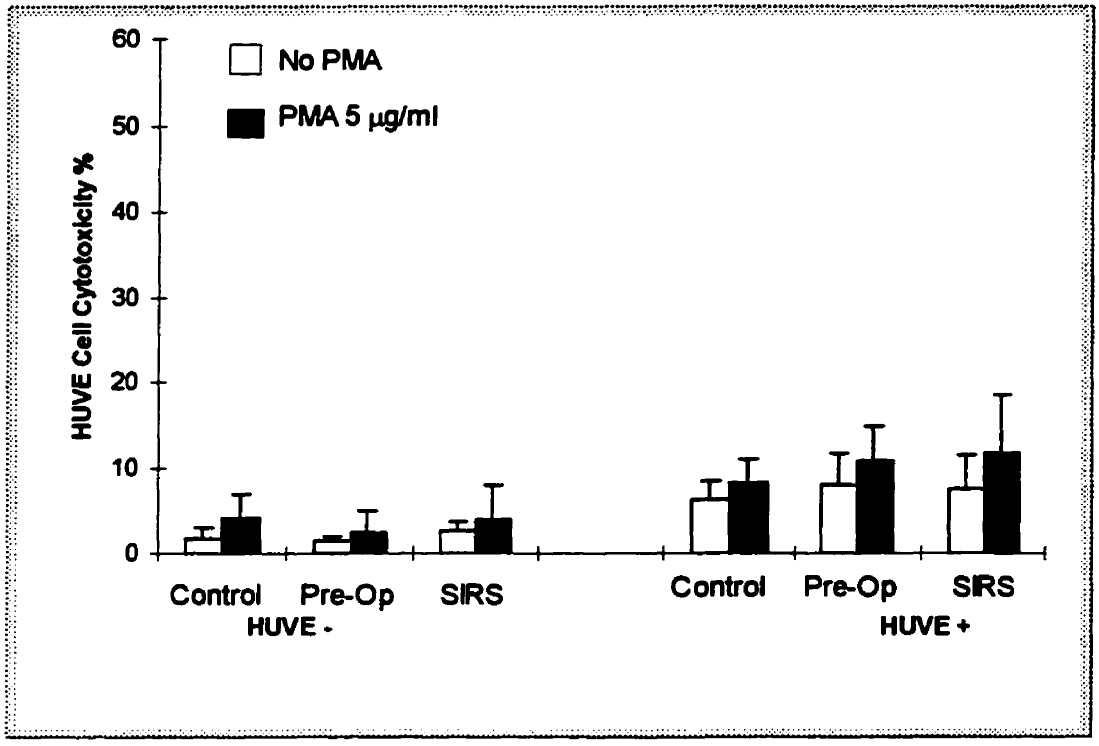


Chart 20 The effect of plasma on the cytotoxicity of unstimulated PMNs towards unstimulated or stimulated (TNF-α and IL-1β for 3 hours) HUVE cells, with the PMN stimulating factor in the form of PMA 5 um/ml.

	HUVE unstimulated	**HUVE stimulated**	*p* value HUVE- vs. HUVE+
Control	4.0% ± 2.8%	8.1% ± 2.9%	<0.01
Pre-Op	2.3% ± 2.6%	10.7% ± 4.2%	<0.01
SIRS	3.8% ± 4.2%	11.6% ± 6.9%	<0.01
ANOVA	NS	NS	

