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thesis entitled

HEMORRHAGE, THE STRESS RESPONSE, AND THE HEAT SHOCK RESPONSE.

presented by

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has been accepted towards fulfillment of the requirements for

M.S. degree in **SURGERY**

Major professor

Date 1/11/96

Hemorrhage, the stress response, and the heat shock response

by

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Surgery

1995

ABSTRACT

HEMORRHAGE, THE STRESS RESPONSE, AND THE HEAT SHOCK RESPONSE

By

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The 70kD family of heat shock proteins (HSP70 / HSP72) are purportedly cytoprotective during stress/injury. Although it is known that Kupffer cell (KC) cytokine release and cytotoxicity is increased following hemorrhage but depressed in peritoneal macrophages (pMØ), it is unknown what role HSP's play in these changes.

Mice were bled to and maintained at a mean arterial pressure of 35mmHg for one hour and then adequately resuscitated, followed by KC and pMØ isolation at 2 and 24 hours. Western blot analysis was performed and HSP72 expression quantified densitometrically. In addition, TNF–α and IL–6 productive capacities of the two macrophage populations were measured. Results were compared to those from sham–operated mice.

Hemorrhaged mice exhibited significantly increased expression of HSP 72 and increased productive capacities of both IL–6 and TNF–α at both 2 and 24 hours when compared to shams. The ability to mount a heat shock response may preserve the capacity of KC to release inflammatory cytokines and maintain cytotoxicity.

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III. INTRODUCTION

GENERAL

An organism's response to a potentially injurious external stimulus is usually one of self preservation. This is a core principle that is not only germane to a single being but one that can be extended vertically to the group or social structure in which that being participates or intrinsically to the organ systems that makes up the organism. For example, much in the same way that human societies contain within them and make use of military forces to defend the society from both internal and external threats, homeostatic mechanisms protect an organism from that which may harm the organism. This analogy, albeit simplistic, does have relevance to the way one approaches the study of basic responses to injury. Defensive mechanisms are inherent and pervasive at both the macroscopic and microscopic level and function as part of a greater homeostatic mechanism that involves the interaction of individual organ systems. If the result of marshaling defense forces allows an organism (or society) to maintain or allow a return to normal homeostasis (or normal social order), then that organism (or society) is able to survive.

However, if the insult is so great that normal homeostatic mechanisms are not allowed to return or are sacrificed in order to maintain a defensive posture, it is often the case that the organism will succumb as a result of the response to the injury. In my experience as a surgeon I have seen patients die not only as a result of the initial injury or physiological derangement but secondarily as a result of progressive multi–organ dysfunction syndrome

(MODS) in response to sepsis, trauma, hemorrhage, or a systemic inflammatory response syndrome (SIRS).

The goal of this thesis is to provide an overview of mammalian responses at a cellular and systemic level to an insult such as hemorrhage. Specifically, I will discuss the interaction of the inflammatory response, in the form of cytokines, with a phylogenetically ancient stress response, the heat shock response. In so doing, I will introduce the results of experiments that measure both cytokine release and expression of heat shock proteins in two distinct populations of macrophages in a fixed–pressure murine model of severe hemorrhage followed by resuscitation. I will then discuss the implications of the findings in furthering the understanding of cellular homeostasis and the preservation of the organism.

HEAT SHOCK PROTEINS

Historical aspects:

In 1962, F.M. Ritossa described a phenomenon of chromosomal "puffing" in the polytene chromosomes of salivary glands of the fruit fly *Drosophila melanogaster* which was induced by exposing isolated salivary glands to temperatures warmer than what was believed most optimal for the fruit fly's normal growth and development.[1] These "puffs" appeared at specific sections of the chromosomes as identified by Giemsa staining. This response was consistently seen immediately after exposing the salivary glands to the higher temperatures and the chromosomal changes persisted and increased in size in a reproducible manner [1, 2]. The investigators termed this phenomenon the "heat shock response." Tissieres and Mitchell in 1974 identified the elaboration of specific, unique protein sequences associated with these chromosomal puffs and coined the term, "heat shock proteins" [3]. Eventually it was found that the heat shock response is an elemental function in all organisms and that in many cases the response is triggered by exposure to temperatures only slightly higher than the usual ambient temperature in which that organism exists, such as that seen in Ritossa's fruit flies or in the mammalian response to fever [4]. As work progressed in the years following Tissieres' work, it became evident that this general response of all cells was not limited only to thermal stimuli. Evidence accumulated that a number of different stimuli induced a heat shock response through the common pathway of disruption of the protein tertiary sequence. These various stimuli include arsenite poisoning, ethanol, heavy metal poisoning, oxygen toxicity, and oxygen

free radicals [2, 5, 6, 7]. Ischemia – reperfusion injury, anoxia, and hemorrhage have also been found to be strong inducers of the heat shock response.

Eventually an entire group of related proteins have been identified that are highly conserved among species and even kingdoms; are constitutively produced as well as inducible; and are important to many cellular homeostatic functions including protein folding and unfolding, protein assembly, transport across intracellular membranes, and rescue/transport of improperly folded, partially denatured, and abnormal proteins.[4, 8] In their constitutive form they are considered "chaperone proteins" or "housekeeping proteins" because they accompany proteins through major phases of production, assembly, and transport and as such they have a major role in baseline cellular homeostasis. It is now clear that the stress proteins fall within certain "families" based on their size. The best –known are the 28 kD, 60 kD, 70 kD, 90 kD, and 110 kD families of heat shock proteins (hsp28, hsp60, hsp70, hsp90, and hsp110, respectively).

Inducers of the heat shock response, thermotolerance, and protection against stress:

Recent evidence indicates a possible role of inducible stress proteins, especially members of the hsp70 family, in protecting the cell against external insults. Blockimg the activity of hsp70 of cells in vitro, via antibody binding or competitive inhibition, prior to heat shock renders previously normal cells thermosensitive.[9, 10] Thermotolerance is the appropriate term for the protective effect the heat shock response confers in reaction to a non–lethal thermal insult. Interestingly, a number of studies have shown that thermotolerance is also conferred when the heat shock response (especially increases in hsp72) is initiated chemically such as in sodium arsenite poisoning. Naturally, the focus

then switches to whether induction of the heat shock response will also protect cells and organisms from other insults. Investigative work in such matters have found promising results and extends the role of the heat shock response in maintenance of cellular homeostasis beyond the bounds of simple thermotolerance.

Yellon and colleagues found specific improvements in the physiological function of myocardium as a result of the heat shock response.[11, 12] In their series of experiments, rabbit ex–vivo hearts, previously exposed to heat–shock in–vivo, had improved cardiac function, decreased creatine phosphokinase release, and significantly conserved ATP levels and mitochondrial function after ischemia–reperfusion than did nonheat–shocked controls. It was noted that hsp72, the primary inducible form of the hsp70 family, was the principle product of the induced heat shock response. However, it is unlikely that heat shock proteins are involved in classical ischemic myocardial preconditioning since heat stress failed to limit infarct size in the rabbit myocardial reperfusion injury model. [12, 13]

Villar, Ribeiro, and Slutsky found an analogous result when thermal pre–treatment, associated with a rise in a 72 kD member of the hsp70 family, attenuated tissue damage and mortality in a rat model of experimental lung injury.[14] The same group, in more recent experiments, found that both thermal pre–treatment and injection of sodium arsenite induces a heat shock response with measurable and significant increases in hsp72. Interestingly, thermal pre–treatment resulted in decreased mortality and reduced end–organ damage of liver and lung in sepsis–induced lung injury.[15] Sodium arsenite induced a significant increase in hsp72 in lungs of treated rats and correlated with decreased mortality during the time periods hsp72 was expressed above basal values.[16]

It is interesting to note also that the mechanism of protection may be related to protection from cytotoxic forces within the host. This has important implications in disease processes such as sepsis and trauma where the elaboration of inflammatory cytokines, especially interleukin–1 (IL–1), interleukin–6 (IL–6), and tumor necrosis factor alpha (TNF–α), secondary to a systemic inflammatory response syndrome (SIRS) may be cytotoxic to the host's organ systems and progress to multiorgan dysfunction syndrome (MODS) or frank failure. It has been found, for example, that heat–treated mouse C3HA cells were resistant to the cytotoxic effects of TNF–α despite retaining the ability to bind TNF as well as nonheat–treated cells.[17] Transfecting TNF–sensitive murine fibrosarcoma cells with human hsp–70 and hsp–27 genes protected these cells from TNF cytotoxicity, TNF–mediated monocyte cytotoxicity, and oxidative stress.[18]

Therefore, there seems to be strong in vivo evidence of a relative protective effect induced by the heat shock response. Teleologically, this affirms that inherent mechanisms exist to preserve cell function and possibly protect from further insult. Furthermore, because heat shock protein structure identity homology is preserved among eucaryotes (often exceeding 50%), it is clear that this ancient, evolutionarily conserved response was meant to confer a survival advantage.[2, 19] The common inducer of the heat shock response appears to be the presence of abnormal or partially denatured proteins in response to a number of stimuli, as previously stated. It would be overly simplistic to ascribe the responsibility of maintaining what Claude Bernard referred to as the "milieu interieur" (internal environment) and William Cannon later termed "homeostasis" [20] to the heat shock response. Conversely, it is proven that the heat shock response confers more than just thermotolerance. It is most likely that a multimodal stress response works in concert with other

adaptive mechanisms to result in the survival of the organism as a whole. The role that the heat shock response plays in this stress response is discussed in the next two sections.

