Combat Medicine

Basic and Clinical Research in Military, Trauma, and Emergency Medicine

Edited by

George C. Tsokos, MD
James L. Atkins, MD

Humana Press

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Preface

Trauma and exposure to toxic and infectious agents invariably lead to organ damage followed by significant morbidity and mortality. Although these conditions have typically been associated with the battlefield, today they are more prevalent in urban areas. The events of September 11, 2001 have brought this problem to the forefront of national and international concern. The demand for solutions is justifiably high, and the research community needs to adjust its efforts appropriately.

Combat Medicine is meant to be a concise manual for the young clinical or basic investigator who is studying organ injury following trauma or toxic or infectious assaults either in an urban or battlefield setting, with an emphasis on current research issues in emergency and military medicine. The aim of *Combat Medicine* is to inspire surgical and medical residents and fellows, as well as biology and biochemistry students and fellows, to pursue research careers in the fields of military, trauma, and emergency medicine. *Combat Medicine* is not intended to be an exhaustive review; rather it is an introduction to key principles of this field.

The area of combat medicine research is enormously diverse, and in many ways it has not yet been defined. Mechanisms that lead to tissue damage include apoptosis, abnormalities in nitric oxide production, and disturbance of cell biochemistry. The affected organs are equally diverse and include the skin, the lungs, and the nervous system. Accordingly, we elected to divide the book into two parts. In the first, we asked experts to discuss the basic mechanisms that are invariably involved in the development of organ injury, such as apoptosis, nitric oxide production regulation, complement activation, and immune cell response to stressors. For the second part, we asked colleagues to discuss the current concepts that govern research aimed at understanding and reversing damage to major organs.

The authors explore mechanisms involved in the development of injury, review animal and, when available, human studies, and focus attention on future directions for research.

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I Basic Mechanisms

1 Apoptosis

Henry K. Wong, MD, PhD

INTRODUCTION

Injury in military combat develops from various forms of insults, such as blunt trauma from a projectile, ischemia from loss of blood or low perfusion, direct injury from irritants and vesicant chemicals, or radiation damage. Frequently, a subset of these events resembles situations that are also encountered in the acute care setting in the hospital emergency room. Specifically, traumatic injuries can lead to incapacitation, and rapid medical care is necessary to limit further physical deterioration of the wounded individual and to allow the stabilization, healing, and repair required for recovery. An understanding of the molecular mechanisms and consequences of combat injuries is critical to the development of novel therapies focusing on the limitation of tissue damage and the stimulation of rapid repair of injuries. It is therefore important to understand at the fundamental level the biochemical processes that occur during cellular injury.

Over the past decade, rapid advances have been made in our knowledge of the molecular events subsequent to injury at the cellular level that leads to cell death. A precise definition is important to explain better the molecular processes that lead to cell death *(1)*. It has become evident that the demise of organs and cell death occur by way of two molecular mechanisms, necrosis and apoptosis *(2,3)*. Traditionally, cell death was viewed as a passive event caused by increasing disorganization of biochemical processes owing to progressive loss of access to oxygen and nutrients, such as in ischemic injuries. However, it has now become clear that active expenditure of energy, through specialized concerted biochemical programs, is also involved in cell death—

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apoptosis or programmed cell death—particularly during the earliest time following an injury *(4)*. Also, it is clear that apoptosis did not evolve only for the purpose of dealing with traumatic injury. Apoptosis is an integral part of normal development of the heart *(5)* and brain *(6)* during embryogenesis, as well as the development of the immune system *(7)*. In these systems, an excess number of cells is initially generated to permit and ensure the appropriate selection of the functional cells that make up the mature organ. For example, in the brain, many cells are needed to form the correct synaptic connections, and those neurons that do not form appropriate connections are eliminated by apoptosis. In the immune system, only the lymphocyte precursor cells that form the correct antigen receptor by gene rearrangement survive; the cells that fail to form functional antigen receptors are removed by apoptosis. The importance of apoptotic pathways as a mechanism for regulating development can be appreciated by the extent that this process is found across metazoan cells, from the nematode worm *Caenorhabdits elegans* to humans.

In severe cardiovascular collapse, necrosis is the late consequence of ischemia and anoxia, and apoptosis is an early process that is initiated following ischemia *(8,9)*. However, it has become clear that cell death from apoptosis, an energy-dependent process, plays an important role in the early stages of tissue injury. Therefore, with a better understanding of the molecular mechanism of injury, it can be seen that even though the progressive damage of organs noted during autopsy is mediated predominantly by necrosis, such damage was probably initiated and exacerbated by apoptosis. Both processes, apoptosis and necrosis, are involved in tissue damage, and apoptosis may be an early event that subsequently leads to necrosis (**Fig. 1**). For example, hypotension develops in hemorrhagic injury and a hypoxic state follows that leads to the activation of apoptotic pathways in the injured tissue.

Cell death developing from necrosis is a disorganized process with increased entropy and loss of energy. There is release of cellular debris. Apoptosis serves to limit the total injury because it is a managed process that conserves energy by removing damaged tissue for the sake of survival of the greater organism. In ischemic tissue, for example, there is less capacity to maintain tissue integrity. The damaged tissue removed by apoptosis does not lead to disruption of surrounding normal tissue architecture, and one may think of the process as walling off the area of damage. With modern resuscitative care, the ability to restore perfusion

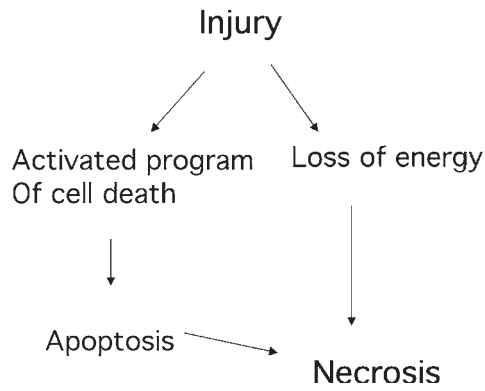

Fig. 1. Pathways that lead to cellular and tissue damage. Apoptosis is an energy-dependent pathway that can lead to cell death. Loss of energy can also lead to demise of the cell.

to injured tissues rapidly allows delivery of energy to the tissue, to begin to permit healing. Apoptosis under these circumstances may be excessive, leading to a greater loss of tissue than is necessary for survival. The control of apoptosis may therefore be vital in reducing the excessive loss of tissue. This chapter reviews the current understanding of mechanisms that are important in apoptosis.

PATHOLOGY

Injury leading to cell death is morphologically defined broadly by necrosis and apoptosis (in ref. *10*). Necrosis is characterized by oncosis, intracellular swelling with enlarged organelles, disruption of the nuclear membrane and plasma membrane, disintegration of nuclear structures and cytoplasmic organelles, cell shrinkage, random fragmentation of DNA, and release of intracellular contents and enzymes. Histology shows is a loss of cellular organelles and degenerative changes, with homogenization of structures, loss of distinct cell borders, and decreased intensity of staining. The process is disorganized and unpredictable, with collateral cellular and tissue damage.

In contrast, apoptosis is a highly ordered process of cell death differentiated histologically by the presence of adjacent cells that are unaffected. One of the earliest changes in apoptosis is alteration of the cell membrane, with movement of phosphotidyl-serine (PS) from the inner

surface of the bi-lipid layer to the extracellular surface. This may mark the dying cell: professional macrophages can phagocytize the cell and remove it in a controlled manner.

Apoptosis, originally described by Carl Vogt as early as 1842, is associated with shrinking of the cell, condensation of the nucleus, and little surrounding inflammation. Enzymatic staining such as terminal deoxythymidine transferase-mediated UTP nick end labeling (TUNEL) can identify cells in the process of apoptosis *(11)*. There is minimal surrounding damage to adjacent cells and no inflammation.

Other assays of apoptosis, such as propidium iodide staining of DNA, can identify specific population of apoptotic cells by measuring the total DNA content within a cell. During apoptosis, there is cleavage of DNA between nucleosomes, where there is a reduction in DNA content to less than the diploid amount of DNA. The population that has less than the normal level of DNA represents cells that have undergone apoptosis.

Annexin V analysis is another useful test for measuring the progression of apoptosis. Annexin V is an anticoagulant that can bind PS in the presence of calcium. In living cells, there is a predominance of PS on the cytoplasmic surface of the lipid membrane. Once apoptosis is initiated, this asymmetry is lost. PS will appear on the outer surface of the cell membrane where it is recognized by annexin V. This test is an early indicator of apoptosis and provides a way to identify apoptotic cells prior to the onset of DNA damage.

MECHANISMS OF APOPTOSIS

The understanding of apoptotic mechanisms has progressed rapidly over the last decade, and these mechanisms vary in location from intracellular sensors, such as the cellular tumor repressor gene p53 that is activated upon DNA damage, to the cell surface receptor Fas that initiates apoptosis upon crosslinking. The first indication that apoptotic mechanisms existed were suggested by early studies in *C. elegans* development that identified the proteins CED3 and CED4 as being essential to appropriate development *(12)*. These gene products were important in the elimination of specific cells *(13)*. Without these genes, there were additional cells in these nematodes that were not present in normal worms. A comparison of the amino acid sequence of these pro-

teins revealed that these *C. elegans* proteins showed similarities to other mammalian proteases. This provided hints to the importance of proteases in the regulation of apoptosis.

Apoptosis is mediated by a cascade of molecular events characterized by DNA cleavage, nuclear condensation, and specific protein degradation. In addition to the apoptotic mechanisms identified in nematodes, one of the best understood systems of apoptosis is that of the immune system *(14)*. Throughout the development and maturation of the immune system, there is ongoing selection of specific subsets of lymphocytes to either proliferate or contract through apoptosis in an effort to maintain homeostatsis during the immune response. During T-cell and B-cell development, those cells that do not rearrange their variable genes to form functional antigen receptors appropriately undergo apoptosis. The peripheral pool of circulating T-cells represents only 10% of the cells that have initially undergone selection. The remaining cells have been eliminated by apoptosis. Thus apoptosis is an ongoing process that is essential for normal function of the immune system. During an infection, antigen-specific B-cells and T-cells proliferate to combat the invading pathogen; upon clearance of the foreign antigen, these cells are eliminated by apoptosis.

Analysis of mechanisms of apoptosis has revealed that regulation exists at many levels. It is now known that apoptosis can be activated through extracellular surface receptors. This mechanism is important in immune surveillance of tumor cells and virally infected cells, which have abnormal proteins on the cell surface. These death signaling receptors themselves interact with adaptor molecules at the plasma membrane that further interact with cytoplasmic executioners, such as the caspases. In addition, there are regulators of apoptosis at the cytoplasmic level that can directly initiate apoptosis in response to excessive cellular stress from free radicals, ultraviolet (UV) irradiation, and γ-irradiation. Once apoptosis is initiated, the process does not require new gene transcription or protein synthesis, and even enucleated cells can undergo apoptosis *(15)*.

Regulation of Apoptosis Through Extracellular Surface Receptors

Apoptosis can be regulated by receptor-mediated signaling. The receptors include Fas/Apo1/CD95, tumor necrosis factor receptor 1

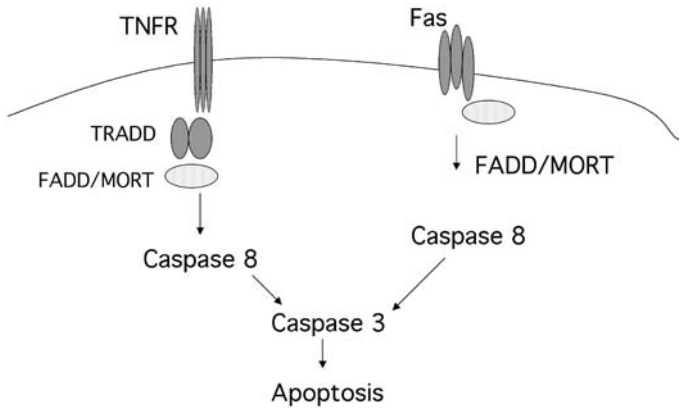

Fig. 2. Receptor-mediated mechanisms for initiation of apoptosis. Examples of two well-characterized apoptotic pathways, the TNF receptor (TNFR) and Fas receptor pathways, are shown. FADD/MORT, Fas-associated death domain/MORT protein; TRADD, TNF-R-associated death domain protein.

(TNF-R1), TNF-R2, DR3, and DR4/APO-2/TRAIL-R. These receptor pathways are illustrated in **Fig. 2.** These receptors are members of the TNF-R superfamily and are characterized by the presence of immunoglobulin-like extracellular domains rich in cysteine residues (reviewed in refs. *16* and *17*.). These proteins share 25–30% amino acid identity. Other surface signaling proteins of the TNF family of receptors include lymphotoxin-α and-β, CD30, CD40, CD27, and TNF-related apoptosis ligand (TRAIL). The intracellular domains of this family of proteins, on the other hand, have only weak homologies and bind to specific adaptor molecules that transduce the death signal. The respective intracellular domains have been termed the death domain, and they mediate protein-protein interaction. Each receptor acts through specific adaptor molecules that are responsible for initiating apoptosis. The variety of different adaptors permits the formation of unique combinations of protein complexes that can mediate specific regulation of apoptosis from many surface receptors.

Since apoptosis is regulated by specific cell surface receptors, probably crosstalk occurs between apoptotic receptors and other cell surface receptors. For example, Fas has been shown to affect intracellular calcium release in Jurkat T-cells in response to stimulation through the T-cell receptor *(18)*. Furthermore, our own experiments demonstrate that Fas signaling also affects transcription regulation, specifically of the

NF-κB transcription factor that is important in the regulation of anti-apoptotic genes. Thus, surface receptor initiation of apoptosis probably blocks multiple levels of intracellular pathways that normally exist to prevent apoptosis.

Fas/CD95/Apo-1

Apoptosis has been studied extensively in the immune system, where maintaining regulation of the total number of immune cells is critical for normal homeostasis. One receptor-regulated pathway needed in the homeostasis of T-cells is that controlled by the Fas/Apo-1/CD95 apoptotic pathway *(19,20)*. Defects or abnormalities in the regulation of this pathway in the immune system can have profound consequences. A defect in the Fas pathway can cause extensive lymphoproliferation and leads to disease *(21)*. Mutations in the Fas gene have been observed in both mouse and human. Murine mutations in the Fas ligand or receptor, the *gld* or *lpr* mutations, respectively, cause lymphoproliferation and autoimmunity. These mutants have been models for the study of systemic lupus erythematosus and autoimmunity. In humans, a recently described syndrome, Canale-Smith or autoimmune lymphoproliferative syndrome, involves genetic defects in Fas *(22,23)*. In the immune system, Fas is primarily needed for T-cell regulation.

Fas, also known as Apo-1/CD95, is a receptor belonging to the TNF family of receptor. The TNF receptor family members act by forming homotrimeric complexes with appropriate ligands to activate transmembrane signaling. Fas binds Fas ligand (FasL), a type 1 membrane protein, which exists as a trimerized complex that induces oligomerization of Fas upon binding. During the immune response, Fas is important in peripheral deletion of activated T-cells after resolution of an immune response. Additionally, Fas is involved in killing virally infected T-cells or cancer cells. Fas has a globular extracellular domain, a transmembrane domain, and an intracellular domain. The intracellular region contains protein motifs that are important for protein-protein interaction. When trimerized by FasL, Fas complex associates in a manner such that the cytoplasmic domains, which mediate protein-protein interaction, can recruit the association of other proteins. These protein interaction domains share similar structures, and some have an affinity for other members of this family. As noted previously, these domains are designated death domains.

The structure of the death domain is characterized by six helical domains that can mediate self-association or association with other proteins with death domains. The Fas death domain recruits adaptor proteins with a death domain known as FADD (Fas-associated death domain/MORT1) *(24)*. FADD itself has an additional domain that interacts with caspase 8/FLICE. The FADD domain that mediates interaction with FLICE is called the death effector domain (DED). Appropriate interaction with DED allows the subsequent activation of caspase 8. The DED has a domain that interacts with caspases. The caspases are the effector enzymes that execute programmed cell death.

When caspase 8 is activated, a cascade of proteolytic activity is initiated that triggers the activation of additional caspases, which are all interleukin-1-(IL-1) converting enzyme-like proteases. These proteases can cleave substrates such as poly-ADP ribose polymerase (PARP), lamin, actin, and others to transform the normal morphology to that a shrinking pyknotic cell (nuclear condensation). The caspases identified thus far are listed in **Table 1.**

Although activation of caspases is important in contributing to the signal to activate apoptosis, there are traditional signaling pathways that are important in activating apoptosis. The use of multiple signaling pathways may be valuable in controlling self-destruction in a regulated manner so that intracellular contents do not disrupt the neighboring cells with proinflammatory contents. One may imagine that this precision is essential in developing organs, such as the brain, heart, and others, or fine structures such as the digits of the hand. Fas has been found to signal through two pathways via death-domain proteins, the c-jun N-terminal kinase (JNK) pathway, mediated by death-associated factor (DAXX), and the caspase pathway, mediated by FADD *(25)*. DAXX was first isolated as a protein that bound to the death domain of Fas, and it was subsequently noted that DAXX activates the intracellular mitogen-activated protein (MAP) kinase signaling pathway, the JNK pathway. The JNK pathway regulates the transcription factor c-jun and upregulate new genes that play a role in initiating apoptosis. Thus there are regulatory mechanisms of apoptosis that can be initiated by the expression of new genes.

TNF Receptor

TNF receptors are ubiquitously expressed, and their activation can lead to diverse cellular responses *(16)*. There are two TNF receptors,

Table 1
Caspase Family Members

Caspase	*Nomenclature*
Caspase 1	ICE
Caspase 2	Nedd2, ICH-1
Caspase 3	CPP32, Yama, apopain
Caspase 4	Ice II, TX, ICH-2
Caspase 5	Ice III, TY
Caspase 6	Mch2
Caspase 7	Mch3, ICE-LAP3, CMH-1
Caspase 8	MACH, FLICE, Mch5
Caspase 9	ICE-LAP6, Mch6
Caspase 10	Mch4

TNF-R1 and TNF-R2, which both bind to unique ligands. Signaling by TNF-R1 leads to activation of proinflammatory pathways and (in certain cells) to apoptosis. For TNF to initiate apoptosis, protein synthesis must be blocked. The role of TNF in mediating apotosis is probably subjected to specific regulation by other signals, and the outcome of TNF receptor activation remains complex. The cytoplasmic domain of TNF-R1 has a death domain that interacts with an adaptor molecule, TNF-R-associated death domain protein (TRADD), which is necessary for apoptosis. TRADD can interact with FADD through death effector domains to activate caspase 8. It is this series of protein-protein interactions that initiates apoptosis. Additionally, TRADD binds to TRAF2, a protein that activates transcription signaling cascades for activation of NF-κB and c-Jun. The interplay of TNF-R and other receptors determines whether the response of a cell is to proliferate or undergo apoptosis.

DR3/DR4/DR5/DR6

At present, it is known that several other members of the TNF receptor superfamily play a role in apoptosis *(26)*. These members are best known for their ability to mediate apoptosis of tumor cells. Unlike Fas, the expression of these death receptor proteins is not restricted to the immune system but is expressed on cells of different tissues. At the present time, six members of this family are known. Common to this family of proteins is the presence of a death domain located in the cytoplasmic portion of the receptor. DR3 (Apo3, LARD, Ws1, TRAMP) is

a protein with similarity to TNF-R1 that can bind TRADD and plays a role in apoptosis. DR3 has cytoplasmic death domains for effector function and can also activate NF-κB. The ligand for DR3 is unidentified at the present time. DR4 is a death receptor in the TNF-R superfamily that binds TRAIL. DR5 shares structural similarities to DR4 and also binds TRAIL. Both DR4 and DR5 utilize similar signaling molecules, FADD, TRADD, and receptor interacting protein (RIP) for apoptosis. DR6 is a recently identified death receptor that mediates apoptosis when overexpressed in HeLa cells. It has been shown to be expressed on T-cells, and when DR6 is absent in knockout mice, these animals develop excessive proliferation of T-cells. The ligand for DR6 is unknown at this time. The mechanism by which DR6 functions (possibly through the FADD/TRADD pathway) remains unknown. In summary, these death receptors have multiple functions and play a role in regulating proliferation and differentiation in addition to apoptosis.

p53

The p53 gene product plays an important role in regulating apoptosis *(27)*. Cellular injury from UV irradiation, ionizing radiation, and excessive oxidative stress activates the p53 mechanism of apoptosis *(27)*. Since p53 resides in the nucleus, it can be viewed as an intracellular sensor of cellular injury. p53 is found in all mammalian cells and also plays an important role in cell cycle progression. From studies using DNA tumor viruses, p53 was identified as a target of viral transforming oncogenes such as the SV40 T-antigen or the adenovirus E1B protein. The interaction of p53 with viral proteins subsequently revealed that the p53 gene functions as a tumor suppressor gene. Thus, mutations of p53 that disrupt its function of repressing cell proliferation can contribute to malignancy. Activation of normal p53 induces cells to arrest in the G1 phase of the cell cycle. The role of p53 is to induce the transcriptional upregulation of p21, an inhibitor of cyclin-dependent kinase inhibitor that plays a role in cell cycle progression and halts further cell division. Also, p53 acts to antagonize the role of Bcl-2 and activates Bax gene expression at the transcriptional level. The regulation of the Bcl-2 family of proteins by p53 is important in inducing apoptosis. Bax activation leads to its translocation from the cytosol to the mitochondrial membrane and the release of cytochrome C. Once cytochrome C is released, caspase activation follows, with the cell programmed to undergo apoptosis.

Protease-Calpains

Calpains are a group of calcium-dependent cysteine proteases that exist in the cytoplasm *(29)*. Other cofactors that regulate calpains include phospholipids. There are two major calpains, I and II. Calpain I is sensitive to low levels of calcium and is active at 3–50 μM, whereas calpain II requires more than 200 μM of free calcium. Calpain activation is mediated by autolytic proteolysis, for example during situations of disruption of membrane integrity. There is rapid N-terminal proteolysis of calpain, which then acts on additional cellular substrates such as the cytoskeleton. The activities of the calpains are highly specific, and targets include protein kinase C, calcium/calmodulin-dependent protein kinase II (CaM-KII), microtubule-associated protein 2, tubulin, spectrin, calcineurin, and eukaryotic elongation factors 4E and 4G. The proteolysis of target substrates often leads to altered function rather than complete inactivation or degradation of the protein. Calpains play a role in cytoskeletal remodeling during migration and wound healing *(30)*. The limited role of calpains in apoptosis suggests that additional functions of other mediators of apoptosis are necessary.

Protease-Caspases

Caspases are a family of cysteine proteases that cleave target motifs after an aspartic residue. This class of enzyme is the executioner of apoptosis (reviewed in *31* and *32*). Caspases were identified as important in apoptosis by the discovery of CED-3, which was required for cell death in the nematode worm *C. elegans*. It has since been found that there are related proteins with similar functions in different organisms across evolution. This family of enzymes can be identified based on the similarity in the amino acid sequence, structure, and substrate specificity (**Table 1**). There are currently 13 members of the caspase family in mamalian cells. They are all expressed as proenzymes, 30–50 kDa in size, and they require enzymatic cleavage at two sites for activation (**Fig. 3**). Caspases contain three domains; upon activation, cleavage takes place between the first two domains. Within the amino acid sequence of caspases, there exist two target sequence sites for caspases themselves, and thus autoactivation is a mechanism by which the full activity of caspases is recruited during apoptosis. The activated subunits then associate to form the activated enzyme. The N-terminal subunit is the most variable in length and sequence and is involved in the regulation of activation.

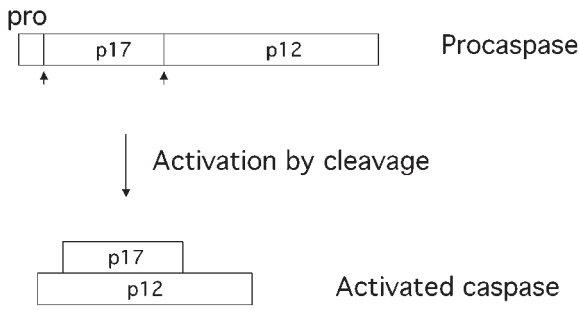

Fig. 3. Caspase structure. The general linear organization of caspase domains is shown. Proteolytic processing at specific sites transforms the inactive protein to active caspase.

Activation of the caspase cascade is dependent on an appropriate signal from the cell surface or from intracellular regulators in the mitochondria, such as apoptosis protease-activating factor (Apaf-1)/cytochrome C. However not all caspases are involved in apoptosis. Caspase 1 and IL-1-converting enzyme (ICE) are involved in the regulation of inflammation. These proteases do not mediate apoptosis. Caspases 3, 7, 8, and 9 are specifically required for apoptosis.