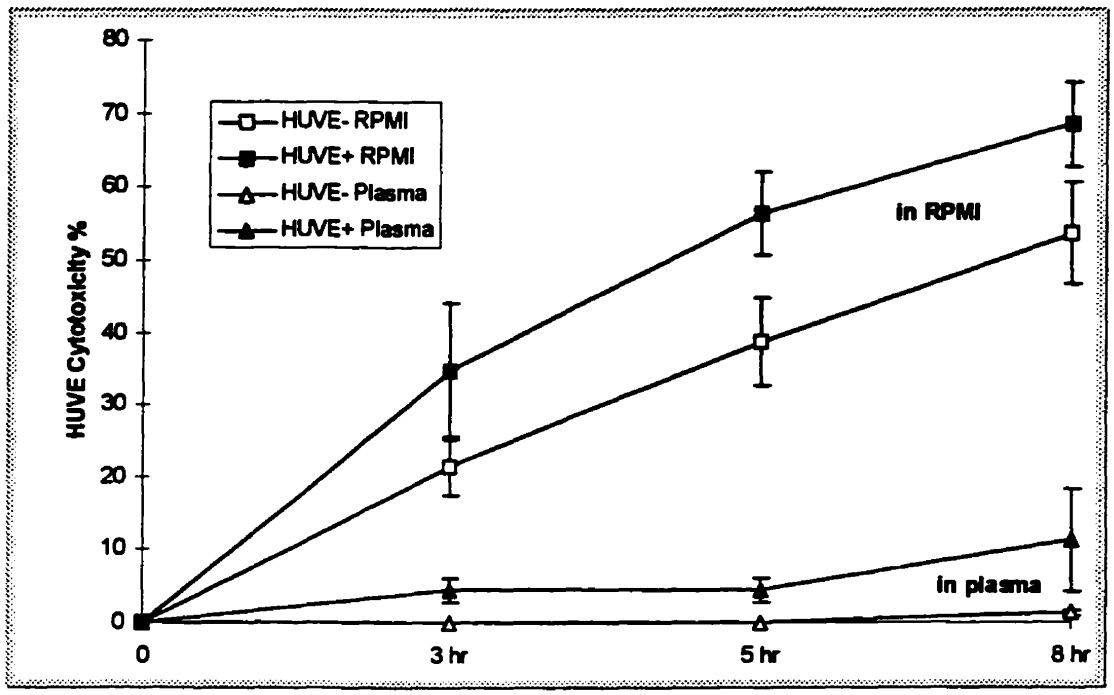


Chart 21: The cytotoxicity time course of control PMNs with unstimulated and stimulated (TNF-α and IL-1β for 3 hour) HUVE cells in RPMI vs. in plasma.

		3 hour	5 hour	8 hour
In RPMI	HUVE-	21.3% ± 3.9%	38.8% ± 6.1%	53.6% ± 6.9%
	HUVE+	34.8% ± 9.3%	56.3% ± 5.6%	68.4% ±5.8%
	p value	<0.01	<0.01	<0.01
In plasma	HUVE-	0%	0 %	1.3% ± 3.4%
	HUVE+	4.4% ± 1.7%	4.5% ± 1.6%	11.3% ± 7.1%
	p value	<0.01	<0.01	<0.01
p value (In RPMI vs. In plasma)		<0.001	<0.001	<0.001

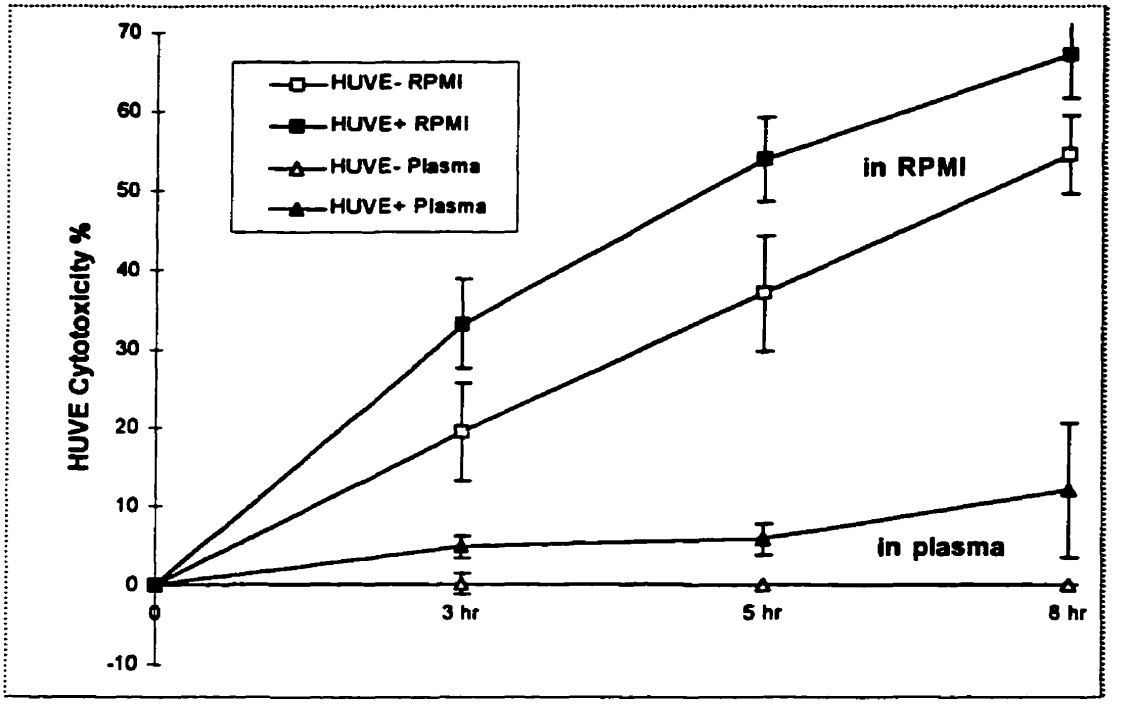


Chart 22: The cytotoxicity time course of control PMNs with unstimulated and stimulated (TNF-α and IL-1β for 3 hour) HUVE cells in RPMI vs.in plasma after PMN stimulation with fMLP 1 nM/ml.