The HSP70 family of proteins

The human hsp70 family is comprised of at least four distinct members [4]. They are, according to the nomenclature of Watowich and Morimoto [21] hsp70, hsp72, p72, and grp78. The major heat inducible 70kD hsp70 is also basally expressed. It combines with hsp90 to form the glucocorticoid receptor [22], is thought to stabilize the cellular oncogene p53 (along with p72), and localizes to the nucleoli in response to stress where its mechanism of action may be to stabilize RNA splicing and structure, especially in response to hyperthermia.[2, 23] Hsp72 is a protein that is stress inducible only and is not basally produced. p72 is slightly heat inducible but to a far lesser degree than hsp70; it is elemental in clathrin uncoating, nuclear matrix / nucleoli binding, and also interacts with p53.[4] In humans, it can be summarized that hsp 70 is the primary inducible form of the gene while p72 is the primary constitutive form but can also be dramatically increased with the appropriate stimulus. grp78 (a.k.a. BiP) is primarily located within the endoplasmic reticulum and functions primarily to fold and unfold proteins as they make their transmembrane crossing to and from the cytosolic sector by binding to them transiently as they assume their correct tertiary structure. It also appears to bind permanently to proteins that are misfolded or unfolded thereby acting as an "editor" of misfit proteins.[24] It should be noted at this time that rodents have three known hsp70 variants very similar in size and function to the human complement, but do not have an equivalent of p72 [4].

The genes for the hsp family of proteins are multiply duplicated and located on human chromosome 6, in association with the major histocompatibility complex, 14, and 21 [4, 25]. Specific and highly restricted heat shock transcription factors (HSFs) mediate the inducible expression of heat shock proteins via binding to the heat shock response element. Since they are restricted, they respond to specific stimuli. For example, HSF1 is the principle mediator of stress induced hsp expression from heat and restraint to ischemia–reperfusion injury [22, 26]. However, the activation of transcription factors and the expression of their resulting gene products is a complex interplay of cellular systems sometimes acting in concert and sometimes in exclusion of others, as will be discussed in the next section.

The stress response:

Some investigators such as William Welch have advocated replacing the terms "heat shock response" and "heat shock proteins" with stress response and proteins, respectively.[19] This is too narrowly defined a term. Historically speaking, the stress response is more correctly defined as a whole body phenomenon involving adaptive responses including those of the autonomic nervous system, the acute phase response, the hypothalamic–pituitary–adrenal axis (HPA) and endocrine responses, and the heat shock response.[22] In fact, within the cell there are at least four genetically discrete responses to stress: the heat shock response, the acute phase response, the oxidative stress response, and the ultra violet stress response.[9, 27, 28, 29, 30] It appears that although some of these responses are triggered by similar stimuli, as in the case of the oxidative stress response and the heat shock response, the acute phase and heat shock responses are discrete and exclusive of each other with

the heat shock response taking precedence as the more primitive and preemptive of the two.

Local tissue injury, such as that seen in surgery and trauma, initiates an inflammatory response with the release of circulating cytokines such as IL–1, IL–6, and TNF–α. These cytokines mediate the systemic effects of the acute phase response such as fever, hypothalamic–pituitary–adrenal axis (HPA) activation, leukocytosis, immune / reticuloendothelial system activation, and production of acute phase proteins.[31, 32] Cytokines, including IL–1, IL–2, TNF, and interferon–γ (INF–γ), have been shown to either induce or decrease the threshold for induction of heat shock proteins.[33, 34] This phenomenon may be a response to the cytotoxic effects of various cytokines, especially TNF, and may also prove to be a protective regulatory effect for host tissues to remain viable in the face of a potentially injurious inflammatory response.[35, 36] One has to be cautious when interpreting the data from experimental conditions where the heat shock response induction is brief, episodic, and artificial. In the clinical setting (a.k.a. "the real world"), the potential stimulus for a heat shock response may be protracted, ongoing, and difficult to reverse. This is true in cases of prolonged hypotension secondary to hypovolemia / hemorrhage, cardiac pump failure, massive tissue injury and ischemia (as in crush injuries and burns), and septic shock. However, there is evidence that critically ill human patients express the inducible hsp72 in granulocytes; ostensibly as a result of circulating factors during a prolonged inflammatory state.

Experimental evidence exists in vivo that severe surgical stress as well as simple restraint stress in rats resulted in specific increases in the expression of inducible forms of hsp70 in the adrenal gland and aorta.[37, 38] In a series of inspired and elegant experiments using chronic hormone therapy (glucocorticoids and antiglucocorticoids), hypophysectomy, and catecholamine

administration in the same model, Udelsman and his associates were able to show that the inducible hsp70 expression in the adrenal cortex and aorta were due to an interaction of the hypothalamic pituitary adrenal axis (i.e., glucocorticoid production) and catecholamines, and that the control of these responses in these two different tissues were the result of a specific transcription factor, heat shock factor 1 (HSF1), which has also been shown to be the mediator of hsp induction in response to heat and other classic stresses.[22, 39] Other organs did not show an appreciable increase in the expression of hsp70 in their models of surgical or restraint stress but their work clearly shows that there is differential organ–specific regulation of hsp expression based, in part, on the effect of the HPA axis in association with catecholamine regulation.

Buchman and colleagues[31 40, 41] showed in *in vivo* models of cardiogenic shock and hemorrhage resuscitation that the expression of hsp72 in the liver as well as two acute phase genes (α1–anti chemotrypsin and inter–α trypsin inhibitor). Additionally, these same investigators were able to show involvement of oxidative stress in the form of superoxide anion generation as an inducer of hsp70.[42] It is unclear in these groups of experiments which cell type in the liver (hepatocyte, Kupffer cell, endothelial cell, etc.) is primarily responsible for the generation of hsp72. Nor is it clear whether the phenomenon is due to ischemia alone or reperfusion through the splanchnic bed. However, what is remarkable is that while hsp72 transcription is initiated by the low flow state, it continues long after blood flow is restored.

In summary, the stress response involves at least four distinct genetic responses that are regulated, in part, by the host's stressed internal environment. Mediators of these responses include the hypothalamic–pituitary–adrenal axis, catecholamines, and inflammatory cytokines. The

control of these responses is most likely located at a transcriptional level and, as in the case of the acute phase and heat shock response, may be exclusive of each other.

PHYSIOLOGICAL ASPECTS OF HEMORRHAGE

Hemorrhage and the stress response:

Sepsis, and the antecedent phenomena of multiorgan dysfunction syndrome (MODS) and failure, is of continued interest and frustration in surgical intensive care units world–wide. It remains the leading cause of mortality and morbidity in surgical intensive care units.[43, 44, 45, 46] These derangements are significantly represented in the surgical, trauma, and cardiac critical care patient populations. Of interest in these patient populations is their susceptibility to overwhelming infections and sepsis which may be due to a breakdown in host immune response and intrinsic defenses. Although modern surgical critical care and trauma care has significantly improved our ability to initially treat these patients, it is increasingly evident that a significant number die of these sequelae. Therefore, a significant amount of investigative effort has been spent in dissecting out the various causes and consequences of the breakdown of host defenses resulting from injurious insults.

Modern medicine is limited by its inability to act effectively in a proactive manner. Although it is possible to support a critically ill patient (volume and inotropic support in the face of shock, ventilators and positive pressure ventilation in the face of acute respiratory distress syndrome (ARDS), antibiotics in the face of infection, total parenteral nutrition, and surgical debridement), overall mortality in the surgical intensive care unit has changed only incrementally. Part of this may be selection: as more patients survive their first insult they gain the opportunity to succumb to the more insidious process of the inability to overcome anti–homeostatic forces set off by the initial insult.[43, 44, 46]

In hemorrhagic shock, the initial result of hemodynamically significant hypovolemia results in the host's response to facilitate maximal support of vital structures (i.e., the central nervous system) at the expense of others (e.g., skin and integument, visceral organs, kidneys). Restoration of volume often returns a semblance of homeostasis to the previously deranged system but this is not universal. In trauma, for example, there are recognized patient subsets stratified on the basis of age (pediatric; elderly), injury severity scores, and with concomitant disease processes (immunodeficient; severe pre–existing circulatory or cardiac problems) that tend to do poorly.[47] Patients within these groups are more likely to succumb to not only the initial insult, but the sequelae of that insult as well: from simple opportunistic and nosocomial infections to multiorgan failure.

Cytokines and immune deficiency:

It is known that burn and soft tissue injury results in a profound suppression of cell–mediated as well as humoral immunity.[44, 47, 48] Hemorrhage, with and without associated tissue injury, has also been proven (in the same non–lethal murine hemorrhage model described here) to significantly depress macrophage immune function, increases susceptibility to sepsis, decreases splenocyte proliferation and lymphokine release[49, 50], and yet is associated with a concurrent increase in Kupffer cell TNF and IL–6 release as well as increased circulating levels of TNF at 2 hours post–hemorrhage[50, 51, 52, 53].

It appears that inflammatory cytokines, especially IL–6 and TNF–α, may have a direct effect in causing depressed Kupffer cell antigen presenting ability as a result of increased levels of these cytokines in both the local environment and in blood.[50, 53] Prostaglandins (especially PGE_2) may play a significant role

since blockade by chloroquine (at phospholipase A_2) and ibuprofen (at cyclooxygenase) at least partially restores Kupffer cell antigen presentation and prevents depression of macrophage functions[51, 55, 56]. Moreover, it has been shown that ibuprofen also decreases the incidence of sepsis in the hemorrhagic rodent model.[57] However, the applicability to humans is limited due the fact that pretreatment of humans is sometimes not even possible and overall this modality has not made a significant decrease in the mortality of the sequlae of hemorrhage.

There is an interesting anomaly in the results of these previous studies. Although macrophages from liver, peritoneum, and spleen are all shown to be relatively immunosuppressed as a consequence of hemorrhage and resuscitation, only Kupffer cells have a significantly elevated capacity to express the inflammatory cytokines IL–6 and TNF–α. This disparity may be due a difference in the relative stress that these cell populations are subjected to. Questions about this form the basis of my experiment as described next.