Caspase 9 is one of the earliest effector caspases activated in response to altered mitochondrial membrane permeability disruption (*32*). The altered mitochondria permeability releases cytochrome C, which binds Apaf-1, and, in the presence of ATP, associates with caspase 9 through specific domains and converts procaspase to the active form, which then activates caspase 3 to initiate a cascade of caspase activation.

In response to cell surface signals that activate apoptosis, adaptor proteins for cell surface receptors located in the cytoplasm are able to activate caspase 8. Adaptor proteins such as FADD have caspase recruitment domains (CARDs) that bind caspase 8. The activation of caspase 8 subsequently leads to the activation of the caspase cascade and apoptosis.

Caspases lead to apoptosis presumably by degrading essential proteins and proteins that normally function to inhibit apoptosis. Caspases directly disassemble cell structures such as nuclear lamins and other cytoskeletal proteins. Apoptosis leads to fragmentation of DNA, chromatin condensation, and membrane and other changes. The role of cas-

pases is to cleave proteins in a targeted manner, leading to the disintegration of structural and regulatory proteins. Apoptosis then proceeds by shrinkage of the cell with precise control of cellular content and debris rather than spilling cellular contents. The targeted cleavage of proteins plays an important role in this concerted and well-orchestrated process since mutations of critical caspases lead to defects in apoptosis. Some of the best characterized targets of caspases are PARP, lamins, and U1 ribonuclear proteins. Others include proteins that regulate cell cycle progression and proliferation. Recognition of caspase target sites requires four amino acids N-terminal to the cleavage site. In fact, caspases are one of the most specific proteases. Because of their high specificity, caspases can be inhibited by the use of tetrapeptides that fit the active site of the enzymes.

MITOCHONDRIAL PERMEABILITY AND APOPTOSIS

Mitochondria proteins are intimately involved in apoptosis, and these components, when activated can lead to apoptosis independently of other apoptotic proteins such as caspases *(33)*. Therefore the mitochondria is not only necessary for ATP energy production through the electron transport chain but is also important for choreographing cell death. The several mechanisms by which the mitochondria is important for apoptosis are also probably interdependent (**Fig. 4**). One mechanism is disruption of the electron transport pathway. Several inducers of apoptosis affect electron transport, such as γ-irradiation, ceramide signaling, and receptor-mediated apoptosis via Fas. Apoptosis can be initiated by the mitochondria when a disruption of the mitochondrial membrane integrity inhibits electron transport and the generation of ATP. The second mechanism is release of cytosolic cytochrome C, caused by membrane disruption. When released into the cytosol, cytochrome C, a potent pro-apoptotic component that activates caspases, forms a complex with Apaf-1 and procaspase 9 leading to the activation of caspase 9, which subsequently directs the enzymatic execution of the cell. Caspases play an important part in regulating apoptosis and there is a large family of caspase members that are necessary for apoptosis.

Normally, cytochrome C is held within the mitochondria by an intact membrane, which only permits the movement of molecules less than 1000 Daltons in size. Cytochrome C is 10 kDa and cannot move freely. In situations of severe oxidative stress, another mechanism that can

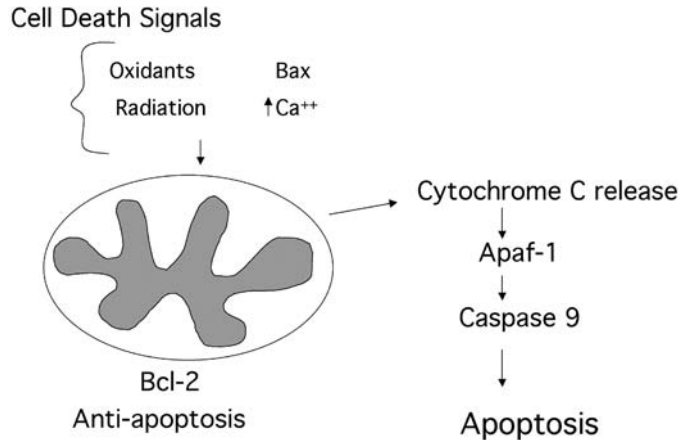

Fig. 4. Mitochondrial components involved in apoptosis, Apaf-1, showing the roles of different stresses that activate mitochondrial apoptotic pathways.

lead to apoptosis, the generation of superoxide anions has toxic effects and can lead to apoptosis. Direct injury or a lack of energy can also lead to mitochondrial swelling, rupture of the outer mitochondrial membrane, and release of proteins from the intracellular space of the mitochondria. This collapse also disrupts the electron transport chain and therefore leads to loss of ATP. In these situations, release of cytochrome C is not mediated by a controlled process, which is also the case in necrosis.

Bcl-2 and Mitochondria

Bcl-2 and cytoplasmic proteins with shared structural homology play an important role in regulating the functions of mitochondrial proteins during apoptosis *(27)*. The release of cytochrome C in a regulated manner is seen during apoptosis and is the end result of activated biochemical cascade. The release of cytochrome C is controlled by the Bcl-2/Bax family of gene products. The Bcl proteins were first identified as targets of rearranged genes in B-cell lymphoma. The Bcl-2 gene was identified as an oncogene that could confer immortalization to primary rodent cells upon overexpression. Extensive studies have now revealed that these proteins play an important anti-apoptotic role (reviewed in ref. *34*). The large Bcl-2 family has more than than 20 members, defined by the presence of structurally related Bcl-2 homol-

ogy domains (BH1–BH4) that mediate protein-protein association to regulate diverse responses to death-inducing stimuli (**Table 2**). There are proapoptotic members of this family, such as Bad and Bax, and anti-apoptotic members, such as Bcl-2 and Bcl-Xl. The *C. elegans* homolog of Bcl-2 is CED-9. The Bcl-2 family is activated in response to cellular stresses, deprivation of growth factors, steroids, UV irradiation, and receptor-mediated signaling. The exact mechanism by which Bax/Bak functions is unclear, but interaction with members of the Bcl-2 family by heterodimerization is an important function in apoptosis. Thus, by binding to the Bcl-2 family of proteins, the balance is weighted toward the activation of apoptosis.

Overexpression of Bax leads to the formation of Bax homodimers, which induces apoptosis through the activation of ICE and ICE-independent pathways. A recent finding using animals that lack Bax or Bak is that these provide redundant functions. Both genes must be eliminated to prevent apoptosis *(35)*. Another interesting finding from these studies is that a lack of Bax and Bak prevents apoptosis initiated by other pathways, such as Fas, UV light, and the caspase pathway *(36)*. This was a revelation in that it was thought that apoptosis can be initiated by several pathways. Bax and Bak promote the release of cytochrome C from the mitochondria, but the mechanism remains unclear. It is likely that complex proteins regulate how Bax and Bad regulate cytochrome C release.

The Bcl-2 family members function by forming interactions among partner proteins as homodimers or as heterodimers with Bax *(34)*. The domains that mediate protein-protein interaction are the BH1–BH4, which are α-helical structural motifs that are dependent on the formation of a hydrophobic pocket. Bcl-2 proteins are situated in the outer mitochondrial membrane, anchored by their carboxy-terminal hydrophobic domains. Their location is important in preventing apoptosis. The pro-apoptotic members (e.g., Bad) lack a membrane-anchoring domain and thus when Bad dimerizes with Bcl-2, the mitochondrial membrane loses Bcl-2 proteins. Other Bcl family members such as CED-9, from *C. elegans*, inhibit apoptosis by binding CED-3 caspases; however this has not been observed in mammalian Bcl proteins.

Another level of regulation of the Bcl-2 family is through phosphorylation on serine residues *(34)*. Phosphorylation of Bcl-2 at serine leads to inactivation and destabilizes the ability of the protein to anchor

Table 2
Bcl-2 Family of Apoptosis Regulators

Anti-apoptotic	Pro-apoptotic
Bcl-2	Bax
Bcl-X_L	Bak
Bcl-W	BOK/MTD
Mcl-1	Bcl-XS
A1/BFL-1	BID
BOO/DIVA	BAD
NR-13	BIK/NBK
CED-9	BLK
	HRK
	BIM/BOD
	NIP3
	NIX/BNIP3

in the mitochondrial membrane *(39)*. The altered localization thus predisposes the mitochondria to release cytochrome C. In a similar regulatory manner, phosphorylation of pro-apoptotic members such as Bad inhibits apoptosis. When Bad is phosphorylated at serine residues, it is unable to bind to Bcl-2 family members *(38)*. Bad is phosphorylated by Akt kinase in response to signaling from receptor growth factors such as epidermal growth factor or insulin-like-growth factor. Phosphorylated Bad associates with 14-3-3 protein, which resides in the cytosol and fails to bind Bcl-2.

Also Bcl-2 proteins link surface apoptotic signaling to mitochondrial release of cytochrome C. Fas or TNF signaling leads to the activation of caspase 8, which cleaves Bid. The terminal fragment of Bid binds the mitochondria and leads to the release of cytochrome C.

CURRENT QUESTIONS

Given that apoptosis is delicately regulated by a balance of activators such as Bax and inhibitors of cell death such as Bcl-2, which are being synthesize continuously, the decision of whether a cell dies or lives is constantly being reevaluated; disruption of that balance leads to apoptosis. Surface receptors that activate diverse signaling cascades can tip this balance, or traumatic insults that disrupt intracellular structural integrity are activated to initiate apoptosis. This raises the hope that intervention re-establishing the balance between pro-apoptotic and

anti-apoptotic pathways can lead to inhibition of apoptosis. Thwarting apoptosis, even temporarily while maintaining normal cellular function, may lessen the overall tissue damage. It has been demonstrated that inhibiting the activity of caspases can retard the progression of cell death. By understanding the pathway for apoptosis, specifically the mechanistic steps, we can identify targets for inhibition that may have therapeutic potential.

During injury, some damaged cells are fragmented irreversibly from physical trauma, similar to the damage from sonication, and certain cells are destined to die by being activated to undergo apoptosis. Inhibiting the progression of events in apoptosis caused by injury will be beneficial for cell survival and may restore enough cellular function so that adjacent viable cells are not disrupted in their function. Inhibiting apoptosis should therefore reduce inflammation. A reduction in total injury can only promote a more rapid recovery.

There are many pathways that lead to apoptosis. Although these pathways have different points of initiation, there are common factors that mediate the execution of cell death. The Bcl-2 pathway and caspases are terminal mediators of apoptosis, and they may serve as ideal targets of early inhibition to allow the balance of molecules that regulate apoptosis to reach a state that prevents cell death programs, rather than initiate and propagate apoptosis. The identification of both positive and negative networks that regulate apoptosis suggests that controlling this balance is essential and may be useful in limiting injuries that develop from apoptosis. This newfound understanding of cell death permits novel approaches to the treatment of acute injury. Areas that need further research include identification of essential regulatory steps in apoptosis that may be important in traumatic injuries.

Modulating the function of cell death proteins is attractive for reducing injury. Blocking apoptosis has implications beyond traumatic injury and benefits injury from diseases. The interest in inhibitors of apoptosis has led to development of pharmacologic targets to disrupt proteins in the cell death pathway. One area with significant progress is the use of agents that affect malignancies. Cancer cells often lack appropriate control of cell death pathways and are associated with abnormal expression of the Bcl-2 family of proteins. One focus is to disrupt the function of Bcl-2 to sensitize cells toward apoptosis in order to treat malignancies through the use of antisense Bcl-2. Preclinical studies in mice have shown that antisense Bcl-2

decreases the level of Bcl-2 and sensitizes transplanted tumors to chemotherapy (*39*).

The most obvious molecular targets of apoptosis inhibition are caspase inhibitors. At present, none of them has reached clinical trials. However, promising research points to the potential of caspase inhibitors to regulate apoptosis. In many models of ischemia reperfusion injury, such as liver, cardiac, renal, and cerebral injuries, administration of caspase inhibitors using tetrapeptides has demonstrated the effective decrease of apoptosis with a marked improvement of organ survival from injury. For example, the use of zVAD (benzyloxycarbomyl-val-ala-asp), a peptide caspase inhibitor, during reperfusion after ischemia has led to decreased renal apoptosis, inflammation, and tissue injury *(40)*.

FUTURE DIRECTIONS

An understanding of the specific mechanisms of apoptosis is essential in developing approaches to regulate programmed cell death. Preliminary studies using peptide inhibitors of apoptosis to specifically target enzymes that play a role in apoptosis have shown remarkable outcomes in animal models *(39)*. Further understanding of the pathways of apoptosis and the use of inhibitors may define targets better, for control of apoptosis. The effectiveness of these inhibitors will depend not only on their molecular specificity but also on their effectiveness at blocking the initiation of apoptosis at the earliest stages. In addition, problems of cellular penetrability must also be addressed. Combining the use of novel apoptosis inhibitors with state of the art tissue reperfusion technology would be beneficial in acute injuries from trauma, stroke, myocardial infarction, and inflammatory diseases.

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2 Nitric Oxide

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INTRODUCTION

Nitric oxide (NO) is involved in numerous physiologic functions ranging from regulation of cardiovascular functions to participating in memory *(1–4)*. In the immune system, this diatomic radical is involved in host defense and has tumoricidal functions *(5,6)* However, despite these properties, which are critical in maintaining homeostasis, NO has been implicated as a participant or causative agent in a variety of pathophysiologic conditions *(7,8)*. Defining the exact role of NO under pathophysiologic conditions is further complicated by the fact that it has been shown to be both protective as well as deleterious even in the context of the same biologic setting. Therefore, the search for mechanistic explanations to account for these differing effects is ongoing.

Unlike other biologic mediators, the main determinants of the biologic effects of NO are its chemical and physical properties. In addition to the numerous potential reactions of NO in biologic systems, many of its effects can be attributed to additional reactive nitrogen oxide species (RNOS) that are formed and that have their own selective reactivity. Although a large variety of chemical reactions relate to NO in biologic systems, it is difficult to determine which ones are pertinent. In an attempt to sort out the diverse chemical reactions and their importance, we have developed the concept of the "chemical biology of nitric oxide" *(9–11)*. The chemical biology of NO sepa-

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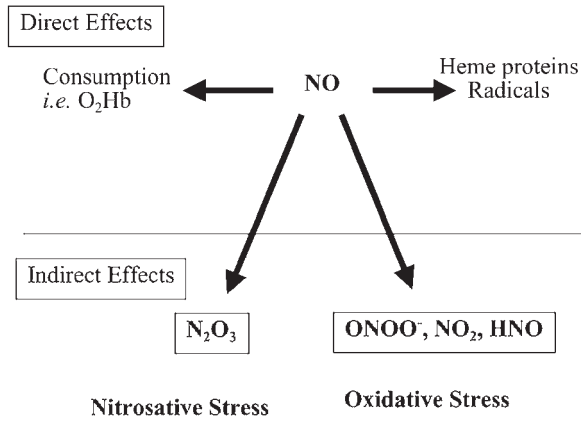

Fig. 1. The chemical biology of nitric oxide (NO).

rates the chemical reactions into two basic categories, direct and indirect effects (**Fig. 1**).

Direct chemical reactions are those in which NO directly interacts with biologic targets. The most common chemical reactions are those between NO and heme-containing proteins. These reactions are generally rapid, require low concentrations of NO, and are the genesis of most of the physiologic effects of NO. Conversely, indirect effects do not involve the direct reaction of NO with biologic targets but rather rely on the formation of other RNOS formed from the reactions of NO with oxygen or superoxide. These chemical species can then react with cellular targets, which may lead to the modification of critical macromolecules. It appears that indirect effects require much higher concentrations of NO than direct effects. This suggests that NO produced at low concentrations for short periods primarily mediates direct effects, whereas higher local NO concentrations sustained over prolonged periods mediate indirect reactions.

Indirect reactions can be further broken down into two subgroups: nitrosative and oxidative *(9)*. A variety of chemical reactions result in nitrosative or oxidative chemistry depending on the species involved. The chemistry of nitrosation in vivo appears to be mediated primarily by N_2O_3, whereas oxidative chemistry is mediated by $ONOO^-$ as well as HNO/NO^- *(12)*. The chemistry of these reactive intermediates shows that they interact to produce either nitrosative or oxidative stress. Furthermore, several studies suggest that nitrosative stress is orthogonal to

oxidative stress with respect to the RNOS chemistry, implying that nitrosative and oxidative stress are produced by different mechanisms *(9,13)*. It also appears that biologic effects such as cytotoxicity are different under nitrosative and oxidative stress *(9)*. A balance exists between these two types of stresses, and therefore the functional outcome, such as cell death and signal transduction, may differ depending on which one predominates.

This separation of chemical reactions into direct and indirect effects is analogous to the function of the different isozymes of nitric oxide synthase (NOS) *(14,15)*. NOS can be found constitutively in a variety of cells such as endothelial and neuronal cells. Constitutively expressed NOS (ecNOS) is thought to generate low amounts of NO at concentrations in the submicromolar range for short durations. On the other hand, inducible NOS (iNOS) generates NO for prolonged periods and in some cases at local concentrations as high as 1–5 μM. Since, in general, the chemistry and the biologic outcome can be a function of NO concentration, it will be dictated ultimately by the type of isozyme present. An important consideration is the proximity of a biologic target to the NO source. Targets close to a source such as macrophages producing high levels of NO will be subjected to both direct and indirect reactions, whereas cells farther away will experience mostly direct effects as the primary mode of NO action. We explore here the different chemical reactions in the chemical biology of NO and discuss them in the context of oxidative and nitrosative stress.

DIRECT EFFECTS

Most direct reactions of NO with biologically relevant substrates are not rapid enough to play a significant role in vivo. Hence at low concentrations of NO, there are a few reactions to be considered. Most of these reactions involve metals or other free radicals. The direct reaction of NO with thiols is far too slow to occur in biologic systems. The major metal-mediated reactions involve either the formation of metal nitrosyls, oxidation by dioxygen complexes, or metal-oxo complexes. Iron is the primary metal involved, in particular, reaction sites containing heme. Heme-containing proteins react with NO the fastest of the bio-organic complexes and should be the first to be implicated in any mechanism involving NO. In addition to metals, organic radicals can react with NO at diffusion-controlled rates. The production of lipid rad-

icals and carbon-centered radicals formed during exposure to ionizing radiation can be important under specific conditions.

The basic regulatory NO reactions involve enzymes containing metal heme complexes such as guanylate cyclase, cytochrome P450, NOS, and hemoglobin. These reactions are facile enough that the NO source can be at greater distances from the target protein. On the other hand, some enzymes such as aconitase require higher NO concentrations and therefore must be in close proximity to the NO source. Under conditions of oxidative stress, NO can react rapidly with redox active metals and can catalyze the formation of oxidants such as hydroxyl radical. In the case of lipoxygenase and cyclo-oxygenase, NO can react with lipid radicals that are formed at low concentrations, whereas at higher concentrations they form metal nitrosyl complex, which inhibits the enzymes. Therefore the inhibitory effects of NO on arachadonic acid metabolism can occur at low or high concentrations of NO. As in the reactions of aconitase and those in the respiratory chain of the mitochondria, indirect as well as direct effects can be involved. We discuss below some of the relevant reactions and their relevance at different concentrations of NO.

Reactions Between NO and Metal Complexes

The three major types of reactions between NO and biologic metals are the direct reaction of NO with metal centers (to form a metal nitrosyl complexes) and NO redox reactions with dioxygen complexes or high valent oxo-complexes (**Fig. 2**). These reactions are extremely rapid at near diffusion-limited rates, making them relevant under almost any physiologic or pathophysiologic condition.

NO may react with a variety of metal complexes to form metal nitrosyls. The vast majority of the reactions in vivo are with iron-containing proteins. Most copper complexes will react to form the nitrosyl but are too slow to be of major importance in the biology of NO. Cobalamin has been shown to react with NO, resulting in nitrosation catalysis *(16)*. Other biologically important transition metals such as zinc do not react with NO under biologic conditions.

The most notable protein to form metal nitrosyl complexes in vivo is guanylate cyclase. The formation of heme nitrosyl adduct in guanylate cyclase causes the removal of the distal histidine, resulting in a 5-coordinate nitrosyl complex, which activates the enzyme *(17,18)*. This alteration in protein configuration leads to the conversion of guanosinetriphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in

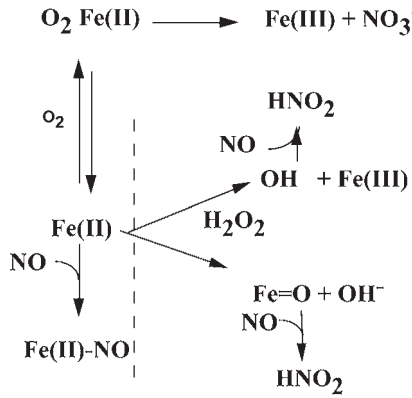

Fig. 2. Important reactions of nitric oxide (NO) with iron complexes.

another domain of the enzyme. The formation of cGMP has many ramifications in a number of tissues, in particular vascular smooth muscle, in which NO mediates the vasodilation *(19)*. The concentration of NO required to activate guanylate cyclase is relatively low [medium effective concentration (EC_{50}) 100 n*M*] *(20)*. The influence of NO on soluble guanylate cyclase has profound effects on vascular tone, platelet function, neurotransmission, and a variety of other intercellular interactions. Recent reports suggest that NO can prevent some apoptotic processes by activating guanylate cyclase.

In contrast to the activation of enzymes such as guanylate cyclase, NO can have an inhibitory effect as well; cytochrome P450 as well as other heme mono-oxygenases are inhibited (**Fig. 2**) *(21–23)*. The inhibition of cytochrome P450 has important pathophysiologic sequelae. During chronic infection or septic shock, NO can be produced in copious amounts. Inhibition of liver cytochrome P450s *(21,23)* inhibits drug metabolism *(22)*. Furthermore, the chronic exposure of NO to the heme domain of cytochrome P450 can result in release of free heme and the activation of heme oxygenase in hepatocytes *(24)*. The activation of hemeoxygenase may serve as a protective mechanism against a variety of pathophysiologic conditions *(25,26)*. The interaction of NO with cytochrome P450 can thus have a regulatory function as well as a positive or negative influence on pathophysiology.

Another important outcome of heme nitrosyl formation is in the regulation of NOS activity. NOS is a cytochrome P450-like enzyme that at

the heme domain catalyzes the oxidation of arginine to form citrulline and NO, similar to substrate oxidation by cytochrome P450 *(14)*. However, it has been shown that NO will inhibit the oxidation of arginine, suggesting that the amount of NO produced from the enzyme is actually controlled by a negative feedback mechanism analogous to that of cytochrome P450 (**Fig. 2**) *(27–30)*. Comparison of the different isozymes of NOS shows that ecNOS and neuronal (n)NOS are more susceptible to inhibition by NO than iNOS *(30)*. This helps explain why significantly higher NO fluxes can be achieved with iNOS than with either ecNOS or nNOS. The difference in NO-mediated inhibition of NOS activity is apparently owing to the relative reactivity of NO and the stability of the resultant Fe-NO complex within NOS. The stable Fe-NO complex restricts the potential concentration of NO that can be produced by nNOS, and probably ecNOS. Even under conditions of hyper-intracellular calcium concentrations, this feedback mechanism will protect against the production of significant amounts of RNOS. Therefore, the predominant source of RNOS (indirect effects) in vivo may be from iNOS.

The competitive inhibition of NOS activity through a heme nitrosyl complex can serve to regulate tissue blood flow. Oxidation of arginine under catalytic conditions results in the formation of a Fe-NO complex in nNOS *(28,29)*. This Fe-NO/nNOS complex competitively inhibits the binding of oxygen to the active site and prevents the oxidation of arginine. This binding of NO to nNOS increases the K_m for oxygen such that there exists a linear relationship in the range of physiologic oxygen concentrations. This suggests that NOS may serve as an oxygen sensor as well as attenuating oxygen supply to tissue *(31)*. In recent studies, it was shown that NO produced from NOS in the lung alveoli responded to different concentrations of oxygen *(32)*. This could regulate the blood flow in the lung depending on the oxygen tension. Since NO and oxygen compete for the heme binding site, the relative stability of the nitrosyl versus the dioxygen adduct determines the level of NO produced. It is thought that this mechanism may play a crucial role in regulation of blood flow through different tissues.

The iron metal nitrosyl complexes discussed above have involved heme-containing proteins. Other metals can also form nitrosyl complexes, such as the reaction of NO with cobaltamine, a cofactor in methionine synthase. The oxidized aquo form of cobaltamine reacts with NO to form

a nitrosyl, whereas the cyano and methyl adenosyl forms do not. The interaction of NO with cobaltamine reduces its ability to serve as a cofactor in methionine synthase. Studies show that the addition of cobaltamine prevents the loss of mean arterial blood pressure induced by lipopolysaccharide (LPS), suggesting that it could be an effective NO scavenger in vivo. In cell culture experiments, cobaltamine was shown to scavenge NO, thus preventing NO's inhibitor effects on cell proliferation. Nitrosyl cobaltamine can nitrosate thiols of protein and glutathione, providing a means for NO to form S-nitrosothiols. Taken together, this indicated that cobaltamine could modulate NO metabolism.