		3 hour	5 hour	8 hour
In RPMI	HUVE-	19.5% ± 6.2%	37.0% ± 7.2%	54.5% ± 4.9%
	HUVE+	33.2% ± 5.5%	54.0% ± 5.3%	67.1% ±5.5%
	p value	<0.01	<0.01	<0.01
In plasma	HUVE-	0.2% ±1.3%	0%	0%
	HUVE+	4.9% ± 1.4%	5.8% ± 1.9%	12.1% ± 8.6%
	p value	<0.01	<0.01	<0.01
p value (In RPMI vs. In plasma)		<0.001	<0.001	<0.001

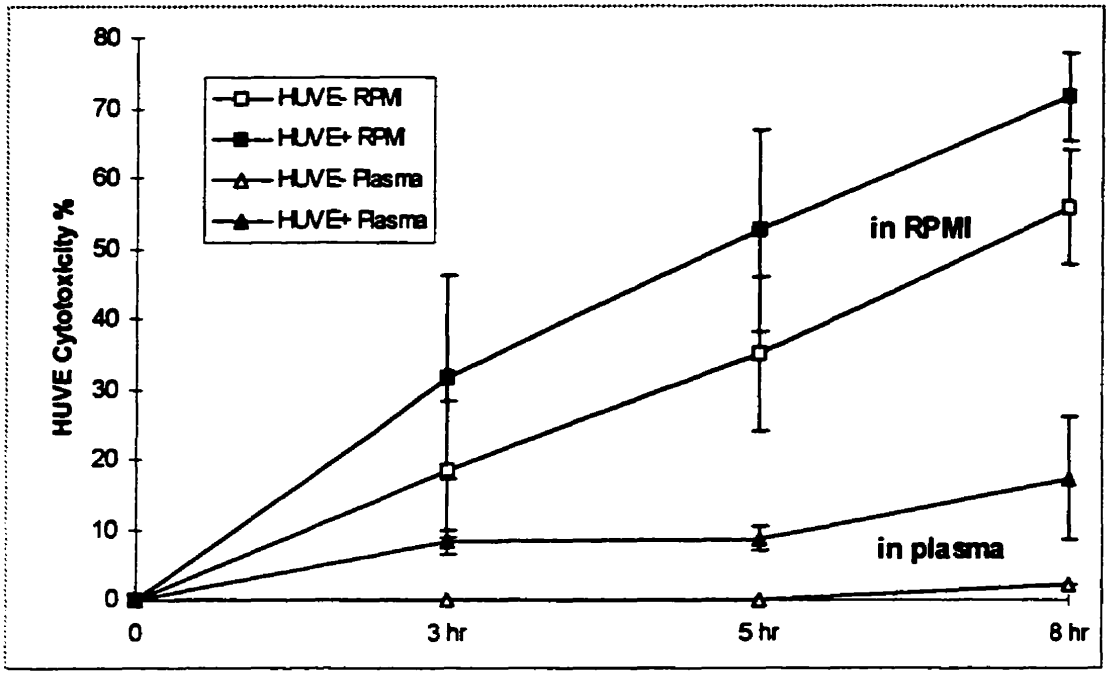


Chart 23: *The cytotoxicity time course of Pre-op subject PMNs with unstimulated and stimulated (TNF-α and IL-1β for 3 hour) HUVE cells in RPMI vs.in plasma.*

		3 hour	5 hour	8 hour
In RPMI	HUVE-	18.5% ± 9.7%	35.0% ± 11.0%	55.8% ± 8.2%
	HUVE+	31.7% ± 14.5%	52.5% ± 14.3%	71.6% ± 6.3%
	p value	<0.01	<0.01	<0.01
In plasma	HUVE-	0%	0%	2.1% ± 3.1%
	HUVE+	8.3% ± 1.7%	8.7% ± 1.7%	17.3% ± 8.8%
	p value	<0.01	<0.01	<0.01
p value (In RPMI vs. In plasma)		<0.001	<0.001	<0.001