HEAT SHOCK PROTEIN 72 EXPRESSION IN A MURINE HEMORRHAGE MODEL

Hypothesis:

The differential expression of inflammatory cytokines between PMØ and KC in a murine fixed pressure model of hemorrhage may be due to a difference in the amount of relative stress seen between these two macrophage populations. This stress is reflected by a difference in the expression of the inducible 72 kD heat shock protein (hsp72). Therefore, increased hsp72 expression may preserve the capacity of KC to mount a cytotoxic response and release inflammatory cytokines in response to hemorrhage.

Specific aims:

The previous discussion leads to some interesting general questions regarding heat shock protein expression and function during as a result of hemorrhage:

- Is there a measurable difference in the inducible 72 kD heat shock protein (hsp72) expression during hemorrhage from baseline?
- What is the relationship between cytokines synthesis and heat shock protein expression?
- What is the role, protective or otherwise, of heat shock proteins in hemorrhage?
- Is there co–induction of gene expression of some cytokines and the stress proteins during hemorrhage and/or sepsis and what is the role of stress proteins in this response?

In this experiment we hope to:

1.) Compare differences in inducible murine hsp72 expression of peritoneal macrophages (PMØ) and Kupffer Cells (KC) in a fixed pressure model of hemorrhage and the relationship to known differences in cytokine expression (i.e., TNF–alpha and IL–6) in the same model

2.) Discuss the teleological and possible functional significance of this difference in response and how it impacts on the global immunosuppression that results from pure hemorrhagic shock.

IV. MATERIALS AND METHODS

HEMORRHAGE MODEL:

A fixed–pressure murine model of hemorrhagic shock was used as previously described.[49, 50] All protocols were carried out in accordance with the guidelines set forth in the Animal Welfare Act and as outlined in the Guide for Care and Use of Laboratory animals by the National Institutes of Health Publications. This project was approved by the Institutional Review Board and Research Committee of Butterworth Hospital and the Institutional Animal Care and Use Committee of Michigan State University.

Briefly, 8–10 week old inbred male C3H/HeN mice (Charles River Laboratories, Portage, MI) were randomly divided into hemorrhage (HEM) and control sham operation groups (SHAM). Prior to randomization and experimentation, the animals were fasted overnight but allowed water *ad libitum*. The HEM group was anesthetized with methoxyflurane inhalation, shaved, prepped sterilely, and underwent bifemoral artery cannulation with P–10 tubing. They were then bled to and maintained at a mean arterial pressure of 35 mm Hg for one hour as measured by calibrated pressure tracings. After one full hour at stable hypovolemia the HEM group was adequately resuscitated with the shed blood plus twice that volume balanced electrolyte solution (Ringer's lactate). SHAM groups underwent cannulation of both femoral arteries in a similar manner without hemorrhage / resuscitation. HEM and SHAM groups were then randomly divided into groups for procurement of KC and PMØ at 2 and 24 hours.

MACROPHAGE CELL PREPARATIONS:

KC and PMØ are harvested at the time of sacrifice for each of the groups of animals using aseptic techniques and isolated as follows:

Preparation of peritoneal macrophages:

A peritoneal lavage technique was utilized as previously described by Ayala et al.[50] Briefly, this consisted of harvesting the PMØ by peritoneal lavage using ice–cold Dulbecco's minimal essential media with 10% fetal calf serum (DMEM, Gibco, Grand Island NY, see Appendix A). Cells were washed once (280g, 15 min., 4°C) with Hank's balanced salt solution (HBSS, see Appendix B). 1×10^7 PMØ from animals within each group were plated into tissue culture bottles with 10 ml RPMI–1640 (Gibco, see Appendix C) with 10% fetal calf serum for a final concentration of 1×10^6 cells / ml in preparation for cytokine and Western blot analysis. After 3 h at 37°C in a 5% CO_2 atmosphere, non–viable and non–adherent cells were removed by 4 sequential gentle washings. Cell yields and viability as determined by trypan blue exclusion, were compared for SHAM and HEM groups.

Preparation of Kupffer cells:

An in situ collagenase digestion method was utilized as previously described by Ayala et al. (23). Briefly, after infusion of the liver with ice–cold HBSS, followed by warm DMEM with collagenase, the liver was resected en bloc and incubated 10 minutes at 37°C in a humidified incubator. After mincing and resuspension with, sequentially, a standard 25 ml and 10 ml pipet, the cells were pelleted, collected, and washed (4°C, in RPMI–1640 with 10% fetal calf

serum, at 280 g). Parenchymal and non–parenchymal cells were separated from each other by centrifugation over Metrizamide (Accurate Chem. Corp., Westbury, NY) and the cells at the interface pooled and washed twice by centrifugation (280g, 20 min., 4°C) with cold media with 10% fetal calf serum (FCS). Viability via trypan blue exclusion and yields were analyzed after the cell pellet is dispersed in and resuspended in RPMI–1640 media. Then 1×10^7 KC from animals within the individual groups were plated into tissue culture bottles (1×10^6 cells / ml) in preparation for cytokine , Northern blot , and Western blot analysis as described for PMØ above. After 5 hrs at 37°C in a humidified, 5% CO_2 atmosphere, non–viable and non–adherent cells were removed by 4 sequential gentle washings. Cell yields and viability were compared for SHAM and HEM groups. The cells from each group were washed and then stimulated for 24 hrs with LPS to induce cytokine production. After the supernatants were collected for TNF–α and IL–6 bioassays, the cells were lysed for protein extraction (see below). The cells were then prepared for protein electropheresis and Western blot analysis using a chemiluminescent HSP 70 probe

Cytokine bioassays:

Interleukin-6 activity in the culture supernatants were determined by the amount of proliferation of the murine B-cell hybridoma cell-line 7TD1, which grows only in the presence of IL-6.[50] 4×10^3 7TD1 cells/ml were grown for 72 hours at 37°C in 5% CO_2 after stimulation with serial dilutions of serum or macrophage supernatants. For the last 4 hours of incubation, 20 µl of a 3-(4,5-dimethylthiazol-2-L)-2,5-diphenyltetrazolium-bromide solution (MTT; 5 mg/ml in RPMI-1640, Gibco) were added to each well. Only viable cells incorporate MTT. The assay was stopped by aspiration of 150 µl supernatant from each

well, with subsequent replacement by 150 µl of a 10% solution of sodium dodecyl sulfate in PBS (Lauryl Sulfate, Sigma Chemical Co., St. Louis, MO) to dissolve the dark-blue formazan crystals. Using an automated microplate reader (EL-311, Bio-Tek Instruments Inc., Winooski, VT) the light-absorbance for each well was measured at 595 nm. The amount of IL–6 in supernatant is directly proportional to the proliferation of 7TD1 cells.

TNF in culture supernatants was determined by the inherent cytotoxic effect on the fibrosarcoma cell line WEHI 164 subclone 13.[58, 59] The proliferation of unlysed WEHI cells was measured, as described above, using a MTT assay. The amount of TNF in supernatant is indirectly proportional to the proliferation of WEHI cells.

In the bioassays described, the units of cytokine activity per milliliter of supernatant were determined by comparison to standard curves produced from dilutions of a purified recombinant IL–6 standard (200 U/ml, Amgen Corp., Thousand Oaks, CA) or a murine TNF standard (200 U/ml, Amgen Corp.) after the methods described by Mizel.[60]

Protein extraction and quantitation:

After heat shock or hemorrhage, cell samples are prepared for protein electrophoresis. After washing three times with PBS at the end of any individual treatments, the cell collections are lysed by the addition of ice cold lysis buffer:

50 mM Tris (pH 8.0)	2 mM phenylmethylsulfonylurea
150 mM NaCl	0.5 % Triton X–100
5 mM EDTA	

After addition of lysis buffer, the lysates are incubated on ice for 30 min. The lysates were clarified by microcentrifugation and the supernatants stored at

–70°C until ready for analysis. Protein concentrations were determined using a modified Lowery protein assay.

Tricine PAGE electrophoresis:

Protein samples were prepared for tricine gel electrophoresis by first heating similar quantities of protein for each sample at 85°C in 1X sample buffer and then running in a precast gel (10% acrylamide / 2.6%Bis–acrylamide, Novex®, San Diego, CA) for approximately 1 hr. at 100V constant voltage. After electrophoresis, the gel contents are electroblotted to nitrocellulose membranes for Western blot analysis. The gel is then fixed and stained with Coomassie blue.

Western Blot and quantitation of HSP 70 synthesis:

The prepared protein blots are blocked with a 5% milk solution, washed, and then incubated at 4°C with primary antibody for hsp72 (murine anti hsp72 MAb, IgG_1,SPA 810, StressGen Biotechnologies Corp., Victoria, B.C., Canada). After this, the membranes are again washed and then incubated with a secondary antibody conjugated to peroxidase for a luminol–based chemiluminescence reaction (ECL systems, Amersham, Inc., Manchester, GB.). At the time of chemiluminescence, the membranes were exposed to autoradiography film (X–Omat AR, Eastman Kodak, Rochester, N.Y.). The exposures were assessed for band intensity by densitometric analysis with a JAVA image analyzer (Jandel Scientific, San Rafael, CA). The band intensity was directly proportional to quantity.

Statistics:

Values are presented as means ± SEM for continuous variables or as percentages / ratios for discrete variables. Differences between groups were compared using analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD). Statistical significance was recognized at $p < 0.05$.

V. Results

MACROPHAGE CYTOKINE PRODUCTIVE CAPACITIES IN RESPONSE TO HEMORRHAGE

Cell Yields and Viability of Macrophage Preparation and Isolation:

Trypan blue exclusion for cell viability discriminates viable cells (no dye uptake) from non–viable cells (positive dye uptake). Our analysis revealed that no significant differences existed in the percentage of viable cells of the total number isolated whether the animal was subjected to sham operation or hemorrhage / resuscitation.