BIOLOGICAL RELEVANCE

NO Interaction with Metal-Oxygen and Oxo Complexes

The reactivity of NO with metals is not limited to covalent interactions with metal ions alone. Various metal-oxygen complexes and metallo-oxo complexes react rapidly with NO (**Fig. 2**). As with activation of guanylate cyclase, the reaction between NO and oxyhemoglobin is an equally important determinant of NO behavior in vivo. The reaction between NO and oxyhemoglobin results in met-hemoglobin and nitrate *(33,34)*:

$$\text{Hb (Fe} - \text{O}_2) + \text{NO} \rightarrow \text{met Hb [Fe(III)]} + \text{NO}_3^- \qquad (1)$$

This reaction provides the primary endogenous mechanism to eliminate NO as well as control the movement and concentration of NO in vivo *(35)*. In addition, it exerts important control on the mechanisms of indirect effects.

Another important reaction of NO with metal oxygen adducts is the rapid reaction between NO and metalloxo and peroxo species (discussed in ref. *9*). Agents such as hydrogen peroxide form highly reactive metal complexes through their oxidation. In the absence of NO they can lead to cellular damage such as lipid peroxidation *(36)*. When NO is present, it reacts rapidly with these complexes to abate oxidative chemistry mediated by metallo-oxo species *(37–39)*:

$$\text{Fe} + \text{H}_2\text{O}_2 \rightarrow \text{Fe} = \text{O} + \text{H}_2\text{O} \qquad (2)$$

NO results in the reduction of the hypervalent metal complex to a less oxidizing normal valent state:

$$\text{Fe} = \text{O} + \text{NO} \rightarrow \text{Fe} + \text{NO}_2^- \qquad (3)$$

These antioxidant properties of NO may be a primary mechanism by which this diatomic radical protects tissue from peroxide-mediated damage *(39)*.

Another important reaction is the NO interaction with catalase. Kim et al. *(40)* demonstrated that cytokine-stimulated hepatocytes reduced catalase activity owing to the production of NO. Similar inhibition of hydrogen peroxide consumption by catalase was observed using NO donors *(41)*. Farias-Eisner et al. *(42)* suggested that the NO inhibition of catalase could play a role in the tumoricidal activity of macrophages.

NO can inhibit catalase by two different mechanisms: (1) metal nitrosyl formation and (2) NO reacting with metalloxo species. Hoshino et al. *(43)* showed that NO could bind to the heme moiety, forming a ferric nitrosyl with a rate constant of $3 \times 10^7\ M^{-1}s^{-1}$ and a K_{diss} of $1 \times 10^5\ M^{-1}$. This is analogous to the mechanism for P450 and NOS inhibition. The Fe-NO adduct prevents the binding of hydrogen peroxide to the metal ion by occupying the coordination site. It is estimated that between 10 and 15 μM NO inhibits hydrogen peroxide consumption by 80%, by means of this mechanism *(42)*. Cells that express iNOS have reduced catalase activity, suggesting that local NO concentrations near these cells may reach as high as 10 μM for prolonged periods.

There is a second mechanism by which hydrogen peroxide may attenuate NO levels. During the enzymatic mechanism of catalase, hydrogen peroxide first reacts to form complex I and water (**Fig. 2**). Complex I will react with hydrogen peroxide to form O_2. However, NO can also rapidly react with compound I to form complex II, which reacts with an additional NO. This results in the conversion of 2 mole of NO and 1 mole of hydrogen peroxide to 2 mole of HNO_2. This results in NO consumption while hydrogen peroxide depletion is retarded. Brown *(44)* has shown that NO can partially inhibit hydrogen peroxide consumption while NO is consumed by catalase/peroxide. The K_i for NO in this reaction was 0.18 μM. This finding suggests that submicromolar NO levels, such as those produced by cNOS, may be partly controlled by this mechanism.

The reaction of NO with high valent heme derived from peroxide may be a mechanism to attenuate the NO levels in vivo. Reports have shown that an increase in glutathione peroxidase activity, which does not react with NO, increases the bioavailability of NO (from ecNOS) *(45)*. This implies that hydrogen peroxide, via a mechanism similar to that shown

in **Fig. 2**, may play a crucial role in regulation of the direct effects of NO in vivo. On the other hand, Fe-NO formation would be important under conditions in which NO concentrations are higher than H_2O_2. Both mechanisms may play a role during the sequences of NO and peroxide bursts under some physiologic and pathophysiologic conditions.

Reaction of NO with Radical Species

Another direct effect of NO is its reaction with other radical species. The tyrosyl radical formed during the catalytic turnover of ribonucleotide reductase reacts with NO and inhibits the enzyme *(46–49)*. Inhibition of this enzyme has been proposed to be a factor in the cytostatic properties of NO, owing to the suppression of DNA synthesis.

NO can react with oxyradicals formed during lipid peroxidation *(50,51)*. Lipid peroxidation is an important component of cell death and in stages of inflamation *(52)*. The process of lipid peroxidation results in the formation of variety of lipid oxy and peroxy adducts that perpetuate lipid oxidation. This can result in compromise of cell membranes. The reaction of NO with these peroxy-oxy radicals results in protection against ROS by termination of lipid peroxidation *(53)*:

$$LOO^{\bullet} + NO \rightarrow LOONO \quad (4)$$

Reaction 4 has been proposed to play a role in the abatement of lipid peroxidation by NO that protects cells against peroxide-induced cytotoxicity *(39,54,55)*. Lipid peroxidation induced by oxidants and formed as result of exposure to copper, xanthine oxidase, or azo-bis-amidinopropane is terminated by NO *(51,56)*. The chain termination also prevents oxidation of low-density lipoprotein in both endothelial *(57)* and macrophage cells *(56)*. It is thought that a reduction in oxidized cholesterol reduces initiation of arterioscleroses mediated by foam cells. Other processes in inflammation such as the production of leukotrienes are effected by NO. Lipoxygenase, which mediates a variety of lipid oxidations, is inhibited by NO.

CHEMICAL TOXICOLOGY OF NO AND ROS

Although NO can act as an antioxidant in a number of reactions, there are other reactions affecting cellular processes that make tissue more susceptible to oxidative stress. Thus the formation of oxidants such as peroxynitrite, the proposed product of ROS and NO, is hypoth-

esized to lead to tissue injury. The cytotoxic effects of NO would be caused by formation of peroxynitrite and related species.

The complexity of the potential reactions within a cell has led to some differing opinions on the role of NO in oxidative stress *(58)*. The clonigenic assay can be used to answer these questions at the cellular level. It is the gold standard for cytotoxicity mediated by different chemical substances and takes into account both necrotic and apoptotic death. To sort out these effects, the toxicity of hydrogen peroxide, alkyhydroperoxide, and superoxide was examined in the presence of NO.

Hydrogen peroxide (H_2O_2) mediates oxidation of biologic molecules, which can result in tissue damage. Although NO does not react chemically with H_2O_2 *(59)*, it can protect cells against toxicity mediated by H_2O_2 *(39,41,54,55,59)*. Recent studies on the biology and chemistry of NO have made use of a class of compounds known as NONOates, which release NO in a controlled manner over specific periods *(60)*. In a recent study, lung fibroblasts exposed to increasing concentrations of H_2O_2 exhibited marked increases in cytotoxicity *(59)*. The presence of NONOates resulted in surprising protection against the cytotoxicity of H_2O_2 *(59)*. Treatment with these NO donor complexes before or after exposure to H_2O_2 did not result in protection; in fact, the byproduct of the decomposition of NO, nitrite, increased the cytotoxicity of H_2O_2. Similar observations were made in neuronal *(59)*, hepatoma *(39)*, and endothelial cells *(55,61)*. Other reports suggest that NO derived from endothelial cells is involved in the protection against damage to vascular smooth muscle mediated by H_2O_2 *(62)*.

These protective effects of NO were not restricted to NONOates. Compounds containing *S*-nitroso functional groups also protected against H_2O_2-mediated toxicity *(41)*. However, clinically used nitrovasodilators such as 3-morpholinosydnonimine (SIN-1) and sodium nitroprusside (SNP) increased the toxicity of H_2O_2 *(41,42)*. Angeli's salt ($Na_2N_2O_3$;AS), a compound similar to the NONOates but one that donates nitroxyl (NO^-) instead of NO, significantly potentiated the toxicity of H_2O_2 *(41)*. These results show that different putative NO donors can modulate the toxicity of H_2O_2 differently.

The effects of the different NO donors on cellular antioxidant defenses as well as the amount and flux of NO produced during the experiment may explain the differences exhibited by the various NO donors. One of the major cellular defenses against H_2O_2 is its consump-

tion by the enzymes glutathione (GSH) peroxidase and catalase *(63)*. When the kinetics for the disappearance of H_2O_2 were examined in the presence of the different NO donors, it was noted that several of the compounds inhibited the cellular consumption of H_2O_2 to varying degrees. In these studies, SNP, DEA/NO, AS, and SNAP all increased the amount of time required to decompose 0.75 m*M* H_2O_2 by as much as 30–200% *(41)*. In the case of SIN-1 and GSNO, the consumption of H_2O_2 was retarded by as much as 400% *(41)*. Thus, the enhancement of H_2O_2-mediated toxicity by AS and SIN-1 might be explained partially by the inhibition of H_2O_2 consumption. However, this cannot be the sole mechanism by which NO enhances or protects against H_2O_2, since GSNO, SNAP, and DEA/NO also decreased the rate of decomposition of H_2O_2.

Furthermore, different NO donors reduce intracellular levels of GSH to different degrees. Exposure of V79 cells to 1 m*M* nitrite, SNAP, SIN-1, GSNO, DEA/NO, or AS resulted in varying degrees of depletion of intracellular GSH *(64)*. Exposure to SNAP, GSNO, or DEA/NO resulted in only a modest decrease (<30%), after which the levels of GSH recovered rapidly. However, SIN-1 and AS decreased intracellular GSH levels by as much as 85%. Nitrite (1 m*M*) decreased the levels GSH in these cells by 50% after a 1-h exposure *(64)*. The increased reduction of GSH by SIN-1 and AS suggests a reason why these substances enhanced H_2O_2 toxicity.

The other main explanation for the differences in protective effects among the various chemical NO donors may reflect the actual flux of NO produced by each compound. The temporal profiles of NO release by the different compounds demonstrate that different amounts of NO are released over time *(41)*. Both the NONOates and the *S*-nitrosothiol complexes, which protect against H_2O_2 toxicity, released NO over the time-course of exposure to H_2O_2. However, SIN-1, SNP, and AS did not produce measurable NO (<1 μ*M*) under these experimental conditions, coincident with a lack of protection against H_2O_2 *(41)*.

SNP, however, appears to increase the toxicity of ROS by yet other mechanisms. Chemistry mediated by SNP can result in the formation of chemical species other than NO, such as cyanide (CN^-) and iron. Desferrioxamine (DF) completely protected cells from H_2O_2, yet DF only partially protected against the toxicity mediated by SNP combined with H_2O_2 *(41)*. This discrepancy may be accounted for by the

enhanced CN^- released from SNP. Monocytes and polymorphonuclear leukocytes have been shown to facilitate the release of CN^- from SNP, a phenomenon believed to mediated by H_2O_2. The authors suggested that a transition metal complex with a labile ligand could then further oxidize substrates via Fenton-type catalysis *(65)*. Further evidence supporting this hypothesis comes from Imlay et al. *(66)*, who showed that bacteria became more sensitive to H_2O_2 in the presence of CN^-. The fact that DF completely protected against the toxicity of CN^- suggests that metal-peroxide reactions are required to initiate cytotoxicity. Thus, the DF-insensitive enhancement by SNP of H_2O_2-mediated toxicity could be attributed to an iron complex, which cannot be bound by DF; such a complex could catalyze the Fenton oxidation chemistry of cellular molecules.

Freeman and coworkers *(51)* have investigated the effect of NO on XO-mediated lipid peroxidation and found that NO acts as an antioxidant. We have also examined the effect of NO on organic hydroperoxide-mediated toxicity, thought to be mediated by oxidation of lipophilic membranes *(54)*. Our studies further illustrate the importance of the presence of NO during the exposure to oxidants, showing that it is critical that NO be present during the exposure of the oxidant.

NO may be involved with several potential mechanisms in the protection against organic hydroperoxide-mediated toxicity. Intracellular metalloproteins such as those containing heme moieties react quickly with organic peroxides to form hypervalent complexes. These complexes can decompose and release intracellular iron, which in turn can catalyze damage to macromolecules such as DNA. Nitric oxide can react at near diffusion controlled rate constants with these hypervalent metalloproteins, which may restore these oxidized species to the ferric form *(38,67)*. The reduction of these metallo-oxo proteins prevents both their oxidative chemistry and their decomposition, which releases intracellular iron *(37–39)*, thus limiting intracellular damage mediated by oxidative stress.

Although NO can protect against the toxicity of H_2O_2 to mammalian cells, the opposite effect is observed when the target is *E. coli*. H_2O_2, delivered either as a bolus or through the enzymatic activity of XO, exhibits only modest bactericidal activity *(68)*. However, simultaneous exposure to both H_2O_2 and NO, the latter delivered either as gas or by a NONOate complex, increases bactericidal activity by 4 orders of mag-

nitude. Addition of either catalase or superoxide dismutase demonstrated that NO/H_2O_2 was the chemical species responsible for this bactericidal activity. Thus, the combination of NO and H_2O_2 may be ideally suited for killing *E. coli* owing to the additional protective effect of NO on the host. This mechanism may hold true for other species of bacteria, albeit with different kinetics. Staphylococcal killing by O_2^- was abrogated by NO at early time points, yet NO helped sustain killing at longer time intervals. Maximal killing depended on different timings of exposure to NO, H_2O_2, and O_2^- *(69)*. These findings may explain why NO and ROS are produced by immune effector cells at different times following exposure to different pathogens.

The diametrically opposite responses of mammalian cells and prokaryotes to the combination of NO/H_2O_2 may reflect their different cellular structures and complements of metalloproteins. Bacteria utilize iron sulfur clusters to a greater extent than do mammalian cells, and these types of proteins are especially susceptible to degradation mediated by NO or RNOS *(70,71)*. In *E. coli,* decomposition of iron complexes occurs in the periplasmic space, which is in close proximity to the cytoplasm. This relative lack of compartmentalization may allow iron to bind to and oxidize DNA. However, owing to the organellar structure of mammalian cells, metal labilization may be limited to the cytoplasm and mitochondria. In such a cellular arrangement, metals would be required to travel a large distance in order to reach the nucleus and bind to DNA.

Effect of NO/O_2^- on Cytotoxicity

Treatment of cells with peroxynitrite results in cell death in both the bacterial *(72)* and mammalian systems (see review in ref. *(73)*). However, lung fibroblast and neuronal cells treated with a superoxide source and NO donors showed no appreciable toxicity *(59)*. Other studies have shown that ovarian carcinoma cells exposed to 5 m*M* SIN–1, a simultaneous NO/O_2^- generator, resulted in no appreciable toxicity *(42)*. In fact, cells treated simultaneously with XO-and NO-releasing compounds were protected against XO-mediated toxicity, and no appreciable toxicity owing to $ONOO^-$ formation was observed *(41)*. These results suggest that there is a distinct difference between treating cells with bolus (m*M*) concentrations of peroxynitrite and generating similar amounts from NO and O_2^- systems.

Part of the discrepancy between bolus peroxynitrite treatment and peroxynitrite derived from NO/O_2^--generating systems can be explained in terms of concentrations. Beckman and co-workers *(72)* noted that cells treated with bolus delivery of peroxynitrite required high concentrations of peroxynitrite for penetration. The cell membrane forms a formidable barrier for peroxynitrite penetration into intracellular targets. Generation of NO and superoxide over specific time intervals results in peroxynitrite; however, the short lifetime of this chemical species in solution does not allow high enough concentrations of peroxynitrite to accumulate in order to penetrate the cell. Therefore, the amount of peroxynitrite that could cross the cellular membrane under more biologically relevant conditions, despite product in stoichiometrically high amounts over a prolonged period, is dramatically reduced. Therefore, the cell membrane limits the toxicity of extracellular peroxynitrite.

Another factor to consider in respect to toxicity mediated directly by peroxynitrite is the reaction between NO and peroxynitrite to form NO_2. As was discussed above, competition for superoxide by cellular components such as superoxide dismutase (SOD) and redox proteins increases the amount of NO required to form peroxynitrite. Since NO must outcompete these other reactions for O_2^-, fluxes must become rather large. The peroxynitrite that is thus formed can potentially be converted to potent nitrosating agents since the extracellular chemistry of excess NO reacting with $ONOO^-$, will convert it to nitrite. Thus, direct necrotic cell death mediated by oxidative chemistry of peroxynitrite from exposure of simultaneous NO/O_2^- derived from reduced nicotinamide adenine dinucleotide phosphate (NADPH) is unlikely.

INDIRECT EFFECTS

The indirect effects of NO are thought to result in the chemical species responsible for the etiology of numerous diseases related to NO. However, when determining the mechanisms under conditions that produce indirect effects, it is important to consider direct effects as well. Under conditions of high NO flux in the vicinity of a macrophage, the activity of guanylate cyclase, a direct effect of NO, still occurs.

The indirect effects can be divided into two basic types of chemistry, nitrosation and oxidation. Nitrosation chemistry results primarily in the formation of NO abducts on amines and thiols. Oxidation chemistry

results in the oxidation of different macromolecules ranging from mild reducing agents such as catecholamines and metal centers to those processes requiring higher oxidation potentials that damage DNA, proteins, and lipids. Oxidation and nitrosation chemistry can occur under nontoxic conditions or result in chemical intermediates that are cytotoxic. The terms nitrosative and oxidative stress are used to refer to chemical reactions that result in cytotoxicity. The chemical species responsible for NO-mediated nitrosative and oxidative stress involve RNOS. Under these conditions, molecules not normally associated with regulatory effects can be damaged. During chemical stress, proteins and DNA are often damaged, requiring the cell to repair itself. These chemical reactions are often invoked as the etiology of many diseases.

The ultimate chemical modifications resulting from RNOS species is a function of the intermediates formed under biologic conditions; N_2O_3, $ONOO^-$, NO^-, and NO_2 are the most important (**Fig. 1**). N_2O_3 is a relatively mild oxidant. It will only oxidize substrates with potentials less than +0.7 V and does not oxidize biomolecules such as DNA. However, N_2O_3 readily nitrosates nucleophils and may be the principle nitrosating species in vivo, at high local concentrations *(11)*. On the other hand, peroxynitrite and nitroxyl, which do not nitrosate substrates, readily mediate oxidative chemistry of macromolecules in vivo. *(13,74,75)*. A comparison of the resultant thiol products in the presence of RNOS can illustrate this point. An aerobic NO solution will autooxidize to produce N_2O_3. If GSH is present, it will form nearly 100% of the nitrosative product, GSNO *(76)*. Conversely, if GSH is exposed to either peroxynitrite, nitrogen dioxide, or nitroxyl, the resultant product is oxidized thiols and not nitrosative products *(75,77–79)*.

In addition to these bimolecular reactions, numerous other more complicated reactions of NO and RNOS/ROS can take place. For instance, NO can react with peroxynitrite, nitrogen dioxide, and nitroxyl, thereby inhibiting the oxidation of thiols *(13,78)*. The reaction between NO and nitrogen dioxide or peroxynitrite can result in N_2O_3, which facilities nitrosation of thiols. Since thiols are the primary reactive site for the indirect effects on cells, it can be seen that a balance of numerous reactions is what results in the eventual outcome. In addition to these interactions, NO can interact with ROS to abate oxidative chemistry. Therefore, it is not sufficient to consider just one reaction; they must be placed into perspective relative to each other. With this in

mind, we discuss the sources and conditions whereby nitrosative and oxidative stress might occur and the chemical reactions that are responsible as well as their likely biologic targets.

NITROSATIVE STRESS

The study of nitrosation chemistry dates back to the turn of the century *(80)*. Nitrosation of amines to form nitrosamines derived from nitrite in the gastrointestinal tract became a concern in the mid 1970s as a potential source of carcinogens *(81)*. A decade later, the discovery of nitrosamines and nitrite production under conditions of infection or by activated leukocytes was a critical link in elucidating the formation NO in vivo *(5,82–84)*. Later studies showed that under some types of chronic infection, nitrosamines are produced, thereby confirming that nitrosation does occur in vivo *(85)*. Nitrosation of thiols and their ultimate biologic fate have been extensively studied in a number of diverse conditions from cardiovascular function to cancer *(7,86)*.

The chemistry of nitrosation can occur by several different mechanisms. Nitrosation differs from nitrosylation in that there is the addition of a nitrosonium ion (NO^+) equivalence to a nucleophile. Nitrosylation is defined as the formation of a nitrosyl adduct such as those formed between the reaction of NO and metals, as described for the direct effects. Simple nitrosation may occur from reactions mediated by metals as well as those from RNOS. Metal-mediated nitrosation can occur in the test tube and could have a role in vivo.

Metal-Mediated Nitrosation

The formation of *S*-nitrosothiols and nitrosamines from metal complexes such as SNP rapidly catalyzes nitrosation reactions. This involves a simple transfer of an equivalence of nitrosonium ion to the nucleophile. Heme complexes have also been shown to form *S*-nitrosothiols and nitrosamines. However, nitrosonium ion involves the ferric state of the heme. Ferric nitrosyl complexes are much less stable than their corresponding ferrous state, but the ferrous nitrosyl heme complexes do not nitrosate thiols and amines. Ferric heme can nitrosate some thiols and amines; however, this process requires reactivity of iron (III) porphyrins and heme proteins with nitric oxide. Nitrosyls transfer to carbon, oxygen, nitrogen, and sulfur *(86a)*.

One mechanism in particular that may be important in the physiologic transport of NO as well the formation of *S*-nitrosothiols is the chemistry of iron sulfur nitrosyl complexes. The formation of nonheme nitrosyl complexes seems to occur under high fluxes of NO. Several studies have indicated that ferritin, aconitase, and even metallothionien are responsible for this *(87,88)*. Nitrosation of thiols can occur under anaerobic conditions in the presence of ferrous iron. However, this chemistry is readily reversible. Therefore, this is not likely to account for inactivation of enzymes in cells exposed to high NO concentrations. However, an equilibrium exists among NO, dinitrosyl iron, and RSNO so that even a small fraction of GSNO may be all that is required for physiologic stimulation. These mechanisms remain to be elucidated.

An alternative possibility for the nitrosation of thiols could be catalyzation by proteins such as cobaltamine. As discussed above, a stable cobalt (III) nitrosyl can readily be formed even under physiologic conditions. In the presence of thiols, it was shown that *S*-nitrosothiols could be formed. This may play an important role in NO-mediated metabolism in the vascular system as well as in tumors.

Although some metal-mediated nitrosative chemistry could occur in vivo, the detection of nitrosamines under physiologic conditions is most likely a result of RNOS. Heme-and iron-mediated nitrosation of amines requires at least an atmosphere of NO and an exposure time of days. These are conditions not likely to be encountered in vivo. Hence, the formation of nitrosamines in stimulated cells is mediated by RNOS. The presence of these nitrosative products in cells and in vivo suggests that nitrosative stress is an important biologic effect of NO.

There are basically three potential sources of RNOS-mediated nitrosation: (1) NO autoxidation, (2) nitrite acidification, and (3) the NO/O_2^- reaction under excess fluxes of NO (discussed just below). Except in gastric regions (pH < 1.5), the primary nitrosating intermediates are isomers of N_2O_3. The route of formation of these intermediates is important and determines where and when nitrosation occurs.

The chemical reaction most noted for the formation of N_2O_3 is the reaction between NO and oxygen; this is referred to as autoxidation. This reaction has been studied for decades because of its importance in gas phase atmospheric nitrogen oxide chemistry *(89)*. The autoxidation

of NO in the aqueous, hydrophobic, and gas phases has a third-order rate equation with second-order dependency on NO *(89)*:

$$d[NO]/dt = k_{NO}[NO]^2[O_2] \quad (5)$$

This second-order dependence on NO helps to explain some of the confusion over how a toxic radical species like NO can participate in physiology. Because of the instability of NO in the presence of oxygen, as well as the formation of toxic chemical species like N_2O_3 and NO_2, it was hard to envision why nature would choose NO as physiologic mediator. The second-order NO dependency of this autoxidation reaction dictates that the lifetime of NO is inversely proportional to its concentration (**Eq. 5**) *(90,91)*. Therefore, when NO is formed and moves away from its cellular source, its concentration is diluted. As its concentration decreases, there is a concomitant increase in its lifetime. This allows NO to react with the other biologic targets such as guanylate cyclase without interference from the autoxidation reaction and production of the related RNOS. Conversely, when NO levels are high, the formation of RNOS increases dramatically. In local regions of high NO output, intermediates associated with the autoxidation can occur.