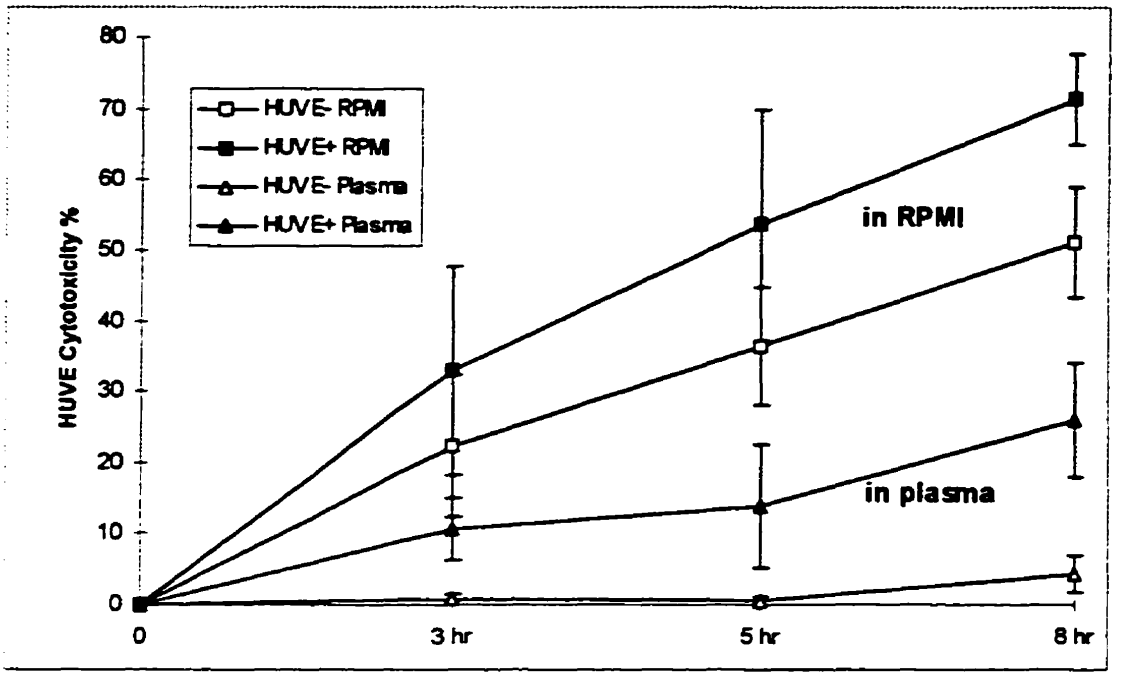


Chart 24: *The cytotoxicity time course of Pre-op subject PMNs with unstimulated and stimulated (TNF-α and IL-1β for 3 hour) HUVE cells in RPMI vs.in plasma after PMN stimulation with fMLP 1 nM/ml.*

		3 hour	5 hour	8 hour
In RPMI	HUVE-	22.3% ± 10.0%	36.4% ± 8.3%	51.1% ± 7.9%
	HUVE+	32.9% ± 14.8%	53.6% ± 16.2%	71.3% ± 6.3%
	p value	<0.05	<0.05	<0.01
In plasma	HUVE-	0.9% ±1.4%	0.6% ± 1.6%	4.4% ± 2.6%
	HUVE+	10.7% ± 4.3%	13.9% ± 8.6%	26.0% ± 8.2%
	p value	<0.01	<0.05	<0.01
p value (In RPMI vs. In plasma)		<0.001	<0.001	<0.001

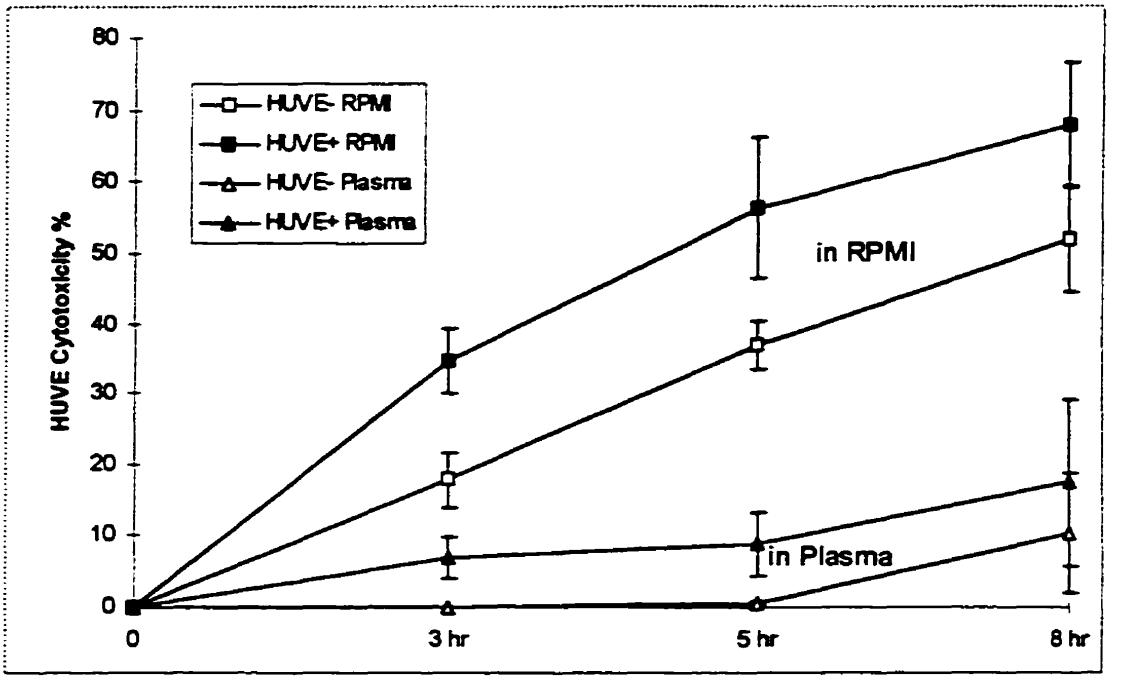


Chart 25: *The cytotoxicity time course of PMNs from patients with SIRS with unstimulated and stimulated (TNF-α and IL-1β for 3 hour) HUVE cells in RPMI vs.in plasma.*

		3 hour	5 hour	8 hour
In RPMI	HUVE-	17.8% ± 3.9%	36.9% ± 3.5%	52.0% ± 7.5%
	HUVE+	34.7% ± 4.6%	56.3% ± 9.7%	67.8% ± 8.6%
	p value	<0.01	<0.01	<0.05
In plasma	HUVE-	0%	0.7% ± 2.6%	10.4% ± 8.5%
	HUVE+	6.8% ± 2.9%	8.9% ± 4.5%	17.5% ± 11.8%
	p value	<0.01	<0.01	<0.05
p value (In RPMI vs. In plasma)		<0.001	<0.001	<0.001