Kupffer Cells (see Table 1):

Kupffer cells isolated from hemorrhaged mice showed an increased capacity to release TNF–α (see Figures 1 and 2) and IL–6 (see Figures 3 and 4) when compared to controls. At 2 hours, Kupffer cell TNF–α productive capacity from hemorrhaged animals (6 animals, mean = .048 ± .0098 units/ml/10^5 cells) was increased 20–fold when compared to sham–operated animals (7 animals, mean = .002 ± .0004). This was statistically significant with $p < 0.05$ (ANOVA, Fisher's PLSD). This was true also at 24 hours for Kupffer cells from hemorrhaged (7 animals, mean = .110 ± .0130) versus sham–operated animals (10 animals, mean = .001 ± .0002) and was also statistically significant with $p < 0.05$ (ANOVA, Fisher's PLSD).

IL–6 productive capacities for Kupffer cells were similarly affected. At 2 hours Kupffer cells from the hemorrhage group (6 animals, mean = 23.5 ± 7.08) had a significantly increased IL–6 productive capacity when compared to shams

shams (7 animals, mean = 4.9 ± 1.03). At 24 hours this difference remained significant as the hemorrhage group (7 animals, mean = 12.6 ± 1.56) had a six–fold increase in IL–6 productive capacity as compared to shams (10 animals, mean = 2.1 ± 0.47).

As previously described in "Methods and Materials," productive capacities are initiated by stimulation with LPS. Therefore, this does not represent circulating inflammatory cytokine levels but is an *in vitro* measure of where the circulating cytokines are produced. Although the absolute values of TNF are much smaller than IL–6 values, the measured values are consistent, as represented by the standard errors of the mean (SEM).

Peritoneal macrophages:

PMØ at 2 and 24 hours showed no significant differences in the capacity to release TNF–α (see figure 5 and 6) and IL–6 (see figure 7 and 8) when comparing hemorrhaged and sham groups.

At 2 hours, peritoneal macrophage TNF–α productive capacity from hemorrhaged animals (6 animals, mean = 17.6 ± 3.71 units/ml/10^5 cells) was not significantly different compared to sham–operated animals (7 animals, mean = 12.4 ± 1.68). At 24 hours, hemorrhaged (7 animals, mean = 3.5 ± 0.31) versus sham–operated animals (10 animals, mean = 3.3 ± 0.64) showed no significant differences in peritoneal macrophage TNF–α productive capacity.

IL–6 productive capacities for peritoneal macrophages were similarly affected. At 2 hours the hemorrhage group (6 animals, mean = 1206.5 ± 100.8) had no significant differences in IL–6 productive capacity when compared to shams (7 animals, mean = 1131.7 ± 222.7). At 24 hours, there were also no significant differences between the hemorrhage group (7 animals, mean = 580.1 ± 72.9) and shams (10 animals, mean = 446.7 ± 122.8).

These results mirror those previously described in our lab. It establishes the validity of this model for the experiments involving the expression of hsp 72, which is described in the next section.

Cell Type	(n)	TNF–α *	IL–6 *
HemPer 2	(6)	17.6 ± 3.71	1206.5 ± 100.77
ShamPer 2	(7)	12.4 ± 1.68	1131.7 ± 222.67
HemPer 24	(7)	3.5 ± 0.31	580.1 ± 72.95
ShamPer 24	(10)	3.3 ± 0.64	446.7 ± 122.77
HemKup 2	(6)	0.0480 ± 0.0098	23.5 ± 7.08
ShamKup2	(7)	0.0022 ± 0.0004	4.9 ± 1.03
HemKup 24	(7)	0.1100 ± 0.0130	12.6 ± 1.56
ShamKup24	(10)	0.0010 ± 0.0002	2.1 ± 0.47

Table 1: Summary of results representing the productive capacities for TNF–α and IL–6 from PMØ (Per) and KC (Kup) in control (Sham) and hemorrhage groups (Hem) at 2 and 24 hours. Number per group in parentheses. * All values means ± SEM in units/ml/10^5 cells.

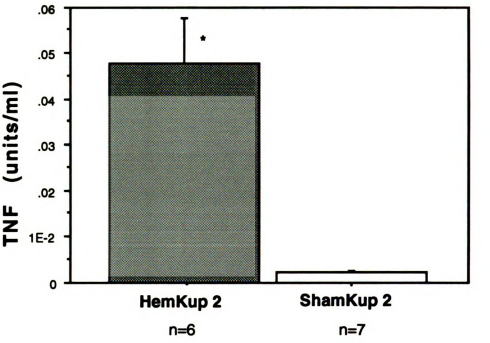

Figure 1: TNF–α productive capacities of Kupffer cells 2 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

*** $p < 0.05$ (ANOVA, Fisher's PLSD).**

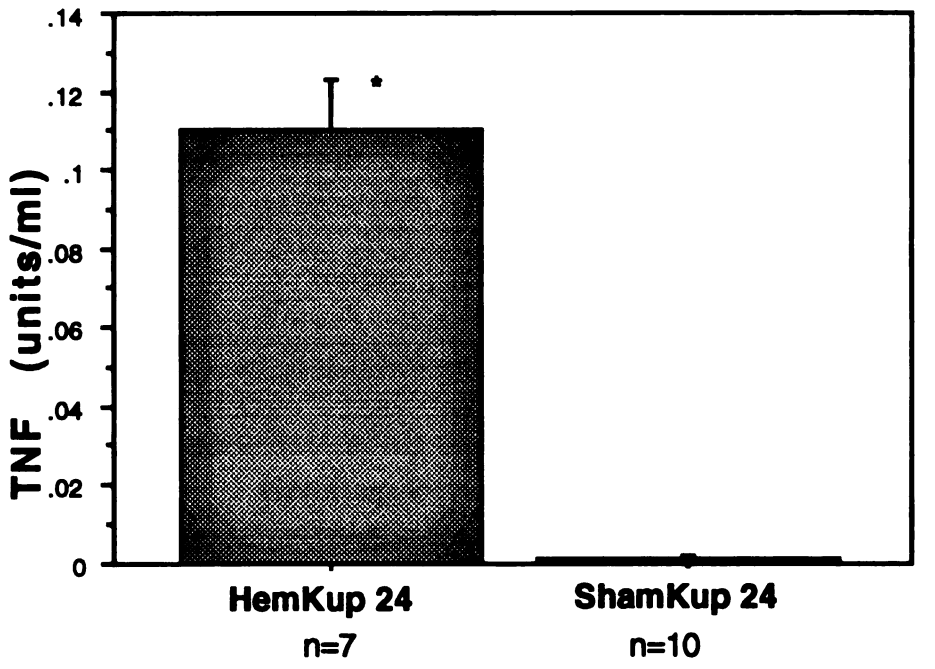

Figure 2: TNF–α productive capacities of Kupffer cells 24 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

*** $p < 0.05$ (ANOVA, Fisher's PLSD).**

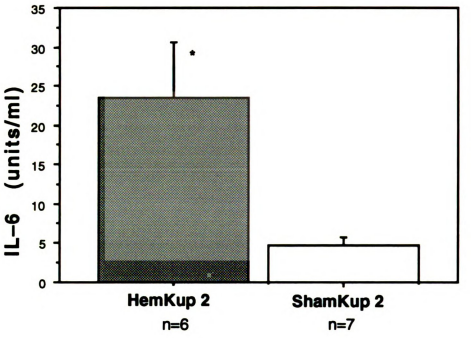

Figure 3: IL–6 productive capacities of Kupffer cells 2 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

*** $p < 0.05$ (ANOVA, Fisher's PLSD).**

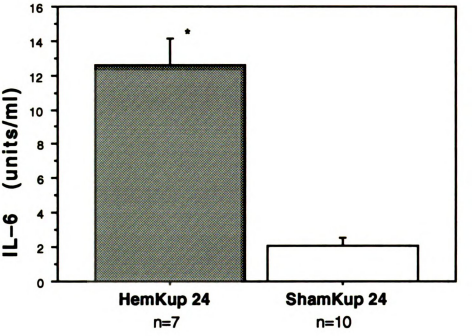

Figure 4: IL–6 productive capacities of Kupffer cells 24 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

*** $p < 0.05$ (ANOVA, Fisher's PLSD).**

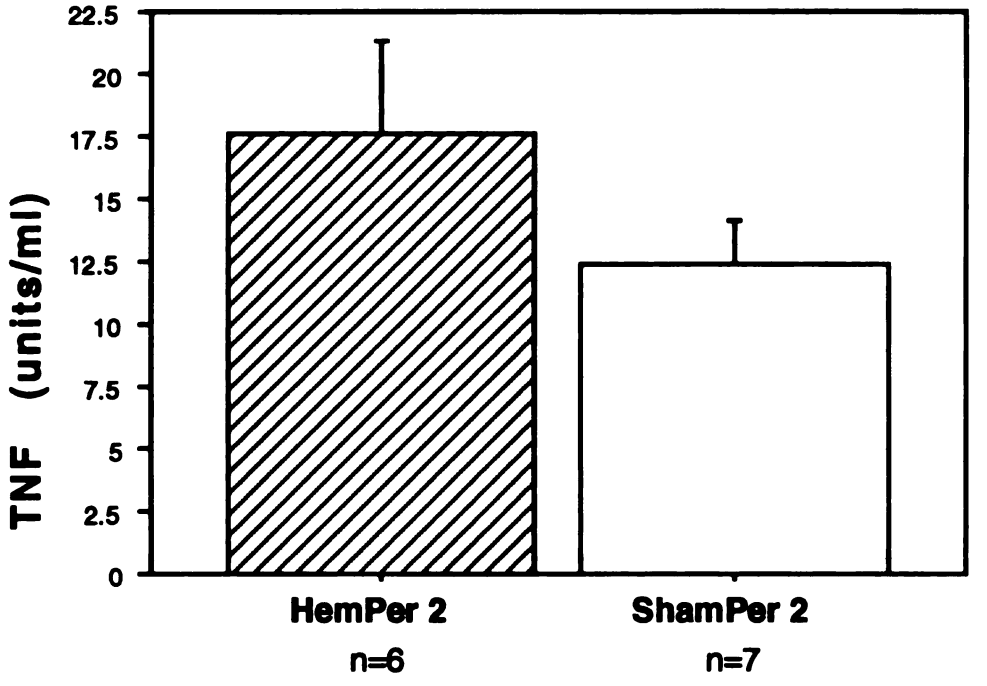

Figure 5: TNF–α productive capacities of peritoneal macrophages 2 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group. No difference, by ANOVA, Fisher's PLSD.