Where in the cell would the autoxidation be likely to occur? Comparisons between the rate constant for the autoxidation reaction in hydrophobic regions versus aqueous solution show a similar rate constant. This finding suggests that the surrounding medium does not influence the rate of autoxidation dramatically. The solubility of the reactants will, however, affect the rate of the reaction since NO and oxygen are more soluble in hydrophobic phases than in aqueous solutions. Reports have shown that NO and oxygen levels are 10–50 times higher in lipid membranes than in aqueous solutions owing to their increased solubility *(92)*. For example, if a cell is exposed to a NO flux from a chemical donor or another cell, NO will partition such that the NO levels will be 10 times greater in the membrane than the surrounding aqueous solution. Therefore, since the rate of the autoxidation reaction is a function of reactant concentration, it should occur much faster in membranes than in aqueous solution based solely on the differences in the relative concentrations of NO and O_2 in each region. A recent study has shown that in the presence of detergent micelles, the rate of autoxidation occurs 300 times faster in the hydrophobic region than in the surrounding aqueous solution *(93)*. This work suggests that the nitrosation reac-

tions mediated by autoxidation would be mostly likely to occur in the membrane. Hence, membrane-bound proteins, which are functionally and structurally dependent on thiols, or amines would be most affected by nitrosative stress.

Another factor to consider in nitrosation chemistry is the mechanism of N_2O_3 formation and whether it requires the intermediacy of NO_2. In gas-phase and hydrophobic solvents, the initial intermediate of autoxidation is the formation of NO_2 *(89)*. Nitrogen dioxide then reacts with another NO to form an equilibrium with N_2O_3 (**Eq. 6**). In the presence of water, N_2O_3 is converted rapidly to nitrite (**Eq. 7**):

$$NO_2 + NO \leftrightarrow N_2O_3 \quad (6)$$

$$N_2O_3 + H_2O \rightarrow 2\ HNO_2 \quad (7)$$

An additional means for N_2O_3 formation is the reaction of nitrite under acidic conditions. The protonation of NO_2^- to form H_2ONO will react with an additional NO_2^- to form N_2O_3 *(80)*. N_2O_3 can then disproportionate into NO_2 according to **Eq. 6**. This is the exact same species, N_2O_3, as is formed in the autoxidation reaction in aqueous solution; however, unlike the autoxidation reaction, there is formation of NO_2. Studies have also suggested that N_2O_3 formed in aqueous solution is different from that in hydrophobic media *(91,94)*. Several studies using competition reactions show that in aqueous solution NO_2 cannot be trapped. This suggests that in aqueous solution the formation of NO_2 from N_2O_3 cannot escape the solvent cage. To illustrate best the differences between nitrosation versus nitration, reactions with phenol and tyrosine were examined *(76,95)*. Nitrotyrosine was the exclusive product when tyrosine was exposed to acidic nitrite, or RNOS formed first in the gas phase. This is thought to occur through the reaction of NO_2 *(76)*. However, when RNOS are formed from the autoxidation in water, no nitrotyrosine is formed. This suggests that the autoxidation in aqueous solution will not produce nitrotyrosine but that in a hydrophobic environment such as that found in membranes it could occur.

The selectivity of the intermediates formed in the autoxidation reaction has been determined. Since N_2O_3 is hydrolyzed to nitrite extremely rapidly (half-life of 1 ms), only substrates that are present in high concentration and have sufficient affinity will react *(96)*. At neutral pH, thiol-containing peptides have an affinity for N_2O_3 1000 times greater than any other amino acid *(76,96)*. In addition, buffers

such as carbonate and phosphate have affinities less than 400 times that of thiol-containing peptides *(96)*. These results suggest that the primary reaction of the NO/O_2 in aqueous solution will be to form *S*-nitrosothiols. *S*-nitrosothiols have been shown to have a variety of effects on biologic functions. This supports the possibility that this reaction can occur from NOS-generated NO *(86)*.

Chemistry of RSNO

The fate as well as the biologic action of *S*-nitrosothiols resulting from the nitrosative stress is important in understanding the biology of NO. In particular, small peptides such as GSNO or CysNO can play important roles in cellular metabolism and influence cardiovascular properties. GSNO has three major reactions that are important to the biologic outcome of nitrosative stress: reaction with other reduced thiols, reaction with metal complexes, and reaction with superoxide. It is important to understand that low molecular weight thiols are usually less stable than their corresponding protein adducts. For instance, CysNO in biologic solutions has a shorter half-life than GSNO, which in turn has a shorter lifetime than protein S-nitrosothiols. Therefore most of the *S*-nitrosothiol will be on proteins.

The reactions of RSNO with reduced thiols can result in two basic reactions, transnitrosation or reductive elimination of nitroxyl. Transnitrosation reactions have been proposed as a mechanism by which NO can be transported through biologic system. This is simply a transfer of a nitrosonium ion (NO+) from one thiol to another. Several reports have investigated the relative rates of these reactions. In general, CysNO will preferentially transfer NO+ equivalence to higher molecular weight thiols, whereas the transfer from GSNO to proteins will be slower. These reactions are often in equilibrium with each other and hence favor the thermodynamically more stable product.

One of the most common and important reactions of RSNO in test tube experiments is the reaction with metal centers. Metals, especially copper in buffered solution, dictate the stability of RSNO. Kinetic analysis has revealed that Cu (I) and not Cu (II) is responsible for the decomposition of NO. The Cu (I) decomposition of GSNO may be important when CuZnSOD is present along with GSH. MnSOD, on the other hand, has no effect. GSNO with copper ions in SOD reacts with GSH to form GSSG and NO as products. The amount of GSH required

was 0.1–10 m*M*, which is in the range of physiologic GSH concentrations in the cytoplasm. This decomposition suggests that CuZnSOD may play a key role in the detoxification of nitrosative stress, either by direct scavenging of N_2O_3 or via transnitrosative reactions.

Surprisingly, superoxide reacts with GSNO. Several papers have shown that the reaction of superoxide and GSNO will produce an oxidizing species resulting in GSSG. The kinetics for this reaction is the requirement of 2 GSNO for every superoxide in the rate-limiting step. GSSG and equal molar nitrite and nitrate are formed. Studies using millimolar GSNO suggest that the oxidant formed may be peroxynitrite. However, other kinetic analyses suggest that 2 NO_2 is the possible intermediate.

OXIDATIVE STRESS

Oxidation is the removal of electrons from substrate and occurs under normal physiologic conditions. However, there is a significant difference between normal cellular redox chemistry and that associated with oxidative stress. Under conditions of oxidative stress, powerful oxidizing agents, resulting in products not normally found under normal physiologic condition. For example, oxidation of DNA results in strand breaks, and oxidation of nucleic acids can occur under oxidative stress *(97)*. Oxidation of lipids results in lipid peroxidation, and oxidation of protein modifies their structure and impedes their function. These processes have been associated with the onset of different pathophysiologic conditions, suggesting that chronic oxidative stress is the etiology of many pathophysiologic states *(97)*.

The chemistry of NO can result in different RNOS that are capable of causing conditions of oxidative stress. The three major RNOS that mediate oxidative stress are nitrogen dioxide, nitroxyl, and peroxynitrite. Nitrogen dioxide primarily originates in the same processes involved in nitrosative stress: autoxidation of NO, the NO/O_2^- reaction (as discussed in the NO/O_2^- Chemistry section below), and acidic nitrite. NO_2 can directly nitrate substances such as tyrosine *(76)* and might be the source of nitrotyrosine observed in vivo. NO_2 does not appear to alter DNA in such forms as strand breaks *(98,99)*, but it can induce lipid peroxidation *(100)*. Under conditions of excess NO, it can react rapidly with NO_2 to form N_2O_3, which mediates nitrosative stress.

Oxidation mediated by NO_2 would probably occur from acidic nitrite. NO_2 produced in membranes from the autoxidation reaction or from NO + HOONO would most likely be converted to N_2O_3 owing to the presence of excess NO *(13)*. Therefore, the oxidative chemistry mediated by NO_2 in vivo is probably limited.

Another nitrogen oxide species, nitroxyl (NO^-), is a chemical intermediate of NO. It has been shown that formation of NO^- can result from different processes under a variety of biologic situations. One primary source of NO^- is the decomposition of *S*-nitrosothiols *(101)*. The nucleophilic attack of thiols to RSNO can result in NO^- and disulfide. Decomposition of dithiothreitol (DTT)-SNO results in NO^- and oxidized DTT *(102)*. Other reports suggest that NO^- can be formed from the decomposition of iron dinitrosyl complexes *(103)*. One intriguing possibility is that NO^- may be derived directly from NOS itself *(104,105)*. Several reports have suggested that one initial product in the conversion of arginine to citrulline is NO^-. Other reports suggest that oxidation of the catalytic intermediate in NOS activity, hydroxyarginine, may result in NO^- *(106)*. Taken together, these processes suggest that NO^- may play a role in the biology of NO.

Substances that release NO^- have provided a method of studying the effects of NO^- in biology *(107)*. One of these is called Angelis's salt or sodium trioxodinitrate ($Na_2N_2O_3$). At neutral pH, this complex releases NO^- and nitrite. Recent studies on AS have shown that NO^- is cytotoxic *(108)*. Comparing survival in clonogenic assays with Chinese hamster V79 cells, AS at 2 and 4 m*M* was toxic. Comparisons between the toxicity of the NO/O_2^- donor SIN-1 and the NO donor DEA/NO, with AS, demonstrated that the toxicity of AS was more than 2 orders of magnitude greater. The toxicity of AS compared with hydrogen peroxide and alkyhydroperoxide was similar. Hypoxia abated the toxicity, suggesting that the RNOS chemistry responsible for cell death requires a reaction between nitroxyl and oxygen. The lack of an effect by metal chelators indicates that ROS via Fenton-type reactions are not involved. It appears that addition of AS results in a dramatic loss of GSH as well as DNA double-strand breaks. Since neither hydrogen peroxide nor peroxynitrite mediates double-strand breaks, they are probably not the chemical intermediate.

We have examined the oxidative, nitrosative, and nitrative properties of AS under aerobic and anaerobic conditions *(109)*. A comparison of dihydrorhodamine (DHR) oxidation by peroxynitrite and N_2O_3, with

oxidation by AS demonstrated that AS had a selectivity similar to that of peroxynitrite. AS was also not quenched by azide, an N_2O_3 scavenger. Despite these similarities, the yield of DHR oxidation was twice that of peroxynitrite. By examining the one-electron oxidation of hydroxyphenylacetic acid to its fluorescence dimer product in the presence of AS, it was shown that oxidation by AS was only minimal, whereas peroxynitrite was very effective. Hydroxylation of benzoic acid was more efficient with AS than with peroxynitrite. However, the nitration of HPA was not detected with AS, but peroxynitrite was readily nitrated under the same conditions. Therefore, it appears that NO^- in the presence of oxygen produces an intermediate other than peroxynitrite.

There appear to be two types of reactivates of nitroxyl in biologic systems, an oxygen-independent and an oxygen-dependent reaction. By using DHR oxidation, which requires oxygen, several substances were examined to test their reactivity with NO^-. It appears that amines such as hydroxylamine and thiols rapid react with NO^- rapidly and directly. In addition, NO, NADPH, and SOD all react with NO^- *(108,110)*. Metalloproteins such as myoglobin and catalase have also been shown to react effectively with NO^-. NO^- oxidation and hydroxylation reactions appear to require oxygen, but NO^- in the presence of O_2 mediates DNA damage or cellular toxicity.

NO/O_2^- CHEMISTRY

The reaction between superoxide and NO has been shown to be very important in the fundamental understanding of NO behavior in biology *(73,111)*. Huie and Padmaja *(112)* showed that NO + O_2^- reacted at near diffusion controlled rate to form peroxynitrite. One of the first observations suggesting that this reaction could be important in biology was that SOD enhanced the effect of endothelium-derived relaxing factor (EDRF) *(113)*. It was thought that NO concentrations might be partly controlled by superoxide. Because of the fast rate of peroxynitrite formation, it was thought that this reaction could play an important role in the contribution of NO to various pathophysiologic conditions *(111)*. As we will discuss below, this reaction is much more complicated than the simple formation of peroxynitrite.

The reaction rate constant suggests that peroxynitrite could be formed in vivo. The question is when and where? One of the most important considerations in predicting whether a reaction will occur in

vivo is the relative pseudo-first-order rate constants and not just the rate constant itself. The concentrations of the reactants are as important as the rate constants in determining the participation of a given reaction (for a summary of RNOS evaluation, see ref. *96*). The cellular concentrations of superoxide and NO under normal conditions are 1 *pM* and 0.1–1 μ*M*, respectively. This suggests that superoxide production is the limiting determinant for the location as well as the amount of peroxynitrite formed between these radicals. The NO concentration determines whether the reaction occurs at any specific locality. The determination of whether this reaction occurs depends on competing reactions of superoxide with NO. Most important is the concentration of SOD. SOD reacts with superoxide at a similar rate constant as NO. Since the intracellular SOD concentration is thought to be between 4 and 10 μ*M* (50 μ*M* in the area of the mitochondria, where most of the cellular production of O_2^- would occur), the NO concentration would be have to be 0.4–5 μ*M* for 10% of the superoxide to be converted to peroxynitrite. In addition, other reactants with superoxide such as aconitase (3×10^7 M^{-1} s^{-1}) and ferricytochrome c (5×10^6 M^{-1} s^{-1}) could play a role in the abatement.

Peroxynitrite at neutral pH has been shown to be a powerful oxidant. It can oxidize thiols, initiate lipid peroxidation, nitrate tyrosine, cleave DNA, nitrate and oxidize guanadine, and oxidize methionine. The oxidant responsible for this is an excited state of peroxynitrous acid *(114)*. Peroxynitrite is in equilibrium with the protonated form, peroxynitrous acid. In the absence of an adequate substrate, this protonated form simply rearranges to nitrate. This can be thought of a detoxication pathway. However, at high enough concentrations, substrates such as tyrosine (1 m*M* to yield 50% yield) can react to give nitrotyrosine. It is thought that most of the nitration and oxidative chemistry proceeds through the HOONO species *(114)*. Metals can react directly with $ONOO^-$ at 6×10^3 M^{-1} s^{-1} *(115)*. In the case of CuSOD and FeEDTA, the metal component enhances nitration reactions. It has also been shown that heme-containing enzymes such as myeloperoxidase (6×10^6 M^{-1} s^{-1}), lactoperoxidase (3.3×10^5 M^{-1} s^{-1}) and horseradish peroxidase (3.2×10^6 M^{-1} s^{-1}) react directly with $OONO^-$ *(116)*.

Another important consideration of the NO/O_2^- reaction is that NO and O_2^- can react with peroxynitrite to form nitrogen dioxide *(74,114,117)*:

$$OONO^- + H^+ + NO \rightarrow NO_2 + HNO_2 \quad (8)$$

This places further restrictions on the chemistry mediated directly by peroxynitrite. To examine the fluxes of NO and superoxide in nitrosative and oxidative chemistry, several studies have examined NO donors with xanthine oxidase (XO) *(74)*. For instance, NO does not alter the oxidation of xanthine by XO but does affect the production of superoxide *(59,118–120)*. XO is considered a model of oxidative stress and generates the reactive oxygen species superoxide and hydrogen peroxide. In the presence of an NO^- releasing agent, the amount of hydrogen peroxide produced by XO is unaffected *(41)*. However, the amount of superoxide formed is dramatically reduced. It has been proposed that NO reacts with the produced superoxide formed from XO to form peroxynitrite, which then isomerizes to nitrate *(59,118–120)*:

$$NO + O_2^- \rightarrow OONO^- \quad (9)$$

$$OONO^- + DHR \rightarrow \text{oxidation} \quad (10)$$

$$OONO^- \rightarrow NO_3^- \quad (11)$$

If the same conditions are used in the presence of DHR *(74)*, an increase in oxidation is observed in the presence of NO/XO. Peroxynitrite oxidizes DHR *(121)*, further supporting the hypothesis that peroxynitrite is an RNOS generated from NO/XO (**Eq. 10**).

What is intriguing about this study is that the fluxes of NO and superoxide were varied relative to each other. It was shown that maximal oxidation through peroxynitrite was only achieved when the reactants were present at a 1:1 ratio. It was shown that excess of either radical quenched the chemistry of peroxynitrite. In the presence of excess NO or superoxide, peroxynitrite is converted to nitrogen dioxide *(74,114,122)*:

$$OONO^- + NO \xrightarrow{H^+} NO_2 + NO_2^- \quad (12)$$

$$OONO^- + O_2^- \xrightarrow{H^+} NO_2 \quad (13)$$

Nitrogen dioxide can rapidly react with NO to form the nitrosating species, N_2O_3:

$$NO_2 + NO \rightarrow N_2O_3 \quad (14)$$

Although nitrosation does not occur directly through peroxynitrite, there are several mechanisms by which they can occur through the NO/O_2^- reaction that may be important in the biology of NO.

MIXED DIRECT AND INDIRECT

NO Inhibition of Mitochondrial Respiration

One of the primary proposed cellular targets for the cytotoxic action of NO is the mitochondrion *(1,87,123)*. Dinitrosyl adducts of aconitase are formed in cells after exposure to NO and may be an important factor in the inhibition of mitochondrial activity leading to cytostasis and cytotoxicity *(87)*. Further studies have shown that NO also de-energizes the mitochondria in a reversible manner *(124,125)*, which, under normal physiologic conditions, involves regulation of intracellular calcium *(126)*. So how does NO inhibit mitochondrial function as part of regulatory processes, yet also mediate cell death?

Inhibition of mitochondria mediated by NO appears to have a reversible and irreversible component. Knowles et al. *(127)* reported that NO derived from GSNO inhibited mitochondrial respiration by a distinctly different mechanism than $OONO^-$. They suggested that NO derived from GSNO reversibly inhibited respiration, whereas $OONO^-$ resulted in irreversible inhibition of respiration. Several studies have shown that NO directly interacts with cytochrome c oxidase to inhibit respiration reversibly *(128–133)*. The interaction with cytochrome c oxidase appears to require low concentrations of NO (submicromolar), characteristic of the NO concentrations resulting from cNOS production. However, under inflammatory conditions, complex I (NADH: ubiquinone oxidoreductase) and complex II (succinate: ubiquinone oxidoreductase) are irreversibly inhibited by NO *(131)*. Under these conditions, RNOS may play a role in irreversible inhibition. Similar to the mechanism described for cytochrome P450, inhibition of mitochondrial respiration appears to have a reversible component mediated by direct effects and an irreversible component mediated by indirect effects.

Modulation of respiration by low amounts of NO at the cytochrome c oxidase will determine tissue oxygen gradients as well as cellular adenosine triphosphate (ATP) levels. Like other heme proteins,

cytochrome c oxidase can react with NO to form a nitrosyl adduct *(134)*. Binding of NO to cytochrome c oxidase may influence the activity of mitochondrial enzymes depending on the oxygen concentration. Under both hypoxic and aerobic conditions, NO is consumed by the mitochondria through direct binding to the cytochrome c oxidase site. After the formation of Fe-NO, additional electrons from the respiratory chain reduce NO to nitrogenous products *(135,136)*. However, under aerobic conditions, it appears that electrons in the respiratory chain are diverted from the reduction of NO at the cytochrome c oxidase site to form superoxide and (thus hydrogen peroxide), which can further react with NO *(137)*. The partitioning between the reduction of NO and oxygen is dependent on oxygen tension versus the rate of electron reduction of the nitrosyl cytochrome c oxidase complex.

The inhibition of oxygen consumption at the mitochondria by low levels of NO may be important in regulating tissue oxygen. ecNOS (NOS-3) has been found in mitochondria *(138)*, indicating that this source of NO may be important in various physiologic mechanisms. The presence of NOS in mitochondria suggests that the chemistry of NO is well regulated within this organelle. Some reports have proposed that NO plays a key role in the regulation of respiration. Other reports suggest that the influence of NO on the mitochondria may play a role in smooth muscle cell relaxation in both physiologic and pathophysiologic conditions *(139,140)*.

As NO concentrations and time of exposure increase, there is an increase in RNOS formed in the mitochondria. The source of RNOS under aerobic conditions has been proposed to involve superoxide derived from decoupling of oxygen reduction at the cytochrome a_1a_3 site, which reacts with NO to form peroxynitrite. However, MnSOD, which exists in the mitochondria at high concentrations, will compete with NO for superoxide, thus limiting the formation of peroxynitrite. The amount of NO required to form peroxynitrite results from NO fluxes that are higher than superoxide fluxes. As discussed above, this should create an imbalance in the NO/O_2^- ratio and would favor the conversion of peroxynitrite to N_2O_3. These conditions indicate that the oxidative chemistry mediated by peroxynitrite probably does not play a significant role in mitochondrial dysfunction but that other RNOS such as NO_2 and N_2O_3 may. Furthermore, according to **Eqs. 7–10**, it is more likely that nitrosative, not oxidative chemistry, would be the predominant indirect effect in mitochondria under high NO fluxes.

Most of the mitochondrial studies have been conducted in cell culture or with isolated mitochondria. However, a comparison of cellular and in vivo inhibition of mitochondria suggests that RNOS-mediated irreversible inhibition is less important in vivo. When cultured hepatocytes are stimulated with interferon-γ and LPS to activate NOS and NO generation, inhibition of respiration results. In contrast, respiration in cells isolated from animals treated with LPS and interferon-γ is not affected *(141,142)*. This may suggest that oxyhemoglobin and diffusion of NO away from NOS-containing cells may play an important role in the extent of mitochondrial inhibition when RNOS formation is limited and reversible inhibition is only transient.

Metal Homeostatics

An important role of NO under either physiologic or pathophysiologic conditions is the regulation of intracellular iron status (see reviews in refs. *70* and *71*). There are different aspects of iron metabolism that NO can affect. NO can influence heme metabolism by activating heme oxygenase, which results in catabolism of heme complexes, as well as inhibiting ferrochelatase, an enzyme that places the iron in the porphyrinic complex *(24)*. The inhibition reduces heme availability and decreases the amount of active NOS, which may serve as a negative self-regulation of NO formation.

In addition to influencing heme metabolism, NO affects the formation of the transferrin receptor and ferritin protein, which regulates the uptake and storage of cellular iron. The iron-responsive elements (IREs) are strands of RNA that are posttranscriptionally regulated (see reviews in refs. *70* and *71*). The iron-responsive binding protein (IRB) that contains an $Fe_{3\text{-}4}S_4$ cluster and possesses aconitase activity regulates the IRE synthesis of ferritin to transferrin receptor protein *(143)*. The iron sulfur cluster within the IRB has two forms, apoprotein Fe_3S_4 and holoprotein Fe_4S_4, in which the fourth iron is in the apical position. If the apical Fe is missing, then binding to the IRE results in the downregulation of ferritin production and upregulation of transferrin receptor. These events result in increased cellular uptake of iron *(143)*. However, if the apical iron is present, then ferritin protein increases and the protein for the transferrin receptors decreases, which results in reduction of iron uptake *(143)*.

NO binds to the apical iron to form a nitrosyl complex, resulting in

inhibition of aconitase activity, but it does stimulate binding to IREs. It should be noted that peroxynitrite and superoxide inactivate both aconitase activity and the ability of the IRB to bind to the IRE *(144,145)*, thus limiting the intake of iron. This inactivation of aconitase by either peroxynitrite or superoxide may be a *protective* mechanism against excessive iron uptake, limiting the iron available to catalyze oxidative chemistry. This may be another mechanism by which NO reduces intracellular oxidative stress. Inactivation of aconitase activity would also reduce the available electrons, thus reducing oxygen by the respiratory chain. Since NO may increase superoxide/hydrogen peroxide formation via inhibition of respiration (cytochrome c oxidase), the reduction of electron flow may also reduce ROS production. The inactivation of aconitase may be protective against intracellular hydrogen peroxide formation.

Iron metabolism and availability has a tremendous effect on oxidative stress as well as cell growth. NO may play a key role in inhibiting the availability of iron by inhibiting release of iron from ferritin. In mammalian cells, iron is released from ferritin by reduction from NADPH oxidase through the intermediacy of superoxide. The conversion of the ferric to ferrous state makes iron accessible to cells. NADPH oxidase assembly, not activity, is inhibited by NO *(119)*. This would limit iron availability to the cell. In addition, NO scavenges superoxide, which inhibits the reduction of ferritin-bound iron. In addition to inhibition of ribonucleotide reductase, these two mechanisms may play an integral part in cytostatic mechanisms in a variety of disease states.