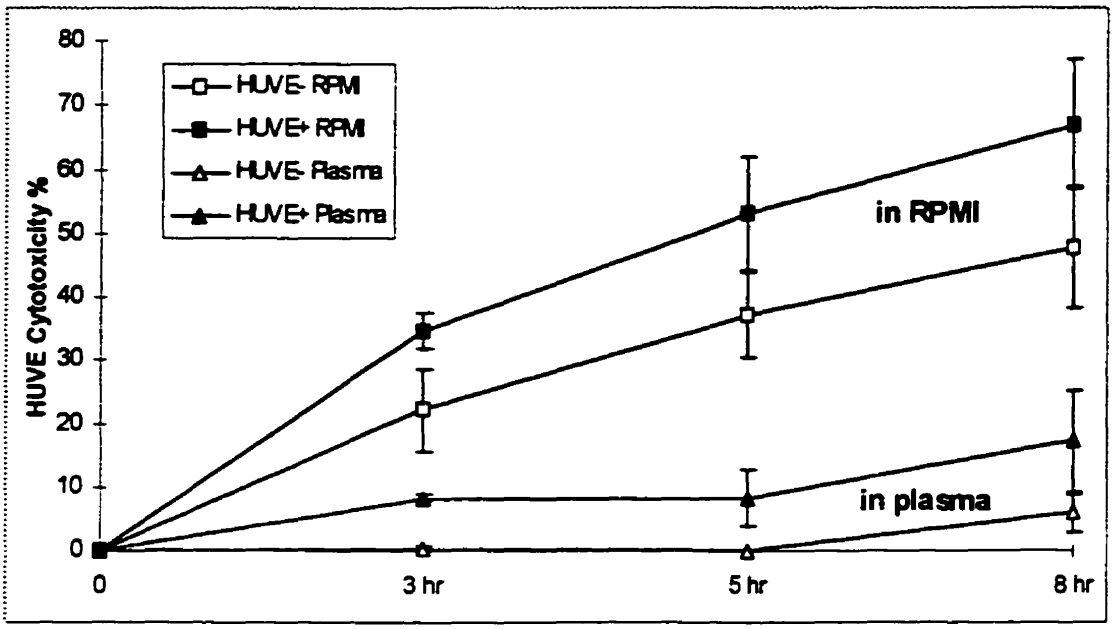


Chart 26: *The cytotoxicity time course of PMNs from patients with SIRS with unstimulated and stimulated (TNF-α and IL-1β for 3 hour) HUVE cells in RPMI vs.in plasma after PMN stimulation with fMLP 1 nM/ml.*

		3 hour	5 hour	8 hour
In RPMI	HUVE-	22.2% ± 3.9%	37.1% ± 6.8%	47.8% ± 9.4%
	HUVE+	34.6% ± 2.9%	53.2% ± 8.9%	67.0% ± 10.1%
	p value	<0.05	<0.05	<0.05
In plasma	HUVE-	0.3% ±1.3%	0%	6.0% ± 6.9%
	HUVE+	8.3% ± 0.6%	8.3% ± 4.5%	17.3% ± 8.0%
	p value	<0.01	<0.01	<0.01
p value (In RPMI vs. In plasma)		<0.001	<0.001	<0.001