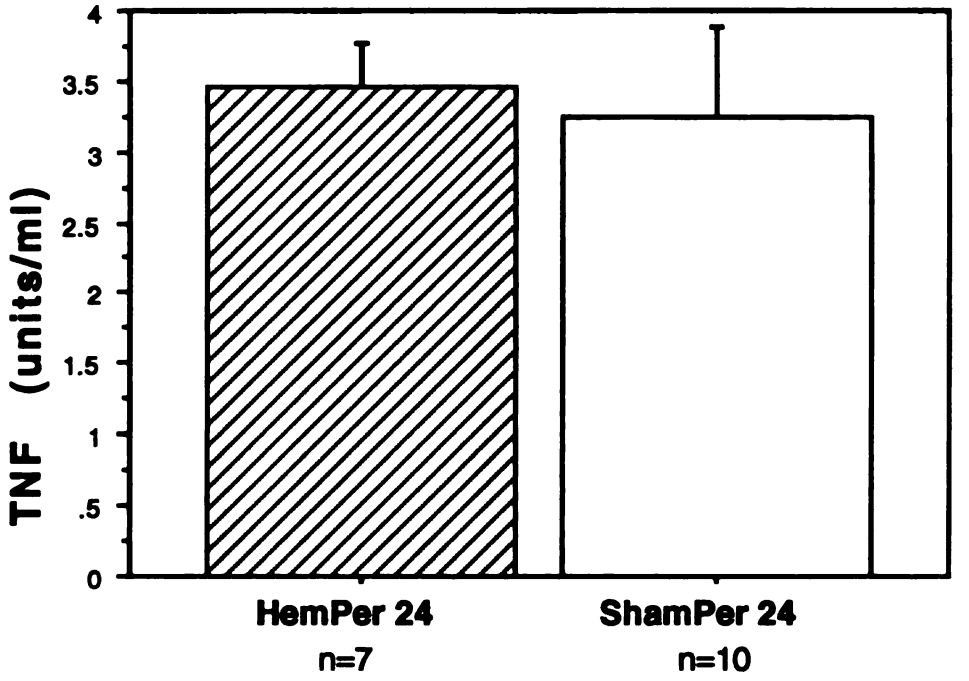

Figure 6: TNF–α productive capacities of peritoneal macrophages 24 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group. No difference, by ANOVA, Fisher's PLSD.

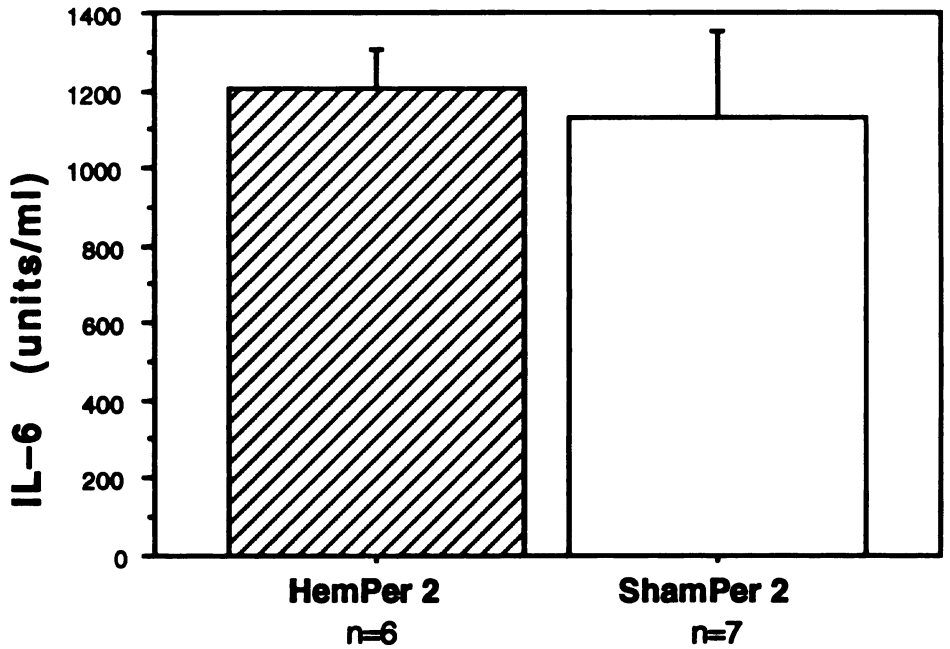

Figure 7: IL–6 productive capacities of peritoneal macrophages 2 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

No difference, by ANOVA, Fisher's PLSD.

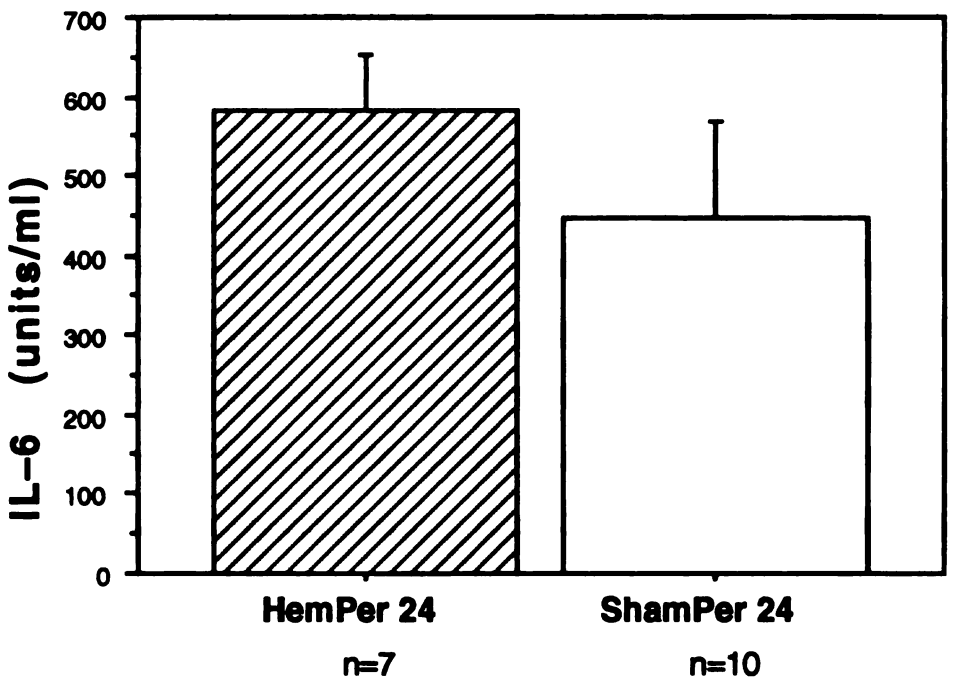

Figure 8: IL–6 productive capacities of peritoneal macrophages 2 4 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group. No difference, by ANOVA, Fisher's PLSD.

HSP72 EXPRESSION IN RESPONSE TO HEMORRHAGE

Kupffer Cells:

Hsp72 levels in Kupffer cells were significantly elevated in response to hemorrhage at both 2 hours (see Figure 9) and 24 hours (see Figure 10) when compared to controls(see Table 2). At 2 hours, hsp72 expression in Kupffer cells were significantly increased in the hemorrhage group (8 animals, mean = 161.6 ± 18.7 intensity units x 1000) versus shams (7 animals, mean = 50.4 ± 5.8). This was statistically significant at $p < 0.05$ (ANOVA, Fisher's PLSD). At 24 hours post–hemorrhage, this difference remained statistically significant between hemorrhage (7 animals, mean = 76.1 ± 15.4) and sham groups (7 animals, mean = 39.9 ± 2.7) for Kupffer cell hsp 72 expression.

Peritoneal macrophages:

No significant differences were seen in PMØ between HEM and SHAM groups at either 2 (Figure 11) or 24 hours (Figure 12). It also appears that although there is some diminution of hsp levels at 24 hours, differences still remain significant. At 2 hours, peritoneal macrophages from hemorrhaged animals (6 animals, mean = 21.9 ± 1.3) exhibited equivalent hsp72 expression when compared to shams (3 animals, mean = 22.9 ± 0.8). At 24 hours, there were still no significant differences noted between hemorrhaged animals (6 animals, mean = 29.9 ±4.9) and shams (6 animals, mean = 28.3 ± 4.8).

Sample Western blots exhibiting the differences in expression of hsp72 in the two cell populations are shown in Figures 13 (Kupffer cells) and 14 (peritoneal macrophages). Reference values from a known molecular weight standard on the original tricine PAGE gel were used to confirm the size of the

band of interest. By setting the background signal to a standard intensity (zero), using consistent amounts of protein per gel lane (5 ng), and standardized exposure times during the zenith of chemiluminescence (5 seconds), we were able to obtain semi–quantitative data which could be used for comparison in our experiments.