CONCLUSIONS

Chemical biology can provide a road map for researchers who are investigating the molecular aspects of NO. The importance of concentration and timing with other reactive oxygen species cannot be overstated. Direct effects may predominate in the physiology of NO, but the indirect effects give NO some of its more pathophysiologic characteristics. Temporal, stoichiometrical, concentration, and spatial considerations must be considered in order to place NO in the context of biologic systems.

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3 The Microcirculation and Adhesion Molecules

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INTRODUCTION

The universe communicates with organisms, and the different components of an organism communicate with each other by a variety of means. Among these is the blood, a substance that in humans flows within the vessels of the cardiovascular system and is composed of red blood cells, white blood cells, and platelets that are suspended in a complex solution known as plasma, which includes water, gases, salts, nutrients, and wastes. As blood aids in the distribution of these substances to cells that are not in close enough proximity to the environment outside the organism to exchange mass, energy, and momentum directly, blood is a major contributor to the homeostasis of an organism. Most of this exchange occurs at the level of the smallest vessels in the cardiovascular system, the microcirculation.

The microcirculation consists of the smallest vessels within the body, the arterioles, venules, and capillaries. Together these vessels form an extensive network that controls the perfusion of specific tissues and the exchange of nutrients and waste products between the blood and the interstitium. This is accomplished by the interplay of the arterioles, which alter the resistance of blood flow, the venules and larger veins, which act as reservoirs or capacitors, and the endothelial capillary bed, which lies between the arterioles and venules.

In addition to the exchange of nutrients and gases, the cells within the blood, particularly those of the immune system, also pass from the blood to the interstitium and the surrounding tissues through the microcirculation. Leukocytes roll on the walls of venules, but not arterioles.

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This process is mediated by specific receptors and their ligands, collectively referred to as adhesion molecules or factors, which are present on both the endothelium and the cells associated with the inflammatory process.

An insult to an organism, severe enough that homeostasis cannot be recovered, causes the intrinsic and extrinsic control systems to fall into disarray as the organism attempts to regain balance. These insults cause a decrease in the perfusion of cells and tissues, be it from loss of circulatory blood volume directly, as in hemorrhage or from obstructive, cardiogenic, neurogenic, or vasogenic factors, or as a side effect of a foreign organism such as in sepsis. In this chapter, the current understanding of both the normal and abnormal microcirculation is described, with an emphasis on adhesion molecules.

THE MICROCIRCULATION

Anatomy and Development

To accommodate the specific demands of each organ system, the microcirculation of a particular organ is different from those found in other organs. However, certain basic structural similarities are common to most of the microcirculation. After branching six to eight times, the arteries become arterioles, which range in thickness from 10 to 150 μm. They are composed of the inner endothelium or intima, which consists of a single layer of endothelial cells that is continual throughout the cardiovascular vessels, the media, which ranges from a single layer of smooth muscle cells in the terminal arterioles to several layers in the larger arterioles, and the adventitia, which contains collagen fibers. The capillary vessels, composed of only the single-cell-thick endothelium, join the arterioles and the venules, which have a larger diameter but a smaller wall thickness to diameter ratio than the corresponding arterioles, to complete the circulation (**Fig. 1**). The fibers between the endothelium and smooth muscle cells comprise the elastica lamina.

The endothelium forms a smooth, nonthrombogenic lining of blood vessels with which the blood is in constant contact. The thickness is 5–10 μm, and each cell is between 500 and 1000 μm long. Adjacent endothelial cells are joined by tight junctions, which allow communication between endothelial cells. Blood is constantly in contact with these cells, and hemostasis is regulated through activation of the coagulation and fibrinolytic pathways and by altering platelet function and vascular

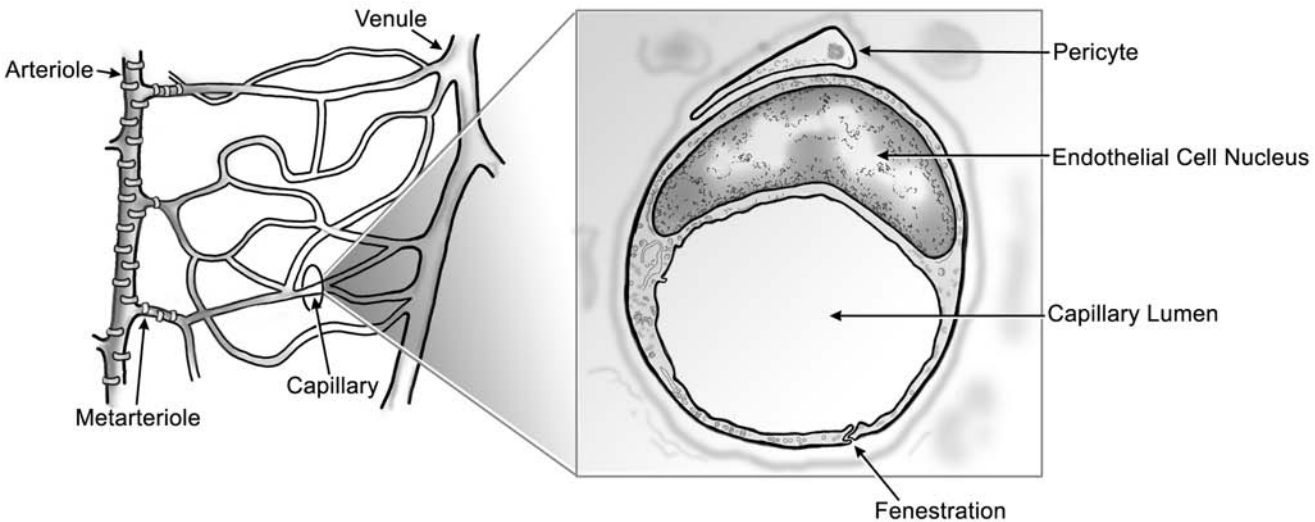

Fig. 1. The microcirculation, consisting of arterioles, venules, and capillaries. Metarterioles help control blood to the capillary bed. The expanded view of the capillary shows an endothelial cell with a large nucleus. The endothelial cell is surrounded by a pericyte, which provides support and controls blood flow. The capillary depicted has pores or fenestrations, which allow substances to pass back and forth from the capillary lumen to the surrounding connective tissue. (Illustration by Annabelle Wright.)

tone. In addition to being involved with the inflammatory process, endothelial cells produce vasoactive substances including prostacylin, endothelin, and nitric oxide (NO).

The development of the microcirculation is a dynamic process, and angiogenesis occurs in response to both biochemical and mechanical stimuli *(1)*. This can occur temporally from moment to moment and over the course of an organism's lifetime. The adhesion factors that are important in embryonic development are expressed in response to signals initiated at different regions and times that result in changes of cellular gene expression and differentiation *(2–4)*. Therefore, there are always fluctuations in the balance between the forces that lead to increases in the diameter and the length of the vessels and the internal forces that resist this growth. Fluid mechanical forces that occur in the presence of blood flow have been shown to influence endothelial cell development through changes in cell morphology and function *(5–7)*.

Physiology

The flow of blood is regulated in the peripheral cardiovascular system through a balance of extrinsic and intrinsic factors that permit the direction of blood flow to areas where it is most needed. These include neural, humoral, and local control mechanisms. Neural influence varies greatly from organ to organ. Although the vessels are innervated by norepinephrine-secreting sympathetic and acetylcholine-secreting parasympathetic nerves, the parasympathetic nerves are less influential in controlling flow, as they do not innervate the skin or skeleton muscle. During hemorrhage, sympathetic nerves cause the resistance vessels to constrict in order to maintain arterial pressure, and norepinephrine secretion leads to the narrowing of capacitance vessels assisting in correction of the decrease in central venous pressure caused by blood loss.

Humoral regulation consists of the release of norepinephrine and epinephrine from the medulla of the adrenal gland in response to sympathetic stimulation. These are both vasoconstrictors that act on the systemic vessels, but epinephrine also vasodilates the coronary vessels. Other humoral vasodilators include angiotensin, which increases total peripheral resistance (therefore increasing arterial pressure) and the body's most potent vasoconstrictor, vasopressin.

In contrast to the neural and humoral factors that are responsible for the general control of blood flow, local factors are responsible for a finer degree of control. The site of local regulation is the microcircula-

tion. Local factors are involved in the special cases of blood flow, active hyperremia, reactive hyperremia, and autoregulation. Active hyperremia is the shunting of blood from less to more active tissues; reactive hyperremia occurs when a vessel becomes anatomically or functionally occluded, whereupon wastes will build up proximal to the site of obstruction. When blood flow returns to the region, flow is increased approximately five times from normal flow rates for the same length of time that the occlusion was present. Autoregulation occurs in response to the metabolic demands of a tissue and the release of vasodilating factors, which increase flow to the region. This increase in flow is accompanied by an increase in nutrients to the tissue plus the washing out of vasodilating factors from the area, resulting in decreased dilation and a return to the baseline flow rate.

Prostacyclin, NO, and various kinins are known to cause vasodilation. Prostacyclin relaxes vascular smooth muscle by increasing cyclic adenosine monophosphate (cAMP) and inhibits platelet adherence to the endothelium. NO also relaxes the endothelium and is converted to NO from L-arginine. NO activates guanyl cyclase in vascular smooth muscle and causes increases in cyclic guanosine monophosphate (cGMP), which decreases cytosolic free calcium, relaxing the vascular smooth muscle.

Kinins, chiefly bradykinin, play a role in regulating blood flow and leakage of fluids in inflamed tissues in addition to the role of blood in skin and salivary and gastrointestinal glands. Maceration of blood leads to tissue inflammation, resulting in the activation of kallikrein. Kallikrein acts on α_2-globulin to release a kinin called kallidin that is converted by enzymes in tissue to bradykinin, which vasodilates tissue. Bradykinin is then digested by converting enzyme. Histamine is released during inflammation from damaged tissues and basophils, and it dilates arterioles and increases capillary porosity. Among the other known substances that play a role in vasodilation are adenosine triphosphate (ATP) and substance P. Endothelin is the best characterized vasoconstrictor.

The resulting interplay between these global and local controls is complex. For instance, the blood supply to skeletal muscle responds primarily to neural control when at rest, but during exercise, intrinsic flow mechanisms assume control, and the dilation of vessels in muscles occurs in response to the local increase of metabolites that override the constrictor impulses. In the case of severe hemorrhage, discharge of the

sympathetic nerves leads to constriction of the blood vessels of the skin, renal, and splanchnic circulation but has little effect on the cerebral and cardiac vessels *(8)*.

Chemical Forces Within the Microenvironment

Diffusion and solvent drag are the major ways that substances pass from the blood to the interstitium and back again. Hydrostatic pressure and oncotic or colloid osmotic forces cause the filtration and absorption that results in mixing of substances within intra- and extravascular spaces. The two forces oppose each other; hydrostatic force is related to vascular pressure, and oncotic force is determined by the amount of osmotically active particles in the vessel. These include ions, small molecules, and various plasma proteins, especially albumen. Albumen has a negative charge and is also able to bind chloride ions, resulting in the retention of sodium ions and therefore the retention of water within the capillary.

The net flow of water across the vessel wall is dependent on the degree of hydrostatic and osmotic pressure. This relationship at the level of the capillaries is referred to as Starling's law. Net flow of water (Q) through the capillaries can be determined using the following formula:

$$Q = k[(P_c + \pi_i) - (P_i + \pi)_c]$$

where k = constant, P_c = hydrostatic pressure of the capillary, π_i = oncotic pressure of the interstitium, P_i = hydrostatic pressure of the interstitium, and π_c = oncotic pressure of the capillary.

Since the hydrostatic force is the principle force and depends on the pressure in the arterioles and venules as well as the pressure in the arteries and veins, it is not a constant force. If the arterial pressure and the venous pressure increase, then the capillary hydrostatic pressure will increase. If the resistance through the arterioles is increased, then the capillary pressure will decrease. Therefore, the driving force of the filtration is through the difference of the capillary pressure minus the interstitium pressure. The velocity of fluid across the capillary wall is approximately 80 times the forward velocity of the fluid within the vessel. Small differences in concentration can result in huge variations in movement. There is an overall excess in fluid flow into the interstitium, and the excess is returned to the vasculature by the lymphatic system.

Sodium ions and other hydrophilic substances pass thought clefts between endothelial cells; hydrophobic substances such as oxygen and

carbon dioxide are able to pass through the cell membrane. In the microcirculation, oxygen molecules are released from hemoglobin in the red blood cells and diffuse through these pores into the extravascular space and into parenchymal cells until they reach the mitochondria, where they are utilized in oxidative phosphorylation *(9)*.

As the metabolic requirements of tissue change in going from rest to activity, there is a need for increased oxygen from increased blood flow to that area. Although a number of biochemical factors *(10)* can alter redox tone *(11)* and immune function *(12,13)*, during hemorrhagic shock, the lack of tissue perfusion initiates multiple cascades to correct tissue perfusion defects. Catecholamines, histamine, bradykinin, and cytokines are released in an attempt to correct permeability of the microcirculation. As the oxygen supply to the cells is depleted, cellular respiration switches from aerobic to anaerobic pathways, depleting available ATP. Normal cellular functions fall into disarray, with consequent loss of electrochemical gradients and swelling and rupture of organelle and cellular membranes, leading to eventual cell death.

Mechanical Forces Within the Microenvironment

To understand blood flow within the microcirculation, one must examine the microcirculation in a dynamic rather than a static mode. Changes in the microcirculation can occur from moment to moment or over the course of an organism's entire lifetime. Blood vessels are formed in a changing environment, which gives rise to an imbalance between the external forces tending to extend the vessel diameter and length and the internal forces tending to resist the vessel extension *(14)*. This process can be accentuated during growth and can decrease during aging, as is seen in the carotid artery *(15)*. This imbalance may stimulate elastin and collagen formation and reduce the stresses of the underlying tissue. Because of these conditions, a residual stress state exists when the vessel is fully retracted and free of external tractions.

Of importance to microvascular mechanics is the interaction of a vessel with the surrounding environment of connective tissue, parenchyma cells, and extracellular fluid. Mechanical forces can be generated when the vessels contract or dilate or when the surrounding tissue is in motion. Vasomotion or spontaneous oscillations of vessel diameter are predicted from theoretical analysis of the resulting nonlinear equations derived from temporal derivatives of these variables *(9,14)*.

The microvascular network in different tissues and organs is capable of changing its structural and functional characteristics by angiogenesis and rarefaction; therefore differences in blood flow distribution can be expected to occur, resulting in normal and pathologic changes. Blood flow rate in arterioles decreases toward capillaries in inverse proportion to the number of parallel vessels *(9,16)*.

Capillaries have a thin-walled lumen, so they can withstand high internal pressures. This relationship is explained by the law of Laplace:

$$T = Pr$$

where T = the tension in the vessel wall, P = the transmural pressure, and r = the radius of the vessel.

The microcirculation is important from a hemodynamic standpoint in that most of the hydrodynamic resistance of the circulatory system lies in the smallest vessels, especially the arterioles. Most of the exchange of nutrients and waste products occurs at the level of the smallest microvessels *(9)*.

The vessels of the microcirculation are small enough that effects caused by the cells that comprise the blood are significant. Although blood plasma behaves as a Newtonian fluid, the viscosity of whole blood decreases in the presence of increases in shear rate. The rheologic properties of blood in the larger vessels are determined primarily by the erythrocytes, whereas leukocytes play an important role in the mechanics within capillaries and small venules. The apparent viscosity of a fluid with a pressure difference (ΔP) that is causing it to flow within a cylindrical vessel is:

$$\eta_a = (\pi \Delta P r^4)/8QL$$

where r = radius of the cylinder, Q = flow rate, and L = length of the cylinder. When the fluid is a newtonian fluid, the apparent viscosity becomes the dynamic viscosity and the equation represents Poiseulle's law *(9)*.

Blood flow is often laminar but can become turbulent at bifurcations and high velocity in areas of occlusion (**Fig. 2**). Flow associated forces within the vessels have effects on the cells of the vascular system. Among these forces are the normal stress caused by the hydrodynamic pressure differences across the vessel wall, shear stress caused by the flow of blood across the endothelium, and tensile stress caused by circumferential vessel wall deformations *(17)* **(Fig. 2**). Changes in these

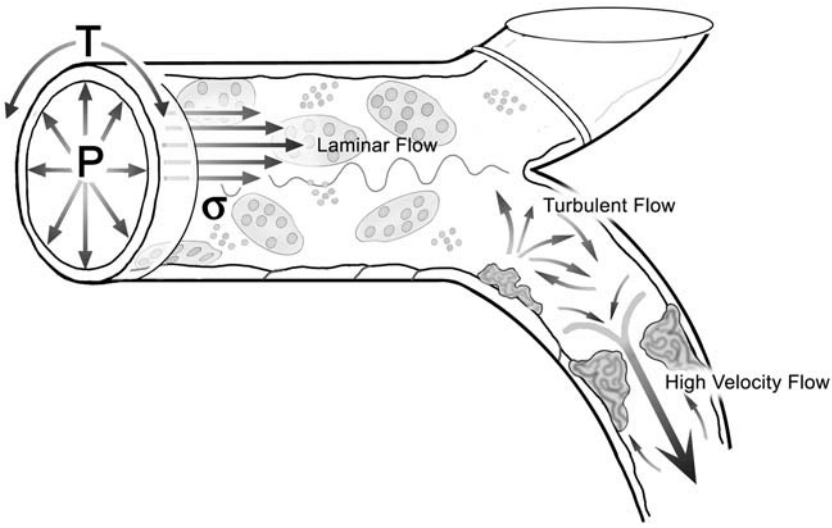

Fig. 2. Hemodynamic forces in the vasculature. The major hemodynamic forces that act on the vasculature are the normal pressure or blood pressure (P), the tensile force (T), and the shear stress (σ). Laminar flow becomes turbulent in areas of bifurcation, and high-velocity flow can result in regions where partial occlusions are present. (Illustration by Annabelle Wright.)

forces can precipitate increases in the release of adhesion molecules, matrix proteins, cytokines, and fibrinolytic factors; such changes can also cause neovascularization *(18)*. A number of diseases have been implicated in changes in shear stress, principally artherosclerotic plaque formation in areas of low shear and an increase in the deposition of procoagulants *(19)*.

An increase in blood flow to arteries and arterioles leads to increases in vasodilation caused by NO release from vessels *(20)*. The increase in blood flow increases shear stress at the vessel wall, which causes NO to be released, which causes vasodilation by vascular smooth muscle. There is a chemical and electrical coupling between the cells of the vessel wall that can travel along the vessel in either direction via gap junctions, which lowers the resistance to blood flow in a larger region *(9)*.

Capillary blood flow is characterized by erythrocytes flowing in single file with a small region of plasma in between them. The red blood cells (RBCs) deform into a parachute-like state, with a small plasma

sleeve between the RBC and the endothelium. Since leukocytes are larger than erythrocytes, a single leukocyte may cause an increase in capillary resistance that is 1000 times greater than that caused by one erythrocyte. Following ischemia, plugging by erythrocytes and leukocytes may prevent tissue reperfusion and decrease ischemia reperfusion injury *(9)*.

ADHESION

Adhesion Molecules

Adhesion molecules are present in one form or another on most types of cells. Of primary interest to hemorrhagic shock and the microcirculation are the classes of adhesion factors present on endothelial cells (the major cells of the immune system), and on platelets. Among these classes of adhesion molecules are the selectins, the integrins, and the immunoglobulin G superfamily. These become activated normally through interactions with intravascular factors including cytokines and the extracellular matrix and with each other during the course of an organism's development and function. However, during trauma and hemorrhagic and other types of shock, the damaged endothelium, decreased intravascular volume, and increase in substances that activate adhesion molecules can result in abnormal adhesion molecule activation and further tissue damage. Important classes of adhesion molecules and their ligands are listed in **Table 1.**

Inflammation

Leukocyte binding and traversing the endothelium is a key event in the initial immune response. Currently, this is understood as a four-step process under normal conditions and has become the model for other types of adhesion molecule and cellular interactions (reviewed in ref. *21*). Following activation, the endothelium upregulates a series of adhesion molecules that slow down neutrophils by transient binding or tethering (step 1) and then release, resulting in neutrophil rolling (step 2) in the direction of flow along the endothelium surface (**Fig. 3**). This is mediated primarily by the class of adhesion molecules known as selectins, which bind carbohydrate moieties on neutrophils. The selectin family is made up of E-and P-selectins, which are expressed on activated endothelium, and L-selectin, which is expressed on most leukocytes and can bind to carbohydrates on the surface of endothelial cells.

Table 1
Cell Adhesion Molecules Most Frequently Involved in Hemorrhagic Shock

Adhesion Molecule	*Ligands*	*Expression*	*Function*
Selectins			
P	Sialyl Lewis CHOs	Platelets, endothelium	Rolling (L), binding (P)
L	O-linked CHOs	Leukocytes	Rolling (L)
E	Sialyl Lewis CHOs	Endothelium	Rolling (L)
Integrins			
β_1	ECMP	Widely distributed	Firm adhesion (M,L)
β_2	ICAMS-1,-2, and-3	Leukocytes, macrophages	Firm adhesion (L), transmigration (L)
β_3	ECMP	Platelets, endothelium	Firm adhesion (L)
Immunoglobulins			
ICAMs	LFA-1, β_2-integrin	Endothelium	See β_2-integrin
VCAM	LPAM-1, VLA-4	Endothelium	Firm adhesion (L)
MAdCAM	LPAM-1, (α_4 β_7)	Endothelium	Firm adhesion (I)
PECAM-1	Glycosamino-glycans	P, L, endothelium	Transmigration (L, M)

CHOs, carbohydrates; ECMP, extracellular matrix proteins; L, leukocytes; P, platelets; M, monocytes; L, lymphocytes; ICAM, intercellular cell adhesion molecule; VCAM, vascular cell adhesion molecule; PECAM, platelet/endothelial cell adhesion molecule; LFA, leukocyte function-associated antigen; LPAM, Reproduced with permission from Martinez-Mier G, Toledo-Pereyra LH, Ward PA. Adhesion molecules and hemorrhagic shock. J Trauma 2001;51:408–415.

Furthermore, $\alpha_4\beta_1$ integrins can become upregulated on neutrophils and can initiate primary binding to endothelial cells by binding to vascular cell adhesion molecule-1 (VCAM-1).

During this period of rolling, neutrophils become activated by their exposure to interleukin-8 (IL-8) or platelet-activating factor (PAF), or because adhesion receptors cause an increase in the binding affinity of

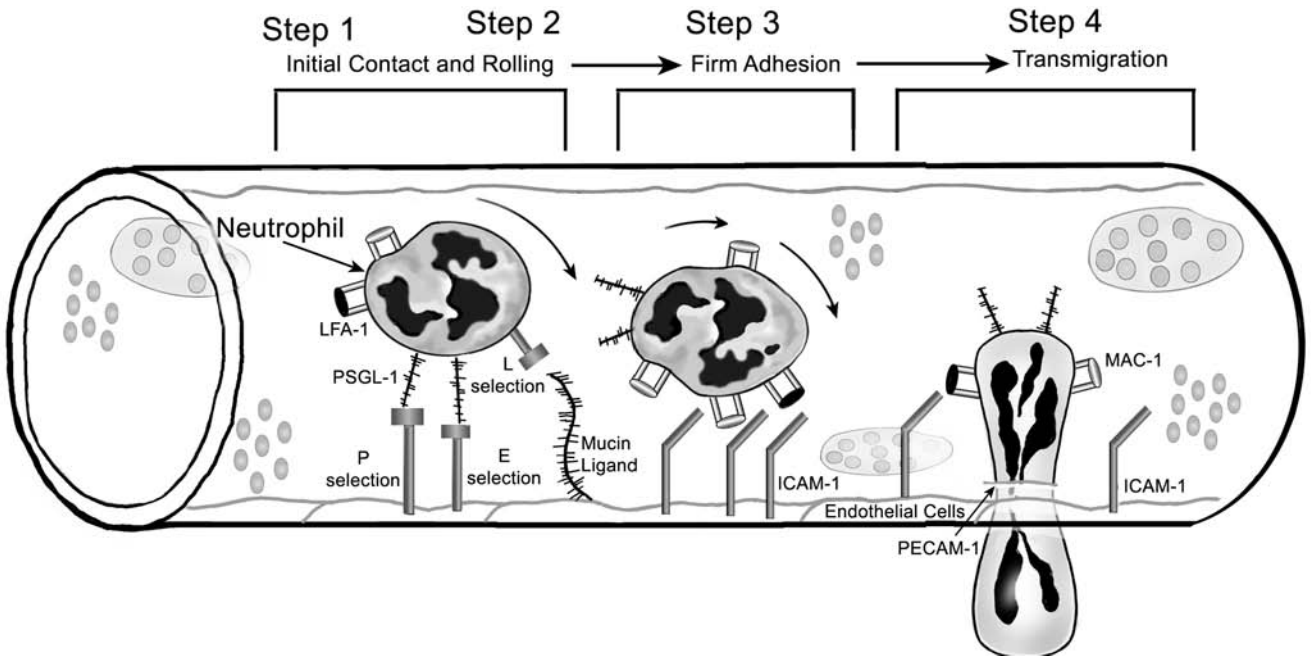

Fig. 3. The model for neutrophil adhesion and transmigration across endothelial cells. Steps 1 and 2. Neutrophil tethering and rolling are mediated by selectin-carbohydrate interactions. Step 3. Firm adhesion will occur if neutrophils receive activating signals while rolling along the endothelium. Attachment of β_2-integrins [leukocyte function-associated antigen (LFA-1) and membrane attack complex-1 (MAC-1)] on neutrophils to endothelial intercellular cell adhesion molecule-1 (ICAM-1) supports this cell adhesion to endothelial cells. Step 4. Transmigration of the neutrophil through the vessel wall will occur if a favorable chemotactic gradient exists. PECAM-1 appears to be required by binding to platelet/endothelial cell adhesion molecule (PECAM-1) on leukocytes. PSGL-1, L-selectin, P-selectin glycoprotein ligand-1. (Illustration by Annabelle Wright, based on ref. *21*.)

the integrin class of adhesion molecules. The integrins undergo a conformational change and an alteration in cytoskeleton binding, which allows their attachment to members of the immunoglobulin superfamily, principally intracellular adhesion molecule-1 (ICAM-1), on the endothelial cells *(22–24)*. This stops the rolling of the neutrophil and results in firm adhesion (step 3) (**Fig. 3**).