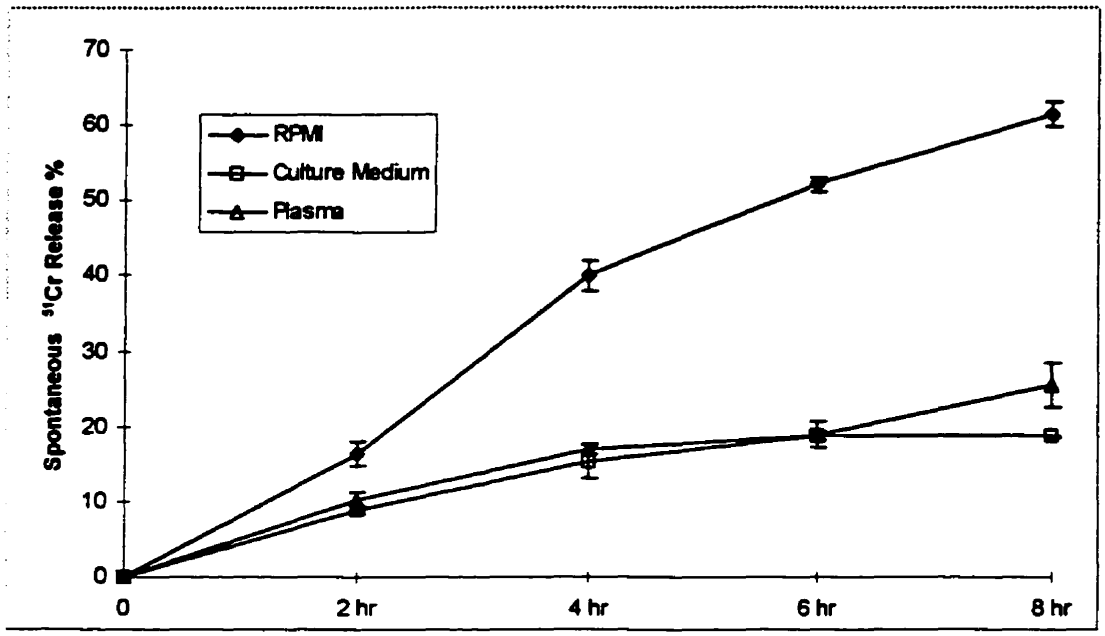


Chart 27: *The rate of spontanneous ^{51}Cr release from labeled unstimulated HUVE cells overlaid with RPMI, culture medium or plasma obtained from healthy subjects.*

	2 hour	4 hour	6 hour	8 hour
RPMI	16.5% ± 1.6%	40.0% ± 2.0%	52.1% ± 0.9%	61.4% ± 1.6%
Culture Medium	8.8% ± 0.5%	15.4% ± 2.3%	18.9% ± 1.8%	18.9% ± 0.1%
Plasma	10.1% ± 1.2%	16.9% ± 0.6%	18.9% ± 0.6%	25.5% ± 3.0%
Culture Medium vs. Plasma - NS RPMI vs. Culture Medium or Plasma - $p < 0.05$.				

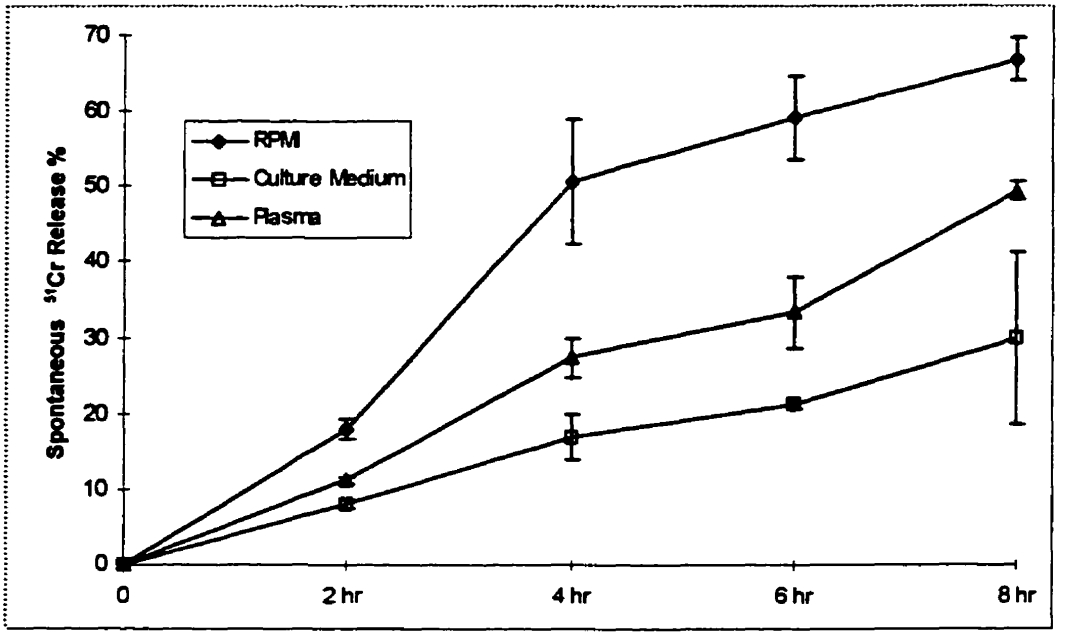


Chart 28: *The rate of spontanneous ^{51}Cr release from labeled activated HUVE cells overlaid with RPMI, culture medium or plasma obtained from healthy subjects.*

	2 hour	4 hour	6 hour	8 hour
RPMI	18.0% ± 1.4%	50.6% ± 8.3%	59.1% ± 5.5%	66.9% ± 2.8%
Culture Medium	8.0% ± 0.5%	17.0% ± 3.0%	21.2% ± 0.4%	29.9% ± 11.3%
Plasma	11.2% ± 0.5%	27.4% ± 2.5%	33.3% ± 4.7%	49.4% ± 1.1%
Culture Medium vs. Plasma - NS; RPMI vs. Culture Medium or Plasma - $p < 0.05$.				