Cell Type	Time (hrs)	Hemorrhage*	(n)	Sham*	(n)	P
KC	2	161.6 ± 18.7	(8)	50.4 ± 5.8	(7)	S
PMØ	2	21.9 ± 1.3	(6)	22.9 ± 0.8	(3)	NS
KC	24	76.1 ± 15.4	(7)	39.9 ± 2.7	(7)	S
PMØ	24	29.9 ± 4.9	(6)	28.3 ± 4.8	(6)	NS

Table 2: †Summary of results comparing the expression of hsp72 at 2 and 24 hours for Kupffer cells (KC) and peritoneal macrophges (PMØ). Number per group in parentheses.* All values means ± SEM in density units x 1000.

P: Significance set at $p < 0.05$ (ANOVA, Fisher's PLSD); S = significant, NS = not significant.

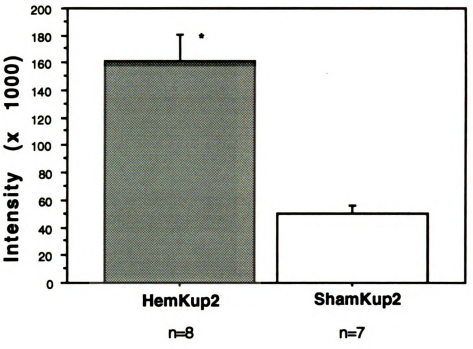

Figure 9: hsp 72 expression of Kupffer cells 2 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

*** $p < 0.05$ (ANOVA, Fisher's PLSD).**

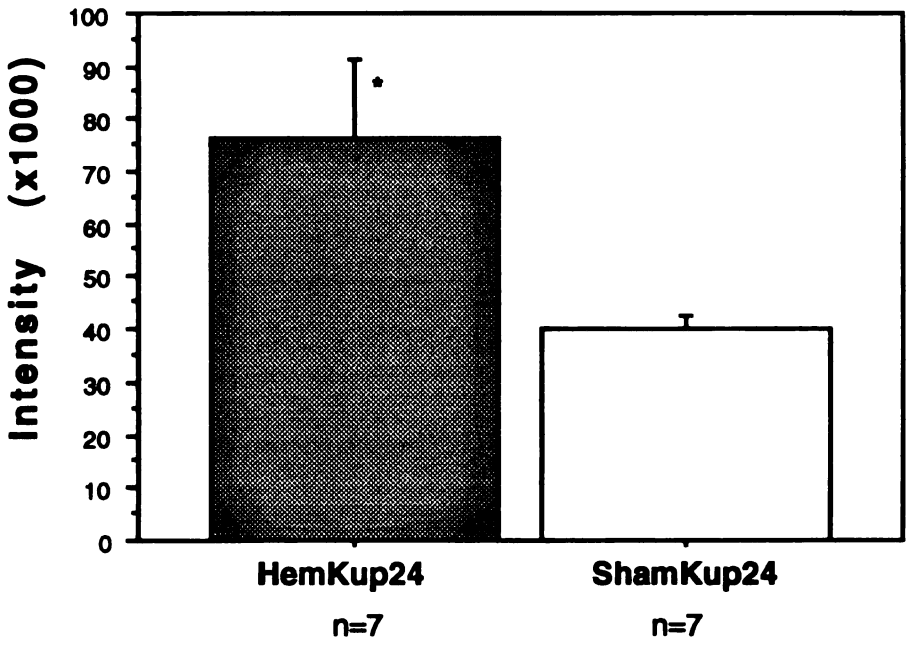

Figure 10: hsp 72 expression of Kupffer cells 24 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

*** $p < 0.05$ (ANOVA, Fisher's PLSD).**

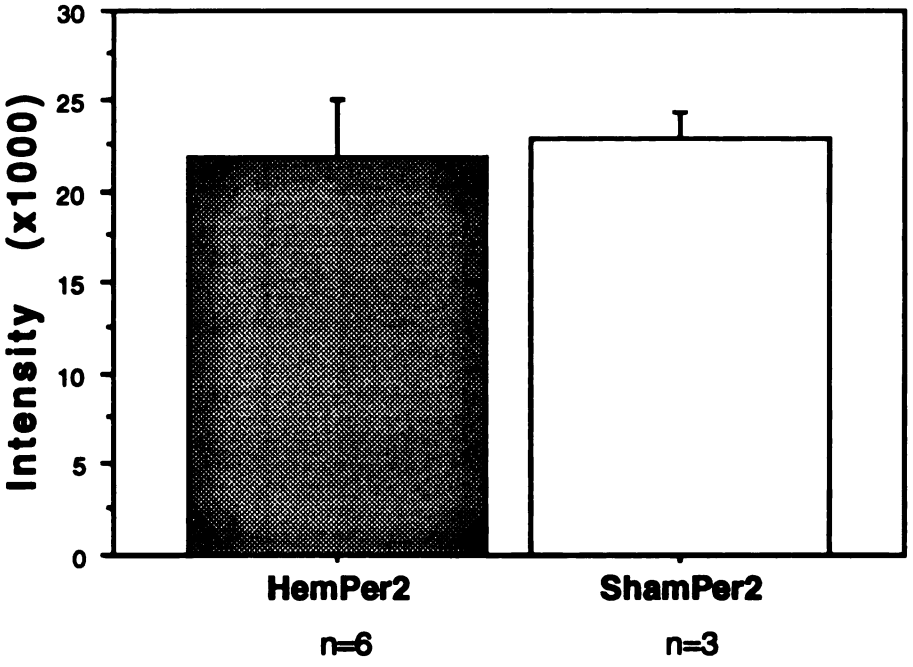

Figure 11: hsp 72 expression of peritoneal macrophages 2 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

No difference by ANOVA, Fisher's PLSD.

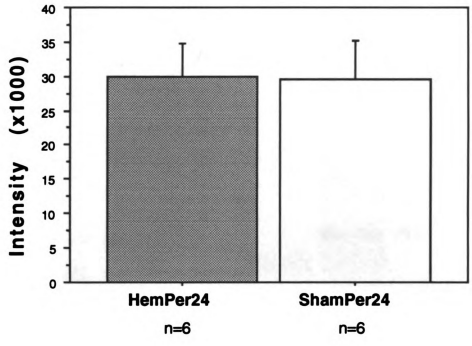

Figure 12: hsp 72 expression of peritoneal macrophages 24 hours after hemorrhage and resuscitation. Mean ± SEM, n = # of animals in each group.

No difference by ANOVA, Fisher's PLSD.

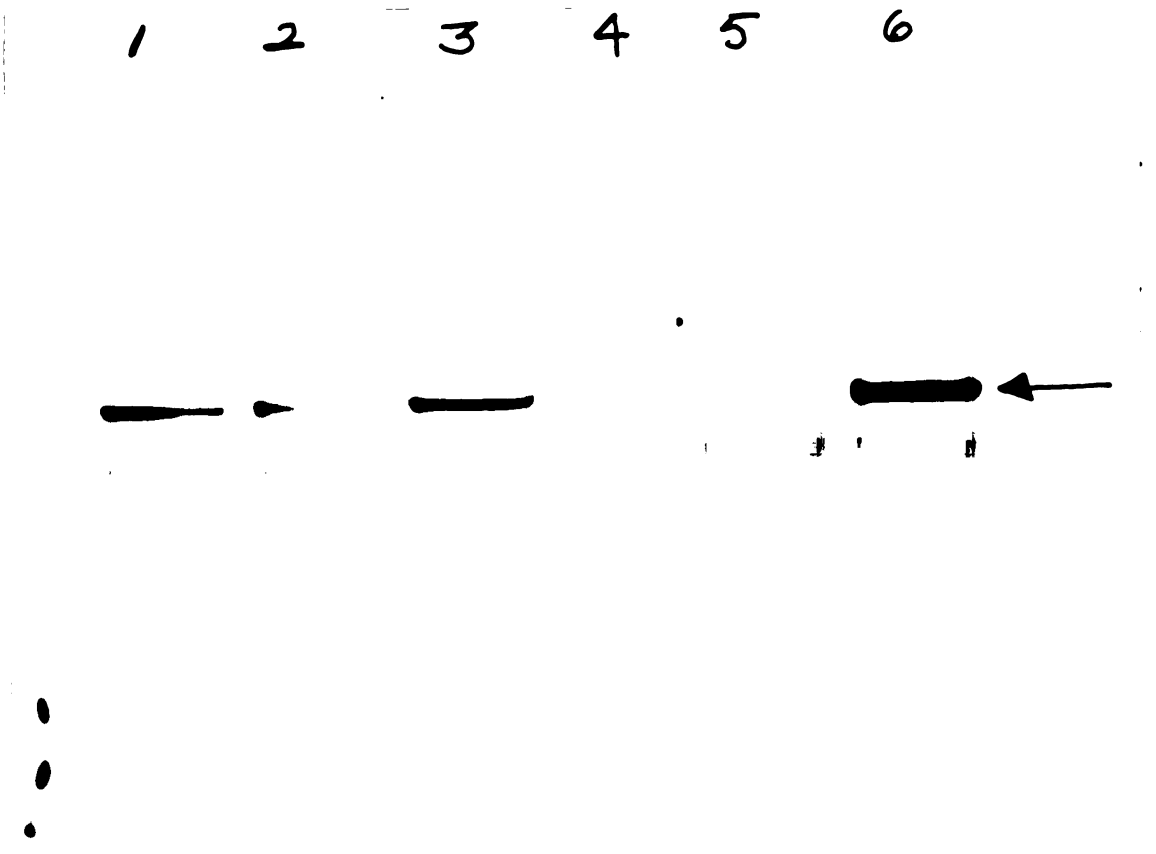

Figure 13: Representative sample of chemiluminescent radiographs from hsp72 Western blot analysis: Kupffer cells. Lanes 1 and 3 – extracts from Kupffer cells 2 hours post – hemorrhage; lane 2 – extract from Kupffer cells 2 hours after sham operation; lane 4 – blank; lane 5 – extract from Kupffer cells 24 hours after sham operation; lane 6 – extract from Kupffer cells 24 hours post – hemorrhage. Arrow – hsp72.