In the process of firm adhesion, neutrophils change shape; they flatten and then bind on the cell surface of the endothelium. This allows the neutrophil to undergo diapedes or transmigrate through the endothelium into the extravascular space. This process is mediated by platelet/endothelial cell adhesion molecule-1 (PECAM-1) on the surface of leukocytes (**Fig. 3**). L-selectins are also capable of binding free-flowing neutrophils to the surface of neutrophils already bound on the endothelial cell and can greatly increase the rate of cell accumulation at a site of inflammation *(25)*.

This is the paradigm used to understand normal adhesion of inflammatory cells under flow conditions. Other inflammatory cells follow a similar pattern. Lymphocytes and monocytes differ from neutrophils in that they express β_1 integrin receptors and bind via the $\alpha_4\beta_1$ integrin to VCAM-1 *(26,27)*.

The receptors involved in this multistep adhesion process tend to be present in specific areas of the neutrophil *(21)*. L-selectin, P-selectin glycoprotein ligand-1 (PSGL-1), and α_4 integrins are concentrated on microvilli, whereas β_2 integrins are present on the cell body. Although microvillous versus cell body location is not important in cell binding under static conditions, it results in a higher rate of cell binding under flow conditions *(28)*.

Thrombosis

The adhesion of platelets to exposed subendothelium at sites of vascular injury initiates thrombosis. This process involves the sequential involvement of distinct receptor molecules analogous to the process of neutrophil binding and diapedesis during inflammation. Glycoprotein (GP)Ibα on the platelet surface mediates initial adhesion with von Willebrand factor (vWF) on the subendothelium. Following platelet contact with the vessel wall, $\alpha_{IIb}\beta_3$ becomes activated and binds to vWF, resulting in permanent platelet arrest on the surface and thrombus formation.

Upon aggregation at sites of vascular injury, platelets express P-selectin on their surfaces. This expressed P-selectin can bind neu-

trophils, leading to their accumulation. Adherent neutrophils are able to migrate across the endothelium. This interaction between neutrophils and platelets may promote thrombosis and vascular occlusion and prevent the return of flow in the microcirculation in ischemic regions *(21)*.

HEMORRHAGIC SHOCK AND THE MICROCIRCULATION

Pathology

The pathology of the microcirculation during hemorrhagic shock is the development of microcirculatory failure, a condition known as systemic inflammatory response syndrome (SIRS). This is a clinical syndrome characterized by a temperature greater than 38°C or less than 36°C, a heart rate greater than 90 beats/min, a respiratory rate greater than 20 breaths/min, and a white blood cell count greater than 12,000 cells/mm^3, less than 4000 cells/mm^3, or greater than 10% immature forms. When SIRS results from a documented infection, it is referred to as sepsis.

In hemorrhagic shock related to trauma, the loss of intravascular volume leads to decreased tissue perfusion and oxygenation, which results in large areas of devitalized tissue. One possible cause for the development of SIRS may be the ischemia/reperfusion injury that is associated with resuscitation. This is a complex pathophysiologic process associated with hemorrhage and resuscitation in which tissue damaged during the ischemia period releases toxic metabolites and cells become activated upon reperfusion. Oxygen free radicals, clotting factors, and cells with activated adhesion factors are then able to damage tissue further.

Mechanisms

The specific mechanisms that govern cell-to-cell interactions and maintain homeostasis in inflammation and coagulation go awry during severe hemorrhage. The leukocytes and complement that guard against invading bacteria and other infectious agents become activated, and hemostasis shifts to thrombosis where orderly wound healing should occur *(29)*. This results in multiorgan failure, interstitial edema, and neutrophil accumulation in all organs. These areas are targets for therapeutics if they are delivered while the normal balance or functioning of these systems is retained or restored.

One major mechanism of ischemia/reperfusion injury is the increased margination of activated leukocytes to the endothelium. The cascade leading to this is initiated during the ischemic period *(30)*. During this time, ATP becomes dephosphorylated to AMP and is further metabolized to hypoxanthine and xanthine. When resuscitation occurs, the restoration of oxygen to the tissue results in the production of hydroxyl and hydrogen peroxide *(31)*. These oxygen free radicals cause injury of cell membranes through lipid peroxidation. Proinflammatory mediators such as tumor necrosis factor-α (TNF-α) and IL-1 become activated. TNF-α plays an important role in the cyclooxygenase pathway, and IL-1 stimulates T-helper cells to produce IL-2, which stimulates cytotoxic T-cells. IL-7 and TNF-α act synergistically to increase PAF, which results in hypotension, increased vascular permeability, and platelet aggregation. NO synthetase becomes upregulated with the sustained release of NO, leading to vasodilation, hypotension, and an increased response to adrenergic agents. Adhesion molecules on leukocytes become activated, resulting in an increase in leukocyte-endothelial cell interactions. Arachidonic acid metabolites, proteases, and more oxygen radicals are released by activated neutrophils, causing further damage to tissue monolayers. The ensuing cell necrosis leads to release of lysosomal enzymes and is directly cytotoxic

Complement activation normally enhances the ability of neutrophils to opsonize and destroy various pathogens. However, in hemorrhagic shock it contributes to cellular leakage by producing peptides that promote capillary leak and leukocyte migration at sites of infection or tissue damage. Acute blood loss leads to the activation of the complement cascade, and the degree of activation correlates with the severity of injury, development of multiorgan failure, and death *(32)*.

MEDICAL TREATMENT

Diagnosis

Hemorrhagic shock remains a clinical diagnosis. Signs and symptoms of SIRS in the setting of major trauma associated with a large amount of blood loss is consistent with hemorrhagic shock. At the present time, several laboratory tests are used that may assist in making the diagnosis of microvascular damage. These include the partial pressure

of arterial oxygen (which decreases), oxygen saturation (which may be normal), and serum lactate level. Several other parameters have been considered in septic shock but are not routinely used in the diagnosis of microvascular damage during hemorrhagic shock. Among these are the D-dimer *(33,34)*. Whereas levels of cortisol, thyroid hormone, calcitonin precursors, arachidonic acid, NO, endothelin, leptin, and adenosine correlate with severity of illness (reviewed in ref. *35*), cytokines are transiently elevated and may be used in the early detection of SIRS. Additional candidates include C-reactive protein, serum amyloid, leukocyte esterase, certain adhesion molecules, PAF, activated protein C, heat shock proteins, and fas ligand *(35)*.

Therapy

The primary treatment for hemorrhagic shock is the restoration of tissue perfusion by stopping the hemorrhage and replacing intravascular volume. To decrease the subsequent damage caused by the ischemia/reperfusion injury associated with the resuscitation process, blocking the adverse sequelae of this process has remained a goal of treating the microcirculatory damage in shock.

As adhesion factors are upregulated during hemorrhagic shock, it is hoped that blocking this process would decrease leukocyte margination and transmigration and subsequent tissue damage. This has been reviewed extensively by Martinez-Mier et al. *(36)*.

Genetically deficient P-selectin mice or wild-type mice given a monoclonal antibody to P-selectin or to soluble PSGL-1 immunoglobulin exhibit decreased leukocyte endothelium binding following hemorrhagic shock *(37)*. Winn et al. *(38)* showed that rabbits given monoclonal antibodies to P-selectin were protected from vascular injury. Similarly, Kushimoto et al. *(39)* showed a decrease of leukocytes into pulmonary tissue after treatment with monoclonal antibodies to P-selectin. Rivera-Chavez et al. *(40)* demonstrated increased survival when monoclonal antibodies to P-selectin or L-selectin *(41)* were given to rats in the setting of hemorrhagic shock. Other groups have shown that treatment with monoclonal antibodies to L-selectin improves survival and increased organ injury following hemorrhagic shock *(36)*. Schlag et al. *(42)* showed a beneficial effect on survival and mortality following anti-L-selectin treatment in primates. Selectin binding has been altered through the use of antibodies to selectin ligands *(37,43)*.

The blocking of β_2-integrins in addition to P-selectin in the liver does not appear to reduce fluid requirements *(44,45)*.

Interestingly, the time and type of fluid used to resuscitate alters expression of selectins, integrins, and ICAM-1 adhesion molecules (reviewed in ref. *36*). P-selectin upregulation is inhibited by the addition of pentoxifylline at 5 in one study *(46)*, and an increase in L-selectin on neutrophils was seen at 2 h when patients were resuscitated with lactated Ringer's solution compared with hypertonic saline *(43)*. Increased ICAM-1 expression was reduced 50% when hypertonic saline was used for resuscitation compared with lactated Ringer's solution *(45)*. Results of phase II clinical trials using monoclonal antibody to CD18 have shown a trend toward decreased intensive care unit stay without significant adverse reactions to the treatment *(47)*.

Prevention

There is evidence that the levels of soluble adhesion molecule expression have prognostic implications in the development of multiorgan failure after shock *(36)*, possibly because complications from the original injury lead to progression of other types of shock. This may play a role in blocking binding to prevent further tissue damage.

FUTURE DIRECTIONS

Each process that is activated during hemorrhagic shock has potential sites for medical intervention. Broadly, these are within the complement, coagulation, or inflammatory cascades. The inflammatory process can be attenuated through blockade of cytokine or xanthine formation, decreasing the shift in oxidative tone through the use of oxygen scavengers.

Adhesion factor activation may be decreased by blocking receptor-ligand binding on neutrophils and endothelial cells. This modality may be the first to be widely used in conjunction with current methods of resuscitation, principally with the use of monoclonal antibodies to selectins, integrins, or members of the immunoglobulin superfamily. Another way that adhesion factors may be effectively downregulated could be by blocking other signal transduction pathways, such as the inside-out pathways that can activate certain adhesion molecules. These approaches need not be limited to the early neutrophil response but may

be important in decreasing damage caused by the activation of monocytes, other antigen-presenting cells, and T-cells.

Although the current approach to blocking adhesion factor binding is through the use of monoclonal antibodies to decrease adhesion factor activation, the use of gene therapy to add genes to circulating cells that have been removed and then returned to a patient in hemorrhagic shock may soon be possible. These genes may be normal genes that downregulate adhesion factors, or they may be engineered to decrease adhesion factor binding to the vasculature in specific tissues. As the basic activation and deactivation steps are understood, it may be possible to develop trauma vaccines or genes that could be inserted with the ability to turn adhesion factors and other molecules on and off as required to benefit the patient.

The microcirculation and the role that adhesion factors play in hemorrhagic shock is a new and exciting field, and the understanding of it is growing at a rapid rate. Much remains to be learned about how adhesion factors function in relation to other byproducts of hemorrhage in the microcirculation. Only then will new treatment modalities become available for the patient in hemorrhagic shock.

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4 Heat Stress

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INTRODUCTION

Epidemiologic studies have shown that the health of military personnel who served in the Persian Gulf War theater is poorer and their mortality rates higher than military personnel who did not serve in that theater *(1,2)*. A number of studies have attributed this observation variously to stress, immunologic abnormalities, and neuroendocrine dysfunctions *(3,4)*. One of the important stressors to which military personnel were exposed was heat. Although little direct evidence currently exists to define the role that heat stress plays in long-term health, the significant effect that heat can exert on normal cell homeostasis suggests the need for such an investigation.

Exposing cultured cells, organs, or animals to sublethal heat stress induces changes in various signal transduction parameters within the cytoplasm and the nucleus. **Figure 1** summarizes some of the most important changes. In the short term, heat stress increases intracellular H^+, Ca^{2+}, Na^+, cyclic adenosine monophosphate (cAMP), and inositol 1,4,5-trisphosphate and activates protein kinase C, phospholipase C, and c-Jun N-terminus kinase. These intracellular changes are thought then to trigger expression of stress genes such as heat shock proteins (HSPs), c-Fos, c-Jun, c-Myc, and CD95 *(5)*.

Sublethal heat stress induces an adaptation in cells, tissues, organs, or animals that protects them not only from a subsequent, otherwise lethal heat stress but also from a variety of other potentially harmful exposures. These protective adaptations are termed thermotolerance

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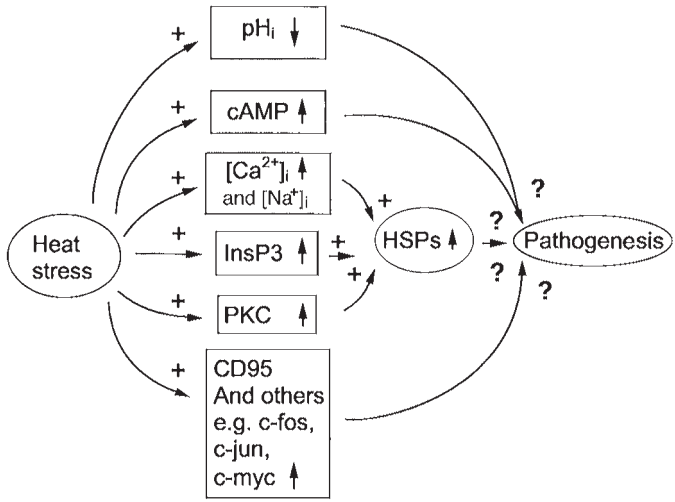

Fig. 1. Effect of heat stress on signal transduction pathways, heat shock proteins, and pathogenesis. Exposure of cells, tissues, or organs to heat stress not only decreases pH_i but also increases cellular cAMP, $[Ca^{2+}]_i$, $[Na^+]_i$, $InsP_3$, PKC, CD95, c-fos, c-jun, and c-myc. Increases in $[Ca^{2+}]_i$, $[Na+]_i$, $InsP_3$, and PKC but not pH_i and cAMP have been shown to mediate HSPs overexpression. It is not clear whether pH_i and cAMP as well as CD95 and oncogene proteins are involved in pathogenesis. However, it is evident that HSP are involved in diseases such as cancer, infections, and autoimmune disorders. +, induce; ↑, increase; ↓, decrease; ?, unknown; $InsP_3$, inositol 1,4,5-trisphosphate; PKC, protein kinase C; HSPs, heat shock proteins

and cross-tolerance, respectively; they are observed within hours of the initial stress and persist for days and weeks *(5,6)*.

The mechanisms by which thermotolerance and cross-tolerance develop remain unclear. It is thought that overexpression of HSPs plays an important role. In 1962, Ferussio Ritosa *(7)* first identified HSPs in heat-stressed *Drosophila* salivary gland cells. He found that heat triggers the production of HSPs in these cells and induces the development of spectacular chromosomal puffs. His observations were largely ignored for many years, but in the late 1980s the level of interest in the remarkable cellular response to heat stress and the process by which that response protects cells expanded dramatically.

Since then, a wealth of information has accumulated about the cellular processes involved. Important classes of proteins induced by heat

stress have been identified that prevent protein aggregation and protect critical biochemical processes in cells. Among these are the family of HSPs with molecular masses of 20, 60, 70, 90, and 110 kDa. In this review we discuss how HSPs are involved in various pathologic (clinical) conditions related to heat stress, with a special emphasis on signal transduction events.

PATHOLOGY

A synergistic relationship is known to exist between environmental and pathologic stressors *(5,6)*. Although our scientific understanding of such relationships is far from complete, accumulating evidence indicates that HSPs induced by elevated temperatures affect physiologic processes as varied as carcinogenesis, autoimmunity, and the response to infectious agents.

Cancer

HSPs are often upregulated in tumor cells exposed to various stressors including heat, acidic pH, low nutrition, and hypoxia. Overexpression of HSP-70 and-90 is found in various tumor cells, including acute leukemias, melanomas, ovarian cancers, pancreatic cancer, endometric cancer, colon cancer, and breast cancers *(8)*. Some of these cells exhibit resistance to treatment with doxorubicin, actinomycin D, and amphotericin *(9–12)*. It has also been documented that cancer cells exposed simultaneously to heat stress and chemotherapeutic agents die at a higher rate than cells treated with chemotherapeutic agents alone. However, cells exposed to heat stress prior to treatment with the chemotherapeutic agents exhibit resistance to drug treatment *(12)*.

Autoimmune Diseases

The view that HSPs play a role in autoimmune diseases remains controversial *(13)*. However, numerous studies have observed the presence of anti-HSP antibodies in sera from patients with rheumatic diseases, Graves' disease, and Hashimoto's thyroiditis. It has been reported that T-cell receptor αβ-positive (TCR $\alpha\beta^{+}$) T-cells recognize an epitope of HSP-65. This is thought to be responsible for autoimmune diseases such as adjuvant arthritis *(14)* and non-adjuvant arthritis in mice *(15)* and rats *(16,17)*. TCR $\alpha\beta^{+}$ T-cells also recognize a different epitope of HSP-65, and this recognition results in a modulation of autoimmune

diseases in rats *(16)*. Other studies have shown that HSP-70 may serve as an antigen that is recognized by a subset of T-lymphocytes that express γ and δ chains in place of the α and β chains *(18, 19)*. This T-cell subset is disproportionately increased in patients with the systemic autoimmune disease lupus erythematosus, which suggests that HSP-70 might play a role in this disease *(20)*.

Autoantibodies against HSP-90 and GRP-94 have also been detected in systemic lupus erythematosus, in which the usually constitutive HSP-90β is overexpressed. Antibodies against HSP-90 expressed on the surface of infectious organisms frequently cross-react with the highly homologous human HSP-90 and behave as an autoantibody. The epitopes of these autoantibodies are usually different from those of systemic lupus erythematosus *(8)*.

Despite these very interesting correlations, however, there are still insufficient data to link HSP metabolism casually with the origin and pathogenesis of autoimmune conditions such as human rheumatic disease.

Infectious Diseases

Several studies have raised the possibility that HSP-70, HSP-90, and GRP-94 may be involved in various aspects of the immune response *(13)*. Genes encoding two members of the HSP-70 family are found to reside within the MHC, and a protein-binding motif of HSP-70 is very similar to the peptide-binding cleft of the MHC class I proteins. A peptide-binding protein named PBP 74 (related to the HSP-70 family) is involved in peptide loading of MHC class II molecules. Deoxyspergulain, an immunosuppressant, is known to bind HSP-73 in a specific manner *(21)*. Another immunosuppressant, FK506, appears to bind to HSP-56 *(22)*. Two of 11 self-peptides isolated from purified class I HLA-B27 are derived from HSP-90. The physiologic and pathologic relevance of these observations is not clear. It is likely that they are involved in immune processes such as antigen presentation and cytotoxic cell killing of immune targets, which may lead to the autoimmunity.

In many bacterial infections in animals, the immunodominant antigen is GroEL, an analog of HSP-65. Antibodies raised against GroEL from one bacterial species tend to recognize the protein from all other species of the same genus, but not those of a different genus. In parasitic infections, the parasitic forms of HSP-70 and-90 represent a major target of the immune response. **Table 1** lists the infectious diseases in

Table 1
Infectious Diseases in Which HSP-70s and -90 Are Immunodominant Antigens

Infectious Agent	*Disease*
Borrelia burgdorferi	Lyme disease
Brugia malayi	Lymphatic filariasis
Chlamydia trachomatis	Trachoma
Leishmania donovani	Visceral leishmaniasis
Leishmania major	Leishmaniasis
Mycobacterium tuberculosis	Tuberculosis
Mycobacterium leprae	Leprosy
Onchocerca volvulus	Onchocercosis
Plasmodium falciparum	Malaria
Schistosoma mansoni	Schistosomiasis
Trypanosoma cruzi	Chagas' disease
Trypanosoma brucci brucci	Trypanosomiasis of cattle

Data from refs. *8* and *13*.

which HSP-70 and HSP-90 are the immunodominant antigens of the infecting organisms *(8,13)*.

MECHANISMS OF THE HEAT STRESS RESPONSE

Heat stress causes a number of alterations in various cellular metabolic parameters and overexpression of stress genes. It is known that intracellular pH, cellular cAMP, intracellular Ca^{2+} and Na^{+} concentrations, cellular inositol 1, 4, 5-trisphosphate ($InsP_3$), activity of protein kinase C, HSP-70 and-90 inflammatory mediators are changed significantly. Some of these changes may be involved in the pathogenesis of diseases mentioned above.

Intracellular pH

The resting intracellular pH (pH_i) in most cells ranges between 7.3 and 7.5. Typically, heat stress acidifies the intracellular environment of cells to pH 6.9 *(5)*. It has been documented that increases in pH_i can trigger processes such as DNA replication and cell proliferation *(23)*. Inhibition of intracellular alkalization induced by growth factors leads to blockage of cell growth *(24)*. In 3T3 and Vero cells, an increase in pH_i by 0.2 units is sufficient to induce tumorigenicity and growth *(25)*.

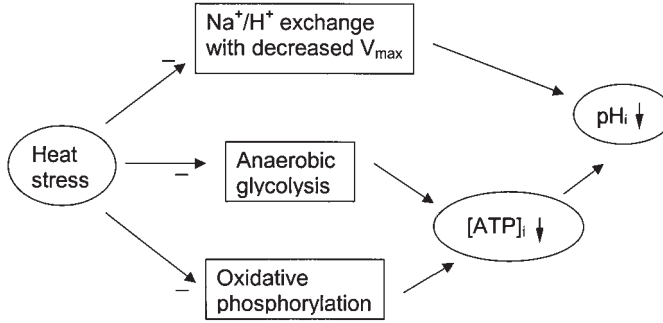

Fig. 2. Mechanism underlying heat stress-induced acidification. Heat stress inhibits Na^+/H^+ exchange by decreasing maximal velocity (V_{max}) without altering the apparent Michaelis constant K_m. Heat stress also blocks anaerobic glycolysis and oxidative phosphorylation, resulting in reduction of ATP production and accumulation of H^+.

Additionally, changes in pH_i can alter second messenger levels. In avian heart fibroblasts *(26)*, rat hepatocytes *(27)*, and human epidermoid A-431 cells *(28)*, intracellular acidification decreases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cellular cAMP. Conversely, both $[Ca^{2+}]_i$ and cAMP are increased by intracellular alkalinization *(28)*.

Figure 2 shows that the intracellular acidification induced by heat stress is by an inhibition of Na^+/H^+ exchange at the cell membrane and an inhibition of oxidative phosphorylation in the cytoplasm. Heat stress decreases the maximal velocity of Na^+/H^+ exchange but not the apparent Michaelis constant, K_m. Decreases in both intracellular ATP and lactic acid are also observed *(29)*.

In extracts of HeLa cells, activation of heat shock transcription factor (HSF, a factor responsible for HSPs production) occurs in cell extracts when the pH is adjusted from 5.8 to 6.4, with maximal activation occurring at pH 6.0 *(5)*. However, studies performed in our laboratory have shown that changes in resting pH_i neither affect the baseline levels of HSP-70 nor alter the ability of heat shock to induce HSP-70 in human A-431 cells *(30)*.