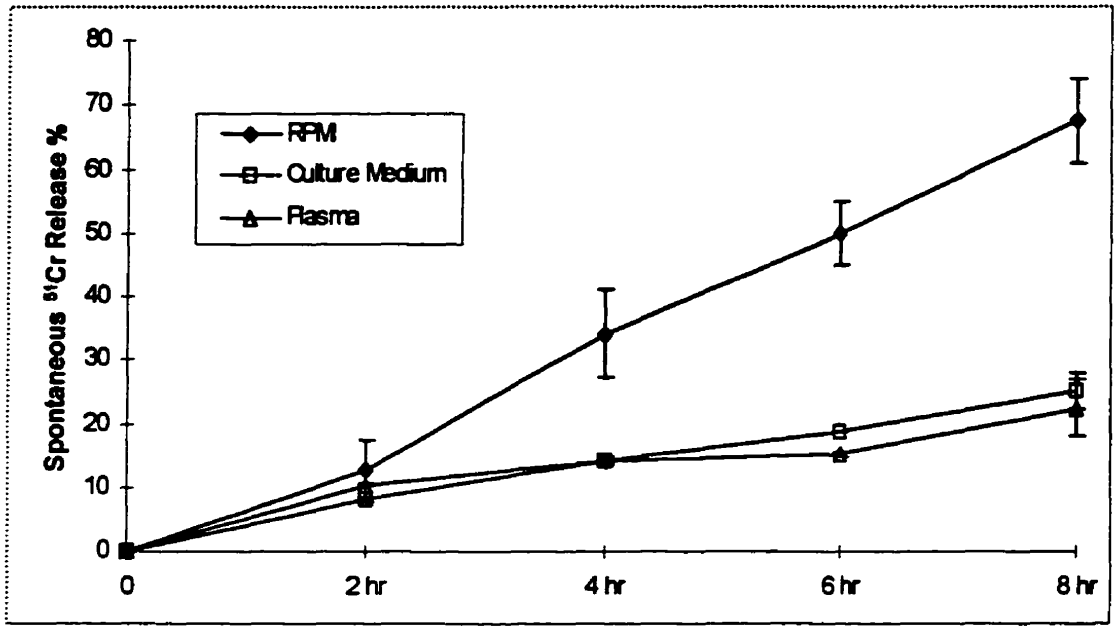


Chart 29: *The rate of spontanneous ^{51}Cr release from labeled unstimulated HUVE cells overlaid with RPMI, culture medium or plasma obtained from patients with SIRS.*

	2 hour	4 hour	6 hour	8 hour
RPMI	12.6% ± 4.8%	34.0% ± 6.9%	49.9% ± 4.8%	67.5% ± 6.5%
Culture Medium	8.2% ± 0.4%	14.1% ± 0.4%	18.7% ± 0%	25.5% ± 2.9%
Plasma	10.3% ± 0.2%	14.3% ± 0.7%	15.3% ± 0.4%	22.4% ± 4.5%
Culture Medium vs. Plasma - NS; RPMI vs. Culture Medium or Plasma - $p < 0.05$.				

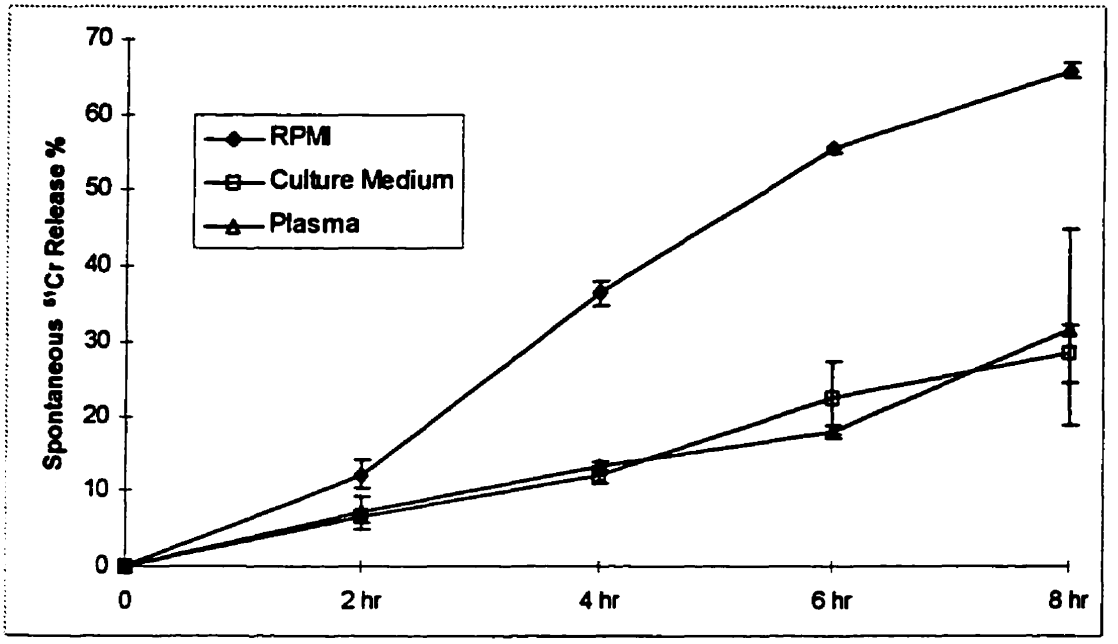
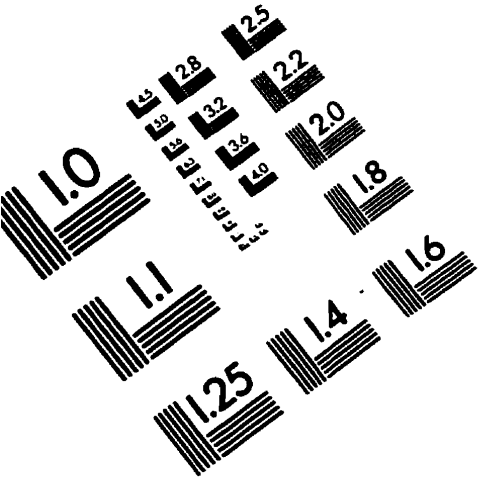
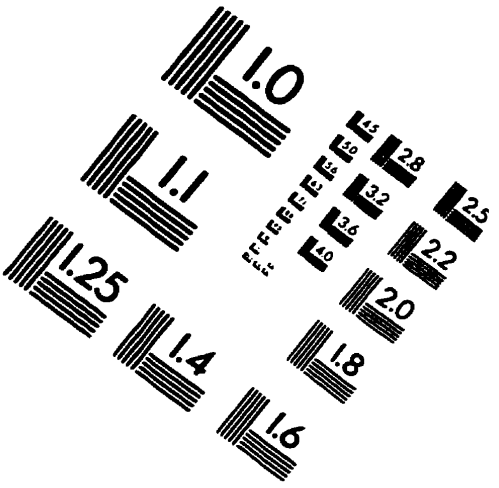