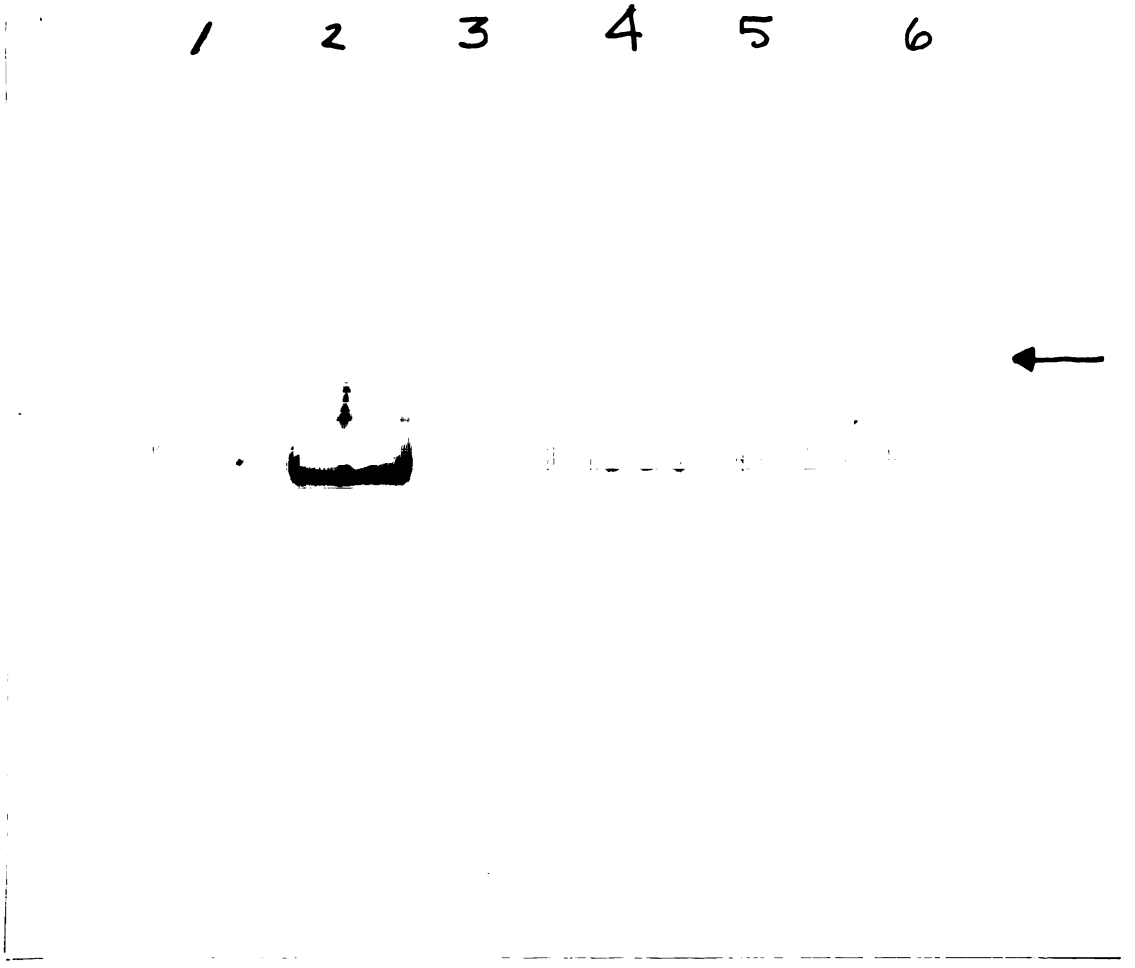

Figure 14: Representative sample of chemiluminescent radiographs from hsp72 Western blot analysis: peritoneal macrophages. Lanes 1 and 3 – extracts from peritoneal macrophages 2 hours after sham operation; lanes 2 and 4 – extracts from peritoneal macrophages 2 hours post – hemorrhage; lane 5 – extract from peritoneal macrophages 24 hours after sham operation; lane 6 – extract from peritoneal macrophages 24 hours post – hemorrhage.

Arrow – hsp72.

VII. DISCUSSION

HEMORRHAGE AND ITS IMMUNOLOGIC CONSEQUENCES

Hemorrhage, and injury in general, results in a cascade of events that are intended to act as a measure of defense for the host. These include, but are not limited to, a metabolic response reflected by an increase in counterregulatory hormones, corticosteroids, and catecholamines; implementation of nonspecific inflammatory responses mediated by kinins, the complement sytem, coagulation, and the reticuloendothelial system; and an immune response mediated by a complex network of macrophages, T–cells, B–cells and their inflammatory mediators (e.g., cytokines, arachidonic acid metabolites, and colony–stimulating factors).[47, 61] However, the result of this activation is often injurious given the correct environment. As such, the final common pathway is often multiorgan failure, with mortality directly related to the number of dysfunctional systems of the affected host.[62]

It appears that many of the functional aspects of the immune sytem that act in a protective manner for the host also act to damage many of the structural elements that support homeostasis. It is clear that when this situation is combined with any element of immune dysfunction, such as depressed antigen presentation by macrophages and depressed phagocytic function, the host is at risk for sepsis caused by secondary infection from endogenous as well as exogenous sources. Chaudry and Ayala have clearly outlined the mechanisms believed to play a role in the breakdown of normal immune function that leads to depressed lymphocyte and macrophage functions and

immunosuppression.[63, 64] Hemorrhage is believed to cause regional hypoxia mediated by decreased oxygen delivery secondary to hypovolemia and preferential shunting away from non–critical organs. This situation is further exacerbated by early systemic mediators (e.g., arachidonic acid metabolites, catecholamines, the acute–phase response) that initially act to counter the initial insult. Regional hypoxia, in turn, initiates a cycle of cellular derangements in energy stores (i.e.: ATP) and calcium homeostasis that may stimulate an increase in inflammatory cytokines which propagate the host's mediator response and amplifies it. It appears that this amplification leads to further suppression of macrophage and lymphocyte function. This is reflected in the decreased ability of macrophages to present antigen, decreased lymphokine activation important for the coordination of a competent cell– and humoral–mediated immune response, and an increase in the elaboration of potent immunosuppresive agents such as transforming growth factor beta (TGF–β) and platelet activating factor (PAF).[65, 66]

It is apparent that the inflammatory cytokines playing large roles in this cascade of events are IL–1, IL–6, and TNF–α. As was indicated earlier, these cytokines are upregulated in response to hemorrhage / resuscitation in a differential manner among two different cell populations: increased productive capacities in Kupffer cells and decreased in peritoneal macrophages. Despite this difference, there is a global effect on many mononuclear cells for decreased antigen presentation.[52, 53, 63] Reasons for these differences may be due to intrinsic as well as environmental factors.

Kupffer cells lie in a matrix rich in immunodulating factors. They are exposed to bacterial by–products from the splanchnic circulation, receive signals from surrounding hepatocytes, clear toxins, and act as accessory cells in the induction of antigen–specific immune responses.[52, 63] As such, they exist

in a much more dynamic immunomodulatory milieu than do peritoneal macrophages. It may be surmised that signals received by the Kupffer cell acting to increase its cytotoxic abilities and ability to elaborate cytokines in an amplified manner are inherently different as a result of this difference in environment. However, it is not clear what mechanisms are affecting the ability of the Kupffer cell to maintain its cytotoxic nature despite a supression of antigen presentation. One mechanism may be its increased expression of hsp72.

DIFFERENTIAL EXPRESSION OF HSP72 FROM KUPFFER CELLS AND PERITONEAL MACROPHAGES IN RESPONSE TO HEMORRHAGE / RESUSCITATION

These experients show that there is a differential expression of the heat shock response, as measured by hsp72, between Kupffer cells and peritoneal macrohages. In response to hemorrhage, shock, and resuscitation, Kupffer cells exhibit an increased expression of hsp72 when compared to shams. Peritoneal macrophages do not exhibit any increase in hsp expression in response to the same insult. When coupled with the findings of differential inflammatory cytokine productive capacities as previously outlined, one can infer that the ability to mount a heat shock response may be a protective mechanism which preserves the cytotoxic abilities of Kupffer cells but not peritoneal macrophages. The reasons for this may again be due to the environment of the Kupffer cell during hemorrhage / resuscitation.

During the resuscitative phase of our model, the liver is exposed to many of the stimuli of the heat shock response including bacterial by products and endotoxin, oxygen radicals, and reperfusion mediators.[41, 42, 67] This is in addition to the potent stimulus of regional hypoxia induced in the liver secondary to the low flow state of acute hemorrhage. Given its metabolic load, it is easy to surmise that significant reductions in blood flow and oxygen delivery to the liver will have more profound effects than a similar reduction to peritoneal cells. Therefore, the effective stress response will be significantly more magnified in the liver parenchyma than in the peritoneal lining.

Teleologically, given the inherent cytoprotective properties of the heat shock response, it is likely that certain cell populations able to mount such a

response will preserve more of their natural cell function than those unable to mount a heat shock response. Therefore, the preservation of cytotoxic responses in Kupffer cells may be related to its ability to express hsp72. What remains to be elucidated are the specific genetic mechanisms that are involved in this phenomenon.