Heat stress still acidifies cells overexpressing HSP-70 as a result of prior exposure to heat stress or HSP-70 gene transfection, suggesting that there is no direct relationship between HSP-70 and pH_i *(5)*. Because cell acidification inhibits various deleterious biochemical processes and thereby promotes cell survival, preservation of the ability of

cells to reduce pH_i after heat stress is probably functionally important. The heat stress-induced reduction of pH_i is thus a defensive mechanism for cell survival.

cAMP

cAMP serves as the second messenger in the cell-signaling process of various hormones and cytokines. Heat stress increases intracellular cAMP levels in rabbit epididymis, human thymocytes, and human A-431 cells *(5)*. It has been reported that reduced intracellular cAMP levels cause the activation of many HSP genes in yeast. If cAMP levels are stimulated in female C57BL/6J Jcl mice by injecting 50 mg/kg dibutyryl cAMP, HSP-70 levels in the liver increase 3–8 h after injection. On the other hand, treatment of human A-431 cells with other cAMP-stimulating agents does not induce HSP-70 production. It is difficult to interpret the significance of these contrasting observations, however, since they involve exposure to the cAMP-stimulating agent for different lengths of time. In the mice, dibutyryl cAMP remained at measurable levels for up to 8 h, whereas A-431 cells were exposed to the cAMP-stimulating agent for only 20 min *(5)*.

Figure 3A shows that heat stress increases adenylate cyclase activity, G proteins, and adenosine levels resulting from heat stress-induced ATP breakdown *(31)*.

Human epidermoid A-431 cells that overexpress HSP-70 as a result of transfection with the HSP-70 gene retain normal basal cAMP levels, but heat stressing these cells results in a greater increase in cAMP than that which is measured in cells not overexpressing HSP-70. This increase in cAMP is a result of an increase in the enzymatic activity of adenylate cyclase and a decrease in phosphodiesterase *(5)*. Since there is now convincing evidence to support the view that HSP-70 protects many cells from otherwise lethal exposures, it may be that cAMP plays an important role in mediating this effect.

Intracellular Free Calcium and Sodium

Heat stress increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ and $[Na^+]_i$) *(32)*. **Figure 3B** shows that heat stress activates and opens tetradotoxin (TTX)-sensitive Na^+ channels. The increase in Na^+ influx in turn activates the reverse mode of Na^+/Ca^{2+} exchangers, which results in increased Ca^{2+} entry. The elevation in Ca^{2+} entry stimulates Ca^{2+} mobilization from intracellular Ca^{2+} pools sensitive to inositol

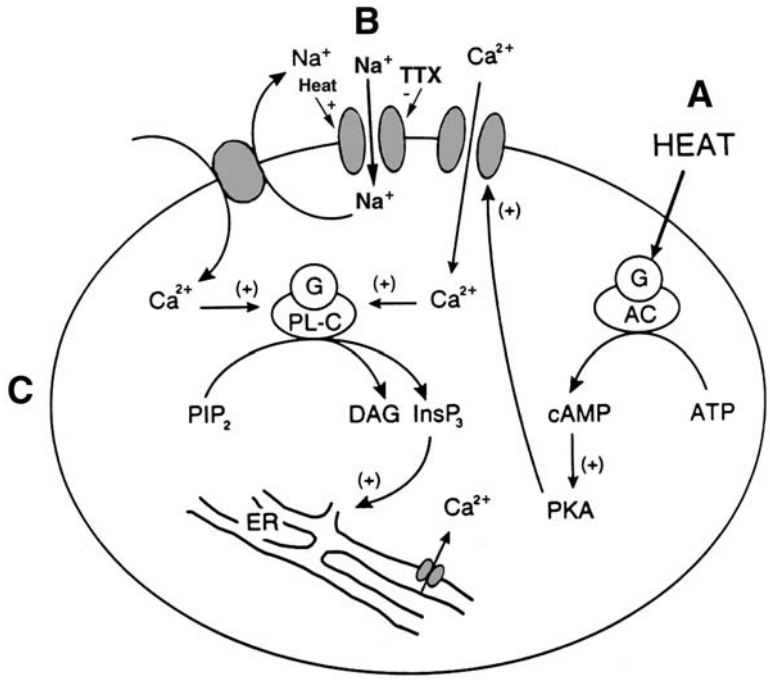

Fig. 3. Mechanisms underlying heat stress-induced increases in cAMP, $[Na^+]_i$, $[Ca^{2+}]_i$, and $InsP_3$. (**A**) Increases in cAMP. Heat stress activates G proteins (G) and adenylate cyclase (AC) so as to increase cAMP production, which activates protein kinase A (PKA). PKA then phosphorylates and opens second messenger-operated Ca^{2+} channels through which Ca^{2+} entry occurs. **(B)** Increases in $[Na^+]_i$ and $[Ca^{2+}]_i$. Heat stress induces increase in $[Na^+]_i$ by activating and opening tetradotoxin-sensitive Na^+ channels. An increase in $[Na^+]_i$ activates the reverse mode of Na^+/Ca^{2+} exchangers to remove excessive Na^+ and allow entry of Ca^{2+}, resulting in increased $[Ca^{2+}]_i$. **(C)** Increases in $InsP_3$. Increased $[Ca^{2+}]_i$ activates G proteins and phospholipase C (PLC), which cleaves PIP_2 to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$). Increased $InsP_3$ mobilizes Ca^{2+} from $InsP_3$-sensitive pools such as endoplasmic reticulum (ER). TTX, tetradotoxin; +, stimulation; –, inhibition.

1,4,5-triphosphate, ryanodine and ionomycin. In addition, increases in cAMP activate protein kinase A (PKA), which phosphorylates messenger-operated Ca^{2+} channels, resulting in greater Ca^{2+} influx *(5)*.

It has been shown that the binding of HSF to heat shock element (HSE) can be activated by Ca^{2+}, Mn^{2+}, and La^{2+} *(5)*. We have shown that increases in $[Ca^{2+}]_i$ induced by ionomycin (a Ca^{2+} ionophore) pro-

mote HSP-70 production in human epidermoid A-431 cells, Madin-Darby canine kidney (MDCK) cells, and rat luteal cells *(5)*. In human epidermoid A-431 cells, ionomycin also increases HSF1 gene expression *(5)*. Increases in $[Ca^{2+}]_i$ are involved in promoting HSP-70 mRNA and protein synthesis induced by heat stress. The role of Ca^{2+} in these processes is fairly clear because inhibiting an increase in $[Ca^{2+}]_i$ by (1) removing external Ca^{2+}, (2) adding ethyleneglycol-bis-β-aminoethylethei-*N*, *N*, *N*', *N*'-tetraacetic acid EGTA (a Ca^{2+} chelator) to the medium, or (3) treating with bis-(*o*-aminophenoxy)-*N*, *N*, *N'*, *N'*-tetraacetic acid (BAPTA; an intracellular Ca^{2+} chelator) greatly attenuates protein synthesis, HSP-70 gene expression, HSF binding to HSE, and HSF translocation from the cytosol to the nucleus *(30,33)*.

In cells that overexpress HSP-70 as a result of heat stress or HSP-70 gene transfection, increases in $[Ca^{2+}]_i$ induced by heat stress, air hypoxia, or chemical hypoxia are attenuated *(5)*. The attenuation may be caused by a desensitization of Na^+/Ca^{2+} exchange systems and other Ca^{2+}-related mechanisms *(5)*. Intracellular Ca^{2+} pools in human A-431 cells are also desensitized. It is not clear whether the size of the pools or their sensitivities to Ca^{2+} mobilizers is modified by HSP-70. Our data show that in human epidermoid A-431 cells, NaCN increases $[Ca^{2+}]_i$ by reducing the K_m and increasing the V_{max} of the Na^+/Ca^{2+} exchangers. In cells overexpressing HSP-70, attenuation of the NaCN-induced $[Ca^{2+}]_i$ increase is a result of a reduction of V_{max} *(5)*. It is possible that HSP-70 binds to the Na^+/Ca^{2+} exchanger to reduce its V_{max}, because others have shown that HSPs stabilize protein molecules such as steroid receptors *(5)*. HSP-70 may also modulate the Na^+/Ca^+ exchanger by altering the activity of PKA, protein kinase c (PKC), or phospholipase A_2 (PLA_2) or affecting the function of Ras, Raf, and pp60v-src kinase *(5)*, but these possibilities have yet to be investigated. Because a sustained elevation of $[Ca^{2+}]_i$ to micromolar levels leads to cell death, this attenuation of the $[Ca^{2+}]_i$ response to heat shock or hypoxia in cells overexpressing HSP-70 is one of the cellular defense mechanisms that promote cell survival.

Intracellular Inositol 1,4,5-Trisphosphate

Heat stress increases intracellular $InsP_3$ levels. **Figure 3C** shows that the increase in $[Ca^{2+}]_i$ induced by heat stress triggers phospholipase C (PLC) activity, thereby increasing both $InsP_3$ and diacylglycerol (DAG) levels *(34)*.

$InsP_3$ is important in the regulation of HSPs expression. We know this because treatment of human epidermoid A-431 cells with pertussis toxin, cholera toxin, or forskolin, which increases production of $InsP_3$, leads to increased levels of HSP-70 mRNA and protein. Conversely, treatment of cells with U-73122, an inhibitor of $InsP_3$ production, diminishes the heat stress-induced increase in the expression of HSP-70 *(30)*. The mechanism by which $InsP_3$ affects HSP-70 production is still not known, but binding of $InsP_3$ to its receptor alters RNA splicing *(5)* and regulates the expression of a number of different gene products *(5,35)*.

In cells overexpressing HSP-70, the basal level of $InsP_3$ is not altered. The heat stress-induced increase in $InsP_3$ is attenuated as a result of a diminished capacity of heat stress to induce increases in $[Ca^{2+}]_i$. We have not found any changes in the level of $InsP_3$ receptor expression and tyrosine phosphorylation in human epidermoid A-431 cells that overexpress HSP-70 *(5)*.

Protein Kinase C and Protein Phosphatases

Heat stress increases PKC activity and decreases protein phosphatase activities *(36,37)*. The changes are attributed to the increase in DAG; a known activator of PKC. Heat stress is known to increase activities of PKC isoforms βI, βII, and ζ in rat thyroid FRTL-5 cells *(38)* and the α, βI, βII, ϵ, and θ isoforms in human Jurkat T-cells (Kiang, unpublished data, 2002).

PKC phosphorylates proteins at serine and threonine residues. It has been shown that activation of PKC induces HSP-70s *(5)*. We have found that treatment of human epidermoid A-431 cells with phorbol 12-myristate 13-acetate (PMA; a potent PKC stimulator) increases the levels of HSP-70 mRNA and protein as well as the levels of HSF1 mRNA. Furthermore, treatment of A-431 cells with PMA increases the translocation of HSF1 from the cytosol to the nucleus and the binding of HSF1 to HSE. The level of HSF1 phosphorylation is also increased *(5)*. PMA also induces new HSP-70 synthesis in MDCK cells and rat luteal cells *(5)*.

When human epidermoid A-431 cells overexpress HSP-70, as a result of heat stress or gene transfection, enzymatic activity of PKC is significantly reduced, whereas the activity of protein phosphatases 1 and 2A is increased *(36)*. Similar changes have been observed in Jurkat cells transfected with the HSP-70 gene *(39)*.

It is not clear whether the reduction of PKC activity or the increase in protein phosphatase activity enhances cell survival. It has been shown that an imbalance of phosphorylation and dephosphorylation leads to apoptotic cell death *(5)*. Although the thermotolerance provided by HSP-70 overexpression is well established, it remains to be determined whether transient or long-lasting increases in HSP-70s benefit cell function and survival. We anticipate, however, that the heat-induced increase in PKC participates in pathogenic processes.

Heat Shock Proteins

Heat stress increases HSPs, especially the HSP-70 and HSP-90 families *(5)*. The increases depend on $[Ca^{2+}]$. It is known that a cytosolic resident HSF bound to HSP-70 or-90 is released after heat stress. PKC phosphorylates the free HSF, which then trimerizes. The HSF trimers enter the nucleus and bind to heat HSEs located on the promoter of the HSP genes, stimulating transcription and, ultimately, translation. The process is regulated by the newly synthesized HSPs, which bind HSF and thereby inhibit initiation of the cycle (**Fig. 4**).

In rats, heat stress increases inducible HSP-70 in the thyroid gland, pituitary gland, adrenal medulla, stomach, ileum, colon, kidney, liver, heart, and lung. The duration of increased HSP-70 expression varies among organs, ranging from 8 h to 4 d (Kiang, unpublished data, 2002). Cells overexpressing HSP-70 fail to respond to heat stress by further increasing HSP-70 production *(6,40)*, a consequence of desensitization of the Ca^{2+} response and PKC phosphorylation. Similar cytoprotective effects are observed in human epidermoid A-431 cells that are heat stressed followed by chemical hypoxia *(5)*. The increase in HSP-70 induction is believed to be responsible for cytoprotection *(5)*. Since this cytoprotection occurs in both normal and tumor cells, it is possible that HSP-70 overexpression may thereby inhibit processes involved in the body's defense against maladies such as cancer, infection, or autoimmune disorders.

Immune Parameters

In rat ileum, heat stress alone does not significantly alter leukotriene B4 generation, neutrophilic infiltrate, circulating neutrophil superoxide production, or prostaglandin E2. When rats are subjected to ischemia/reperfusion *(41)* or acutely induced inflammation *(42)*, leukotrine B4 generation and neutrophilic infiltrate markedly increase,

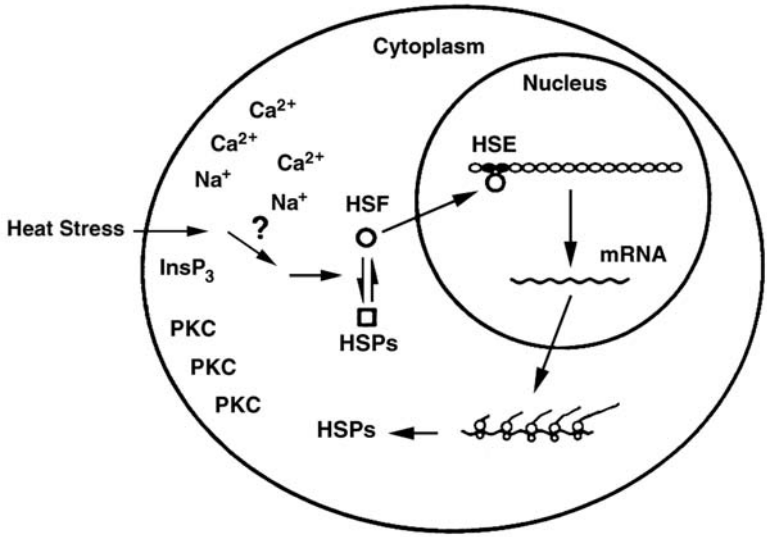

Fig. 4. Possible mechanism underlying heat stress-induced increase in heat shock proteins in cells. Heat shock factors (HSFs) residing in cytosol are normally bound by heat shock proteins (HSPs) and are inactive. After heat stress, increases in $[Ca^{2+}]_i$, $[Na^+]_i$, $InsP_3$, and/or protein kinase C (PKC) may trigger separation of HSF from HSP. Available HSF is phosphorylated by protein kinases such as PKC and forms trimers in cytosol that enter the nucleus to bind heat shock elements (HSEs) in the promoter region of the HSP gene. HSF is phosphorylated further, and HSP mRNA is transcribed and leaves the nucleus for cytosol. In cytosol, new HSP is synthesized. HSF returns to cytosol and is bound once again by HSPs.?, unknown.

villi tips rupture, and their contents are released. Heat stress administered prior to these otherwise harmful treatments results in a significant inhibition of leukotriene B4 generation and neutrophilic infiltrate, which protects villi from damage caused by ischemia/reperfusion *(41)* or acutely induced inflammation *(42)*.

In cultured human Jurkat T-cells, heat stress, but not hypoxia, increases CD95 expression (a protein related to apoptosis) on the cell membrane by 24 h *(43)*. When HSP-70-overexpressing cells were treated with CD3 or-95, the increased HSP-70 enhanced TCR/CD3- and CD95-induced apoptotic cell death. No cytoprotection was observed *(39)*.

In some Persian Gulf War veterans, the diagnosis of chronic fatigue syndrome has been shown to be associated with decreased numbers of

natural killer NK cells, higher total T-cell populations, and elevated concentrations of IL-2, IL-10, interferon-γ, and TNF-α *(44)*. It is possible that heat stress contributes to increases in these inflammatory agents. The evidence is ambiguous, however. HSPs have been shown to inhibit the production of TNF-α in lung endothelial cells, which results in protection against the development of lung injury *(5)*.

THERAPEUTIC APPROACHES

Elevated HSPs promote cell survival by protecting them from various stressors. This can benefit the body by protecting normal, healthy tissues, but HSPs can confer the same benefits to cancerous, autoimmune, and infectious cells. In that sense, elevated HSP expression is not a benefit.

HSPs also exhibit dichotomous effects within the same kind of cell. Macrophages respond to infection by releasing cytokines, oxygen free radicals, and nitric oxide, which are involved in killing the infecting organisms *(45)*. Cytokines, oxygen free radicals, and nitric oxide have been shown to increase HSPs synthesis in macrophages *(46,47)*, but the HSPs act in turn to inhibit the release of these same chemicals *(5)*. This negative feedback role of HSPs may inhibit the host's defense capability.

These examples accent the difficulties encountered in developing useful therapeutic approaches.

Cancer Treatment

It is known that increased expression of the multidrug resistance protein (MDRP) mediates the development of resistance to many cancer chemotherapeutic agents. The gene encoding MDRP contains a heat shock element *(5)*. Therefore, it is possible that modulation of HSP-70 content may enhance the therapeutic efficacy of chemotherapy and perhaps allow the use of lower doses of chemotherapeutic agents.

Studies have shown that certain tumor cell lines express HSP-70 on the cell surface *(5)*. Based on reports that natural killer cells are involved in eliminating cellular targets expressing HSP-70 *(5)*, it is tempting to speculate that increases in natural killer cells, or HSP-70 and HSP-90 vaccination, might have powerful antineoplastic activity *(5)*. However, the hypothetical effectiveness of such a therapy is questionable, since no studies have convincingly demonstrated that HSP-70

proteins are indeed membrane-anchored and not just loosely associated with the outer surface.

The efficacy of chemotherapy might be enhanced by downregulating HSP-70 and other related HSPs. It is known that inhibitors of nitric oxide synthases, PKA, PKC can inhibit overexpression of HSP-70 *(37)*. Incorporation of these inhibitors into current chemotherapy protocols could possibly benefit cancer patients.

Treatment of Autoimmune Diseases

Since anti-HSP autoantibodies are known to be present in sera from patients with rheumatic diseases, Grave's disease, and Hashimoto's thyroiditis *(13)*, it can be speculated that the anti-HSP autoantibodies represent an effort by the body to protect itself from the HSP-like proteins released by stressed cells and infectious pathogens *(48)*. Remedies such as immune-enhancing agents or vaccination with HSPs to increase anti-HSP antibodies in the sera might be useful for treating autoimmune diseases.

Treatment of Infectious Diseases

Like remedies for autoimmune diseases, vaccination and immune enhancement therapies directed against specific bacterial and parasitic HSPs might prove beneficial in combating infection. This requires that circulating host antibodies have access to the infectious HSPs, a process made difficult by the fact that HSPs are generally considered to be exclusively intracellular proteins. However, it is known that cytolysis of infected cells can release pathogen-derived HSPs to the extracellular space, and bacterial or parasitic GroEL is known to be secreted or bound to surface membranes.

The potential for immune therapies that attack infectious HSPs has in fact been demonstrated. Several studies have shown that immunization with HSPs purified from pathogens protects against diseases such as blinding trachoma *(49)*, Legionnaire's disease *(50)*, and malaria *(51)*. In some cases, however, immunity against pathogenic HSPs appears to exacerbate disease (e.g., Lyme disease) *(52,53)*.

CONCLUSIONS

The host response to heat stress is complicated and poorly understood. Many of the heat shock responses are common to all cells, but

other cells exposed to the same heat stimulus demonstrate wide variations in response, including signal transduction pathways and stress gene activation patterns.

The complexity of the cellular response to heat complicates efforts to design approaches to treat or prevent damage resulting from heat exposure. The effort is made even more complex because the heat stress response can sometimes benefit us by protecting healthy cells yet hurt us by protecting diseased cells that we do not want to protect.

PERSPECTIVES

HSPs from heat stress were observed by accident 41 years ago. Only in the late 1980s did the significance of the observation begin to be realized. The effect of HSPs on signal transduction processes and activation of stress-related genes and proteins soon began to be characterized, followed by observations on the role of HSPs in disease. Recent studies of HSPs have advanced our understanding of their roles in a variety of physiologic responses *(5)*. Structural and functional studies of HSPs have defined their function as molecular chaperones in processes such as protein maturation and degradation. Heat stress as well as physiologic stimuli, infectious agents, and environmental stressors can trigger genetic expression of HSPs. Overexpression of HSPs, specifically HSP-70s, can downregulate signal transduction processes, alter enzymatic activities, and protect cells from otherwise lethal insults.

Heat stress appears to play crucial roles in the survival of organisms. Signal transduction processes, stress gene activation, and new protein synthesis occur within minutes to hours after heat stress and can persist for days and weeks. HSPs are ubiquitous in cells under both normal and pathologic conditions, and their structure is evolutionarily conserved. To date, there has also been significant progress in understanding the structure of HSPs.

Despite the advances, little is understood about the role of HSPs in pathogenesis. It is evident that one of the primary roles of HSPs is to protect cells by preventing the harmful aggregation of proteins, which can occur in both normal and disease cells. Other cytoprotective roles involve HSP binding to certain proteins through distinct motifs, modulating signal transduction, including enzymatic actitivities, and triggering other stress protein production. Little is known about HSP genetic

interactions. It is clear that there are many other unrecognized functions for HSPs in healthy and disease states.

It has been shown that HSPs protect cells from noxious stimuli that cause either necrosis or apoptosis, yet no studies have addressed the differential effect of HSPs on these two distinct processes. Heat stress has been shown to induce apoptotic protein CD95 expression on the cell membrane of human Jurkat cells, which might explain how heat stress can lead to apoptosis *(43)*. No studies have investigated whether overexpression of HSPs inhibits CD95 expression on the cell membrane, although one study has shown that HSP-70 overexpression upregulates anti CD3-induced CD95 *(39)*.

Advances in molecular biology techniques will undoubtedly generate new tools to answer many questions about heat stress-related biologic changes. Vaccination against HSPs, immunotherapy to enhance HSPs, downregulation of HSPs by drugs, or modification of signal transduction pathways are possible approaches to solve the subtle puzzles of HSPs in disease progression. Is there any realistic hope of using HSPs therapeutically? The answer awaits further study and a much better understanding of HSP function *(54)*.

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5 Immune System

Madhusoodana P. Nambiar, PhD

INTRODUCTION

The golden hour—that precious 60-minute countdown to stabilize the wounds of soldiers, to save their lives and limbs—extends to days and weeks in field hospitals. After the battle of El Alamein, a surgeon wrote that severe wounds were often followed by illness (more or less serious, lasting for several days) in which many factors other than blood loss or its late effects operated *(1)*. The precise nature of these factors has been a source of debate ever since. Most critically wounded soldiers die immediately from rupture of the heart or major blood vessels or massive neurologic trauma. Around 50% of severely injured trauma patients who survive their initial injuries will succumb days to weeks after injury because of abnormalities of the immune system and infection/septic complications leading to the systemic inflammatory response syndrome (SIRS) and multiple organ system failure (MOF) despite proper therapy. The development of MOF after trauma is associated with remote organ failure (ROF), the dysfunction of organs that were not affected by the injury.

A direct relationship exists among the extent of injury, depression of immune function, and a predisposition of the patients to develop infection/septic complications. Although increasing numbers of well-controlled patient studies remain important in defining the consequences of traumatic injury, experimental models of trauma and hemorrhage continue to play a pivotal role in defining the mechanisms of immunodepression and in characterizing the effects of soft tissue trauma and blood loss on immune function *(2)*. In addition to the cellular effects, accumulating evidence suggests that sex hormones also

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modulate the immune system after severe injury *(3)*. This chapter briefly describes the effect of trauma on the immune response and discusses novel therapeutic approaches aimed at restoring a functional immunologic balance in trauma victims.

The immune system is a remarkable defense mechanism that has been categorized (based on the type of immunity conferred) into innate (natural) immunity (nonspecific) and acquired immunity (specific) (**Fig. 1**). The innate immunity, the body's first line of defense against foreign challenge, is provided by mechanical barriers like the skin, mucous membranes, pH, interferons, and other substances released by leukocytes. Innate immunity also includes phagocytic cells such as granulocytes, macrophages, and monocytes, as well as the hepatic Kupffer cells that comprise the reticuloendothelial system and complement. This system is present from birth and does not require preexposure to a pathogen to be active; it constitutes the first line of defense against foreign challenge. The innate system does not require prior exposure and is not modified by repeated exposures to the pathogen. The acquired immune response is absent on first exposure but increases dramatically with subsequent exposures. It is composed of three distinct populations of lymphocytes, T-cells, B-cells, and accessory cells such as macrophages, to present antigen. T-lymphocytes are responsible for cell-mediated specific immunity, and B-lymphocytes generate the humoral (or antibody) response. The innate and acquired systems act in concert and depend on each other for maximal effectiveness. The acquired immune system is more complex and plays a predominant role in human health and disease.