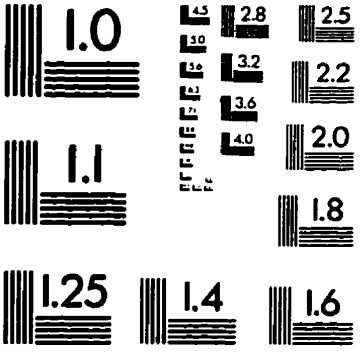
Chart 30: *The rate of spontanneous ^{51}Cr release from labeled stimulated HUVE cells overlaid with RPMI, cculture medium or plasma obtained from patients with SIRS.*

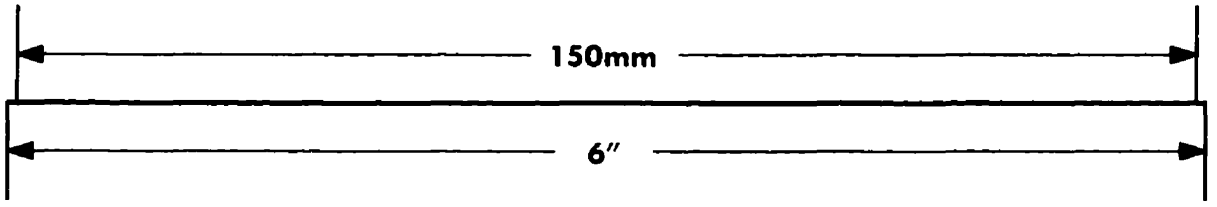
	2 hour	4 hour	6 hour	8 hour
RPMI	12.2% ± 1.9%	36.5% ± 1.6%	55.5% ± 0.4%	65.8% ± 0.9%
Culture Medium	6.7% ± 0.9%	12.1% ± 1.1%	22.5% ± 5.0%	28.4% ± 4.0%
Plasma	7.1% ± 2.1%	13.2% ± 0.6%	17.8% ± 0.9%	31.8% ± 13.1%
Culture Medium vs. Plasma - NS; RPMI vs. Culture Medium or Plasma - $p < 0.05$.				

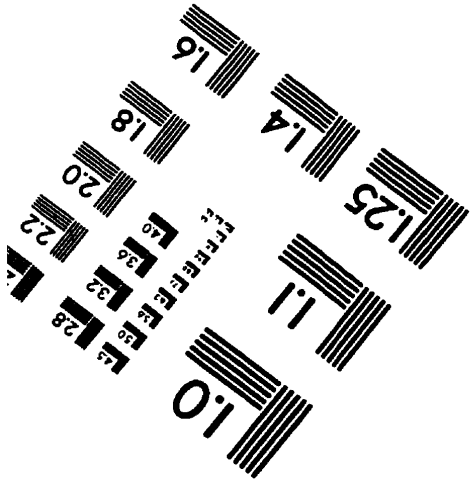
IMAGE EVALUATION
TEST TARGET (QA-3)

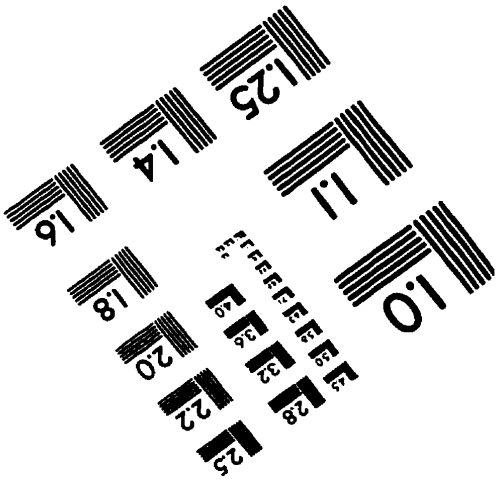












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