Previous work by Udelsman and associates [22, 37, 38, 39] as well as by Buchman and associates [31, 40, 41] had shown measurable increases in the mRNA of the hsp70 family of proteins in response to surgical stress, restraint stress, cardiogenic shock, and hemorrhage (see "The stress response", pp. 8–10). These investigators demonstrated single organ elevations of the message and postulated genetic mechanisms mediated by specific transcription factors such as HSF–1 that are specific to certain stress stimuli. In our study, we have demonstrated that a specific cell population within the reticuloendothelial system, Kupffer cells, exhibit increased expression of hsp72 by Western blot analysis. Our attempts at Northern blot analysis of hsp72 mRNA expression showed that, in some prliminary data, there was an increased expression of this message in response to hemorrhage within liver as a whole and from some of the isolated Kupffer cell samples. Unfortunately, our results were too inconsistent to make any meaningful interpretation possible and are not included in this thesis.

Further work in this area should be directed to genetic mechanisms involved in the elucidation of the heat shock response. It is quite possible that potential mechanism involved in the differences between Kupffer cells and peritoneal macrophages in our specific model may be due to an upregulation in the transcription factors responsible for initiating and amplifying the production of hsp72 in Kupffer cells. The effect of this would be to augment the differences in environmental factors affecting the two cell populations resulting in an

increased heat shock response in Kupffer cells. It is hoped that future investigators with an interest in this area may be able to demonstrate these differences at the molecular level.

VIII. SUMMARY AND CONCLUSIONS

SUMMARY

This project confirmed that hemorrhage and resuscitation in mice resulted in a significantly increased capacity to release TNF–α and IL–6 from Kupffer cells when compared to sham–operated controls. This was not reflected by a similarly significant upregulation of inflammatory cytokine release in peritoneal macrophages from the same animals (see Figures 1 to 8 and Table 1). Similar results were previously described by Ayala et al. in the same hemorrhage and resuscitation model.[50, 53, 54] In these studies, it was shown that hemorrhage and resuscitation resulted in a significantly and similarly decreased capacity for antigen presentation among both PMØ and KC and susceptibility of the subject to early death from sepsis when compared to sham–operated controls.

This study, in addition, showed that differences in cytokine productive capacities are mirrored by differences in hsp72 expression between PMØ and KC in response to hemorrhage. Here the response of KC is an increased expression of hsp72 as a result of hemorrhage and resuscitation in C3H / HeN mice when compared to sham–operated controls (Figures 9 to 12 and Table 2). Although an association between increased cytokine productive capacities and increased hsp72 expression exists temporally within Kupffer cells of mice undergoing hemorrhage / resuscitation, it is uncertain mechanistically whether or not these two phenomena are related.

CONCLUSIONS

These studies demonstrated differential effects of hemorrhage / resuscitation on two specific cell populations. Kupffer cells and peritoneal macrophages experience marked cellular immune dysfunction. However, inflammatory cytokine (IL–6 and TNF–α) production is markedly increased in Kupffer cells compared to peritoneal macrophages. This is mirrored by an increased ability to mount a heat shock response in the former. It is conceivable that the depression of cellular immunity in the face of differential inflammatory cytokine productive capacities occur through separate but temporally related mechanisms. However, in light of the findings of this project, it is also likely that preservation of cytokine productive capacities in Kupffer cells in this model is related to the ability to mount a heat shock response.

Peritoneal macrophages are unable to mount a heat shock response equal to that of Kupffer cells. The reasons for these differences and their consequences may be as follows:

1. There are relative differences in the stress experienced by the two different cell populations based on differences in the environment of each of the cell types and the relative effects of hemorrhage on each environment.
2. Reperfusion injury may have a bigger impact on the liver and its constituents, especially Kupffer cells, which is reflected by the more intense heat shock response in Kupffer cells.
3. The heat shock response elicited in the Kupffer cell population may be a protective mechanism preserving basic cellular functions in the face of an injurious insult.

4. The preservation of function in Kupffer cells is not "complete", resulting in a depression of antigen presentation despite an increased inflammatory cytokine productive capacity and cell cytotoxicity.
5. The critical mechanism for the expression of the heat shock response may be that specific signals required to upregulate transcription factors neede to complete the initiation and amplification of hsp72 mRNA production in our model exist in the splanchnic environment of the liver during hemorrhage / resuscitation but are inadequate within the circulation of the peritoneum.

LIMITATIONS AND FUTURE STUDIES

The findings of this study are unique in that previous work had not demonstrated increased heat shock response in one cell population from an organ as a response to severe stress such as hemorrhage / resuscitation. Furthermore, our findings clearly show a difference in mononuclear cell activity and function depending on the local environment that cell was in during the inception of stress. However, although there is an association of increased cytokine productive capacity to hsp72 expression in response to hemorrhage, the mechanisms and causal relationships need to be worked out.

It can be argued that because LPS stimulation is used in the cytokine bioassays the expression of hsp72 from the two cell populations of interest in this study also need to be treated with LPS. This is a valid point. It would be of vital interest to know if LPS pretreatment has any effect on the expression of hsp72 in our model. This would then show whether or not hsp72 production is also stimulated by the same mechanisms in play during the stimulation phase of the cytokine bioassays. If hsp72 is upregulated equally along all cells and treatments (i.e. sham or hemorrhage), then this upregulation is a background increase that does not necessarily invalidate our conclusions. If there are differential alterations in hsp72 expression due to LPS stimulation that does not parallel our findings, we must rethink the mechanisms involved in the cytokine bioassay and hold off on the notion that cytokine expression and the heat shock response are necessarily linked. However, as a point of reference, our data do show the intrinsic levels of heat shock proteins as a result of the stress of hypovolemic shock due to severe hemorrhage followed by acute resuscitation in peritoneal macrophages and Kupffer cells are significantly different. This

difference may be due to differential responses to a similar stress in two different cell populations insofar as their respective heat shock responses are concerned.

These limitations in our study form the basis of possible future studies in this model:

1. Elucidate the genetic expression of hsp72 production, whether by Northern blot analysis or amplification via polymerase chain reaction (PCR).
2. Gene knockout experiments, whether by antibody binding to hsp72 or antisense RNA prior to stimulation for cytokine release, may be able to delineate whether or not hsp72 is required for cytokine production or if they are even linked mechanistically.
3. Perform the experiments where LPS is used prior to harvesting protein for Western blot analysis and compare to assays where there is no pretreatment with LPS.

These points, in conjunction with studies of the mechanisms of hsp72 upregulation at the transcriptional level, may further our understanding of the reasons why differential alteration of macrophage function in response to hemorrhage / resuscitation leads to a relative global depression of immune function in the affected organism.

APPENDIX A

APPENDIX A

Dulbecco's Modification of Eagle's Essential Media (DMEM)

Amino Acids	mg/l
Arginine HCL	84.0
Cystine	48.0
Glutamine	584.0
Glycine	30.0
Histidine.HCl.H_2O	42.0
Isoleucine	104.8
Leucine	104.8
Lysine.HCl	146.2
Methionine	30.0
Phenylalanine	66.0
Serine	42.0
Threonine	95.2
Tryptophan	16.0
Tyrosine	72.0
Valine	93.6

Vitamins	mg/ml
Choline Chloride	4.0
Folic acid	4.0
Nicotinamide	4.0
Calcium d–pantothenate	4.0
Pyridoxal.HCl	4.0
Riboflavin	0.4

Thiamine HCl	4.0
Inositol	7.0

Inorganic salts	mg/l
NaCl	6400.0
KCl	400.0
$NaH_2PO_4.2H_2O$	125.0
$MgSO_4.7H_2O$	200.0
$CaCl_2$	200.0
$NaHCO_3$	3700.0
$Fe(NO_3)_3.9H_2O$	0.1

Other Components

n–Butyl–p–hydroxybenzoate	0.2 mg/l
Glucose	4500.0 mg/l
Penicillin G potassium	5000 units
Phenol red	15.0 mg/l
Sodium pyruvate	110.0 mg/l
Streptomycin sulfate, equivalent base	100.0 mg/l

APPENDIX B

APPENDIX B

RPMI–1640 MEDIUM

L–amino acids	mg/l
Arginine	200.0
Asparagine	50.0
Aspartate	20.0
Cystine	50.0
Glutamate	20.0
Glutamine	300.0
Glycine	10.0
Histidine	10.0
Hydroxyproline	20.0
Isoleucine	50.0
Leucine	50.0
Lysine.HCl	40.0
Methionine	15.0
Phenylalanine	15.0
Proline	20.0
Serine	30.0
Threonine	20.0
Tryptophan	5.0
Tyrosine	20.0
Valine	20.0

Vitamins	mg/l
Biotin	0.2

B_{12}	0.005
Calcium pantothenate	0.25
Choline Chloride	3.0
Folic acid	1.0
Nicotinamide	1.0
p–aminobenzoic acid	1.0
Pyridoxine.HCl	1.0
Riboflavin	0.2
Thiamine HCl	1.0

Inorganic salts	mg/l
NaCl	6000.0
KCl	400.0
$Na_2HPO_4.7H_2O$	1512.0
$MgSO_4.7H_2O$	100.0
$NaHCO_3$	2000.0
$Ca(NO_3)_2.4H_2O$	100.0
Other Components	
Glucose	2000.0
Glutathione (reduced)	1.0
Phenol red	5.0

APPENDIX C

APPENDIX C

Publications:

Kam DM, Ayala A, Chaudry IH. Insights into the mechanism of increased Kupffer cell cytokine release in response to hemorrhage: increased expression of HSP 72. Surgical Forum, 1995; 46: 152–154.

Presentations and Awards

Kam DM, Ayala A, Chaudry IH. Insights into the mechanism of increased Kupffer cell cytokine release in response to hemorrhage: increased expression of HSP 72. Presented at:

1.) American College of Surgeons Surgical Forum, New Orleans, LA (1995)

2.) Society of University Surgeons, Resident Section, Denver, CO (1995)

3.) Michigan Chapter American College of Surgeons, Troy, MI (1995)

4.) GRAMEC Research Day, Grand Rapids, MI (1995). Winner, Best Trauma Study

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IX. BIBLIOGRAPHY

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