ANIMAL MODELS OF TRAUMA AND HEMORRHAGIC SHOCK

Study of the immune response to trauma is often difficult in clinical settings. Massive blood loss is common in severe injury, making it difficult to assess the relative importance of blood loss versus soft tissue trauma or bone injury in contributing to the immunodepresssion. Experimental models of simple hemorrhage, tissue injury, and fracture are important to dissect the role of severe hemorrhage in overall immune dysfunction in trauma patients. Much of our current understanding of the immune responses to trauma comes from laboratory studies using animal models such as rodents. The expense of large ani-

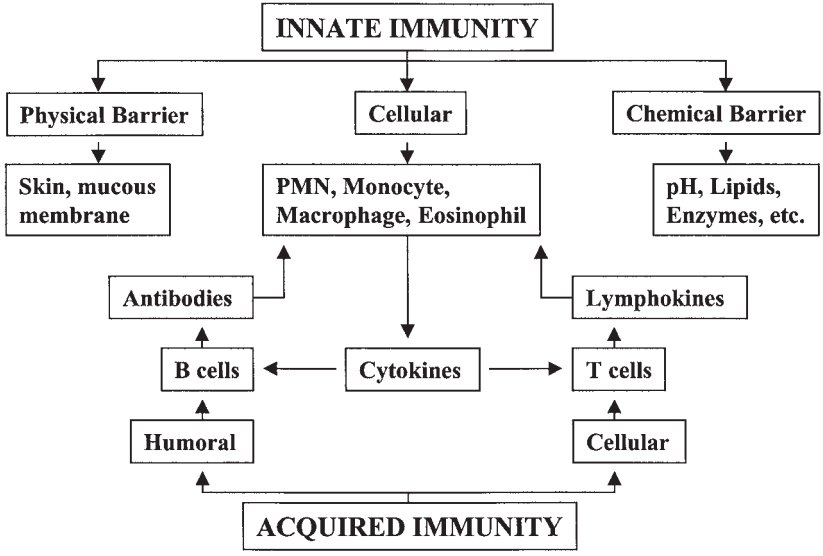

Fig. 1. Schematic view of the immune system. PMN, polymorphonuclear lymphocytes.

mals as well as the unavailability of immunologic assays and relevant reagents has limited the utilization of larger animals such as pigs, dogs, and primates in studies of immune function after hemorrhage. Three hemorrhagic shock models are commonly employed: (1) the fixed pressure or Wiggers model, (2) the fixed volume model, and (3) the continuing hemorrhage model *(4)*. The fixed pressure model involves withdrawal of blood sufficient to lower the mean arterial pressure to a fixed level. In this model, one arterial line is used to monitor arterial pressure continuously and another is used for blood withdrawal and subsequent fluid resuscitation. In the fixed volume model described, a predetermined percent of the circulating blood volume, based on animal body weight, is withdrawn over a 60-s period, and fluid resuscitation is given by injection into the retro-orbital venous plexus. The continuing hemorrhage model, although used extensively to study models of fluid resuscitation, has not been employed in the study of immune function after trauma. Many laboratories employ models of soft tissue trauma induced by midline laparotomy prior to hemorrhage to unravel the effects of trauma and shock on immune function. Various

other insults including bone fracture, cecal ligation and puncture, and pneumonia have been coupled with hemorrhage to study their cumulative effects on immune function *(2)*.

PATHOLOGY

Effects of Injury on the Innate Immune System

Antigenic products from tissue destruction or pathogenic organisms activate cells of the innate immune system, which in turn synthesize potentially toxic mediators including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6, as well as kinins, platelet activation factor, eicosanoids, hydrogen peroxide, and nitric oxide. An overwhelming inflammatory response leads to immune dysfunction and increases susceptibility to infection and sepsis. Innate immune mechanisms play an important role on their own in controlling posttraumatic infection in its early phase while the adaptive immune response is being developed and also through their impact on the adaptive immune response. A schematic view of the effects of trauma and hemorrhage on immune functions is shown in **Fig. 2**.

Complement Activation

Damaged tissue, foreign material, or bacterial lipopolysaccharide can activate the alternate complement pathway. Complement activation results in the assembly of membrane attack complex, C5b-9, on the surface of the bacteria, followed by lysis. Products of complement activation, C3a and C5a, increase capillary permeability and play an important role in the initiation of chemotaxis. Clinically, increased levels of C3a and membrane attack complex are found to be associated with the development of multiple organ dysfunction after trauma. Complement is invariably activated during organ injury if it is suspected to be activated during hemorrhage The complement system in trauma, emergency, and combat medicine is covered in a separate chapter.

Effects of Injury on Monocyte/Macrophage Function

Monocytes are derived from progenitor cells in the bone marrow and then released into the blood, where they migrate and settle in different organs and tissue systems. Once settled, blood monocytes are called tissue macrophages, and they include hepatic Kupffer cells, alveolar macrophages, pleural macrophages, peritoneal macrophages, splenic

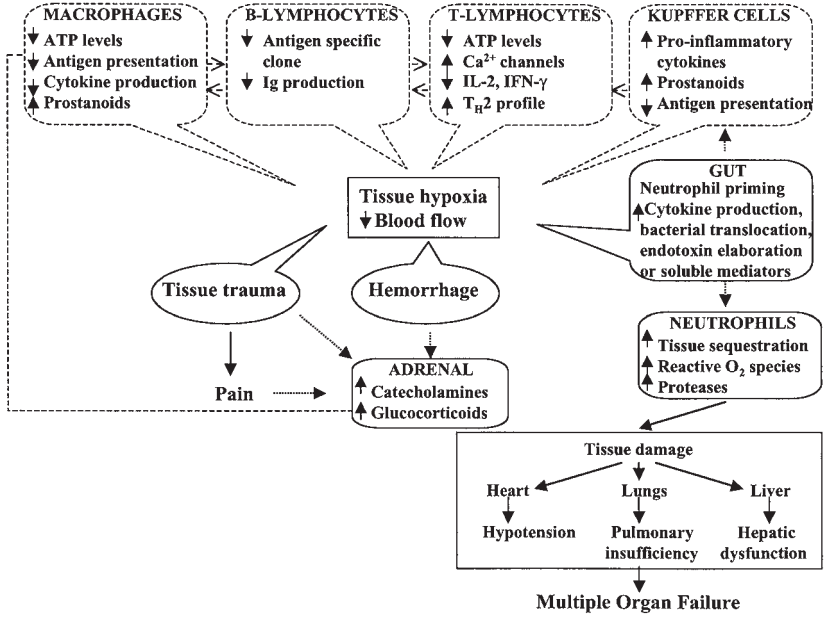

Fig. 2. Overview of the immunologic consequences of trauma and hemorrhage. Arrows with solid lines represent the effect of trauma and hemorrhage on immune function. Arrows with dotted lines depict a stimulatory effect. Call-outs with broken lines represent suppressive effects on the related function. IFN-γ, interferon-γ; IL-2, interleukin-2.

macrophages, Langerhans cells, and others. The mononuclear phagocyte system has two main functions in the immune system, phagocytosis and antigen presentation. Phagocytosis and intracellular killing are the hallmark of the innate immune response. As antigen-presenting cells to both B-and T-lymphocytes, they are also important in regulating the acquired immune system.

Kupffer cells exhibit increased cytotoxicity after hemorrhage, whereas hemorrhage has been shown to depress the cytotoxic ability of both splenic and peritoneal macrophages. Production of the cytokines IL-1 and TNF has been shown to be depressed in splenic and peritoneal macrophages but enhanced in Kupffer cells *(5)*. Superoxide anion synthesis, phagocytosis, and respiratory burst activity in peritoneal macrophages was shown to be depressed following trauma *(6,7)*. Different resident macrophage populations are exposed to different

stimuli based on their microenvironment, and these differences result in divergent immune responses after injury.

Mononuclear cells also secrete numerous cytokines (IL-1, IL-6, IL-8, IL-12, and TNF-α) in response to bacterial pathogens, leading to local inflammaory response that further serve to modulate immune function. Numerous studies have shown elevated prostaglandin E_2 (PGE_2) synthesis by macrophage/monocytes after trauma and or/hemorrhage *(6,8,9)*. PGE_2 is a potent inhibitor of lymphocyte and macrophage function *(10,11)*. Splenic and peritoneal macrophages exhibit a downregulation of proinflammatory cytokine production after injury. Kupffer cells, which behave differently from peritoneal and liver macrophages, show an upregulation of proinflammatory cytokine production after hemorrhage.

The mechanisms responsible for abnormal macrophage/monocyte function after trauma include defects in signal transduction, cyclic adenosine monophosphate (cAMP) regulation, and calcium homeostasis. Depletion of intracellular adenosine triphosphate (ATP) levels in macrophages after hemorrhage correlates with depressed antigen presentation and cytokine production *(12)*. Mediators released from the gut and active in liver and tissue hypoxia can also alter macrophage function *(2)*.

Effects of Injury on Neutrophil Function

Neutrophils are important effectors in the innate immune response and make up an army of phagocytes that respond quickly in vast numbers wherever tissue injury occurs. The mature cells known as polymorphonuclear leukocytes (PMNs) are identified by abundant storage granules containing bactericidal agents and lysosomal enzymes that serve to phagocytize opsonized pathogens. Upon activation, neutrophils synthesize potentially toxic mediators and reactive oxygen species. After injury, neutrophils are sequestered in end organs and serve to mediate host tissue injury *(13–15)*. Neutrophils 'primed' by ischemia in rodent models and activated by low-dose lipopolysaccharide induce pulmonary sequestration and capillary leak and result in mortality.

PMNs isolated from severely injured trauma patients were shown to exhibit enhanced in vitro O_2 release and to upregulate CD11b cell surface expression. Furthermore, there was a rapid clearance of PMNs

from the circulation in patients who went on to develop MOF, suggesting that the upregulation of CD11b leads to tissue sequestration and end-organ injury *(15)*. CD11b-CD18 is the ligand for the endothelial cell-expressed intracellular adhesion molecule 1, and this interaction causes transmigration of neutrophils into surrounding tissue. *(16)*. Reperfusion injury can be minimized by blocking the ability of PMNs to interact with endothelial cells using a monocloual antibody directed against L-selectin *(17)*. PMNs have also been shown to regulate splanchnic blood flow and cardiac function by the release of toxic molecules that inhibit splanchnic PGI_2 release after hemorrhage and resuscitation *(18)*. PMNs from trauma and intensive care unit patients have been shown to be defective owing to auto-oxidative injury to their cell surface receptors that is closely linked to the development of nosocomial infections, suggesting that antioxidant therapy may be useful in the care of the critically ill trauma patient. Thus, after trauma, hemorrhage, and ischemia, alteration in neutrophil function not only serves to mediate the development of MOF but also correlates with increased rates of nosocomial infection.

Effects of Injury on Natural Killer Cells

Natural killer (NK) cells, which are non-T, non-B lymphoid cells bearing no known antigen-recognizing receptors, play an important part in innate immunity. Infected cells bound by antibody are destroyed by NK cells, a phenomenon known as antibody-dependent cell-mediated cytotoxicity through the FcγRII receptor. Suppressed NK cell activity has been reported after both experimental and clinical injury, suggesting that impaired NK cell function may contribute to increased susceptibility to posttraumatic infection *(19)*.

Effects of Injury on Acquired Immune System

T-Lymphocyte Function Following Injury

Numerous investigators have studied the effects of injury on T-lymphocyte function. The functional diversity of T-lymphocytes is determined in large part by the antigens displayed on the cell surface. Based on the expression of T-cell receptor, T-lymphocytes are subdivided into αβ and γδ subsets. The αβ T-cells are further divided into helper ($CD4^+$) cells and cytotoxic/suppressor ($CD8^+$) cells. The CD4 subset has been functionally classified as $CD45RO^+$ and $CD45RA^+$ in

humans. The $CD4^+$ T-cell clones secreting IL-2 and interferon IFN-γ are termed T-helper 1 (Th1) cells; those secreting IL-4, IL-5, IL-6, and IL-10 are termed Th2 cells. Th1 cells mediate several functions associated with cytotoxicity and local inflammatory reactions and are important for combating intracellular pathogens. Th2 cells are more effective at stimulating B-cells to proliferate and produce antibodies.

It is well known that trauma, burns, and hemorrhage cause decreased lymphocyte proliferation in response to mitogenic stimulation. This effect lasts for several days after injury despite maximum resuscitation and normal recovery. The impairment of T-cell proliferative response is associated with an increased susceptibility to infection and death *(20)*. IL-2, an important cytokine required to induce T-cell proliferation, was also found to be depressed following hemorrhage *(21)*. Trauma- and/or hemorrhage-mediated depression of splenocyte proliferation and, IL-2 and IL-3 production was mainly dependent on the severity and not the duration or the resuscitation regimen *(22)*. Although depression in T-cell function is known to occur early following hemorrhage, the duration of the depression correlates with associated injuries. Trauma combined with hemorrhage was able to depress T-cell function beyond 5 d, whereas T-cell function returned to normal by that time following hemorrhage alone *(23)*. Hemorrhage combined with long bone fracture further depressed splenocyte proliferation and IL-2 and IL-3 production; in addition, the normal increase in osteoblast activity was depressed after hemorrhage *(2)*.

There are at least three distinct phases of T-cell depression following multitrauma injury in humans. Some trauma patients do not have a depression in T-cell mitogenic response. Another subset of patients exhibits a monocyte-dependent depression in T-cell function that does not occur in purified T-cell populations. Yet another subset of patients has depressed T-cell function independent of monocyte activity; this group runs the highest risk of mortality *(24)*. Overproduction of PGE_2 by monocytes has been suggested as one mediator of this depression in humans. The principal cellular abnormalities that result in altered IL-2 production remain downstream of the initiation of signal transduction events and involve protein tyrosine phosphorylation and calcium signaling *(25)*.

After injury, there is shift in the Th1/Th2 T-cell balance toward a dominance of Th2, leading to downregulation of cell-mediated immu-

nity and in some cases an upregulation of antibody-mediated immunity *(26)*. In vivo treatment with IL-12, which induces a shift from the type 2 toward the type 1 T-helper cell phenotype increases survival after major trauma. The response to traumatic and thermal injury in humans involves the appearance of immature T-cells in the peripheral blood.

Effects of Injury on Lymphocyte Nitric Oxide

The activation of cytokine inducible nitric oxide synthase (iNOS) during septic injury leads to the production of large quantities of nitric oxide (NO), a recently discovered radical and biologic mediator. Lymphocyte proliferative activity is impaired by macrophage-derived NO *(27)*. The addition of *N*-monomethyl-L-arginine, a competitive inhibitor of NOS, improves the suppressed lymphocyte proliferation after burn injury, indicating a possible immunosuppressive role for NO in major injury. Secretion of IL-2 and IFN-γ by the Th1 cells was enhanced in the presence of NOS inhibitor but was inhibited by the addition of NO donor, suggesting that NO may exert a self-regulatory effect on Th1 cells *(26)*. It has also been proposed that NO may support a Th2 lymphocyte immune response. In animal studies, treatment with a NOS inhibitor caused a significant reduction in T-cell activity, suggesting that NO may promote such activity. The apparent contradiction in the effect of NO on T-cell activity may be owing to the dual effect of NO on T-cell proliferation. Low NO may be required for normal immune response, whereas high concentrations may inhibit cellular proliferation. The effect of trauma on NO is covered in a separate chapter in this book.

Effects of Injury on B-Lymphocyte Function

B-lymphocyte-derived antibodies mediate the humoral component of the acquired immune response. The proportion of B-cells that actively produces immunoglobulins is less than 1% of the total number of plasma cells and is amplified by as much as 10% after systemic infection. Antibodies protect the host from infections by pathogen neutralization, opsonization, or complement activation. Antibody response to antigen by B-cells requires T-cell help. B-cells are recognized by T-helper cells when the antigen is expressed on the cell surface as peptide-bound MHC class II molecules. The interaction of an antigen-binding T-cell with an MHC class II peptide-bound B-cell leads to the

expression of B-cell-stimulatory CD40 ligand on the T-helper cell surface and to the secretion of stimulatory cytokines. B-cell differentiation is regulated mainly by type 2 T-helper cells characterized by the expression of IL-4, IL-5, and IL-6.

Injury has been shown to affect B-cell maturation and response to infection. Spontaneous production of IgG or IgM from B-cells of trauma patients has been variously reported to remain unchanged, enhanced or reduced. Current data suggest that the impaired B-cell response after trauma may be caused by, failure in T-cell help and that it is not PGE_2 mediated *(28)*. Richter et al. *(29)*, in a study evaluating the response of B-cells to trauma and surgery, demonstrated that trauma resulted in depressed immunoglobulin synthesis by circulating B-cells. In trauma patients, depression in immunoglobulin synthesis by peripheral blood mononuclear cells correlates with elevated TNF-α secretion. Partial blocking of TNF-α activity using a neutralizing monoclonal antibody restored immunoglobulin secretion, whereas complete blocking eliminated it, suggesting that some degree of TNF production is necessary for appropriate B-cell function. It has also been demonstrated that in mice immunization within 24-h of hemorrhage results in 50% depression in antigen-specific plasma cells relative to sham controls *(30)*. The spontaneous expression of CD23, a B-lymphocyte activation antigen, is reduced on B-cells from burn patients. In addition, a decreased response to viral antigens occurs in children with blunt trauma. Major trauma and minor injury or stress in mice result in a gradual fall in the level of B-lymphocytes that returns to normal within 24 h. Hemorrhage causes a decrease in serum immunoglobulin levels as well as decreased numbers of B-cell-secreting antibody *(31)*. There may be a change in B-cell repertoire after hemorrhage, so that in the week after the hemorrhage there are fewer pre-B-cells present potentially to become plasma cells that produce antibody.

It is clear that the function of antibody-producing B-lymphocytes is variably affected by traumatic injury. Because B-cell function is directly affected by cytokine production by macrophages and T-lymphocytes, as well as interactions with antigen-specific helper T-cells, abnormalities at any stage of the B-cell activation cascade may result in a diminished B-cell response. Thus it remains unclear whether the impaired B-lymphocyte function could be secondary to alterations in T-lymphocyte and macrophage function following trauma.

Effects of Injury on Antigen-Presenting Monocyte/Macrophage Function

With respect to acquired immunity, macrophages are antigen-presenting cells that are crucial for the activation of antigen-specific T-cells. After both experimental and clinical injury, antigen presentation and MHC class II molecule expression have been shown to be depressed, leading to impaired immune response. Even though MHC class II receptor expression was depressed on macrophages after hemorrhage, these cells were able to present predegraded antigen, suggesting that the defect in antigen presentation was the inability of macrophages to process foreign antigens *(32)*. Increased production of PGE_2 by macrophages after hemorrhage adversely affects antigen presentation by depression of Ia (similar to MHC class) antigen expression *(33)*.

The mechanisms responsible for mediating these alterations in macrophage/monocyte function after trauma and hemorrhage are not clear, but it has been suggested that defects in signal transduction, cAMP regulation, and calcium homeostasis play a role. Depleted intracellular ATP levels have been demonstrated in macrophages after hemorrhage, and this depletion correlates with depressed antigen presentation as well as downregulation of IL-1, IL-6, and TNF-α synthesis. Treatment with ATP-$MgCl_2$ was shown to increase macrophage ATP levels after hemorrhage and also resulted in normalization of each of these measured parameters. It has also been shown that performing a portacaval shunt 2 wk before hemorrhage prevents the depression in antigen presentation by splenic macrophages, indicating that mediators released from the gut and active in the liver play a role as well. Furthermore, hypoxia in the absence of blood loss was shown to enhance peritoneal and Kupffer cell release of IL-1, IL-6, and TNF-α suggesting that tissue hypoxia can also alter macrophage function.

Effects of Gender, Age, and Sex Hormones on the Immune System After Injury

Effects of Gender and Age

Many epidemiologic studies indicate the importance of gender and age as risk factors of sepsis and MOF after trauma *(34)*. Gender differences exist not only in the prevalence of trauma but also in an increased susceptibility to septic complications after trauma. Studies have shown

a higher survival rate in women compared with men after onset of sepsis. However, postmenopausal women show a higher mortality rate after sepsis compared with men. Experimental studies show that the proestrus state of the estrus cycle is characterized by a more vigorous immune response than the diestrus state, suggesting that the state of the estrus cycle influences the immune response after hemorrhage. Thymocyte apoptosis was increased in males but not in proestrus females after trauma and hemorrhage, contributing directly or indirectly to the development of host immunosuppression after trauma and hemorrhage in males through the loss of maturing T-cells. Increased apoptosis in males might also be a mechanism of the immune system to eliminate autoreactive T-cells that might be induced by trauma and hemorrhage. Changes in the levels of male and female sex steroids with age seem to contribute to the loss of immunoprotection present in younger females. Thus it is important to consider age, gender, and state of the estrus cycle in studies evaluating immune responses after trauma and hemorrhage.

Effects of Sex Hormones

Several studies indicate that the gender-specific immune response is regulated by hormones from the gonads, thymus, and hypothalamus-pituitary gland *(3)*. Depletion of male sex steroids by castration prevented the suppression of splenic and macrophage cytokine release and splenic immune response after trauma and hemorrhage (**Fig. 3**). Also, castration normalized the increased proinflammatory cytokine release by Kupffer cells in males. Female mice with artificially elevated testosterone levels displayed a depression that was similar to that in males in splenic and peritoneal macrophage function as well as IL-2 and IFN-γ release after trauma and hemorrhage. It is not known whether the reduction in the level of plasma estradiol induced by the administration of testosterone might contribute to the immunodepression after trauma and hemorrhage. Numerous studies evaluating the effects of sex steroids on the immune system show that estradiol has an immunoprotective effect on cell-mediated immune responses after trauma and hemorrhage. Conversely, depletion of female sex steroids by ovariectomy depressed cell-mediated immune response after trauma and hemorrhage. A high ratio of female-to-male sex steroids seems to exert protective effects for the host after trauma and blood loss.

Administration of prolactin modulates the altered immune response and gene expression after trauma and hemorrhage *(35)*. Prolactin treat-

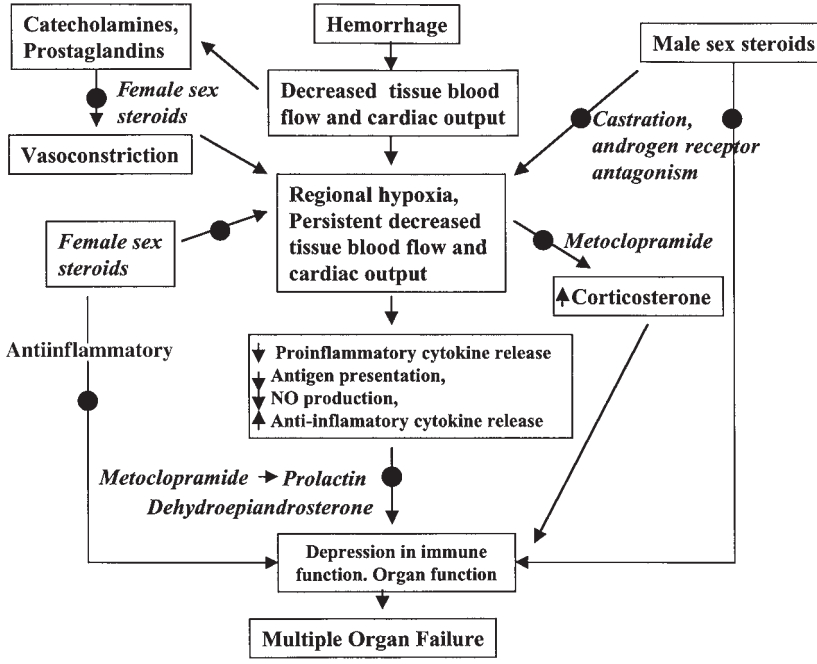

Fig. 3. Schematic view of hormonal modulation of the cascade of events leading to immunodepression after injury. Hormonal/therapeutic agents (depicted in italics) that inhibit multiple steps in the cascade are shown by black circles.

ment restored the splenocyte functions and normalized the increased release of proinflammatory cytokines by Kupffer cells after trauma and hemorrhage. Prolactin also maintains immune cell function at the level of transcription *(36)*. Improved cell-mediated immune response in mice given prolactin was associated with an increased survival rate of animals with hemorrhage subjected to subsequent sepsis.

A given sex steroid can have different effects on the immune system depending on the tissue site. Steroids are also shown to modulate the time-course of plasma proinflammatory cytokine release. Other factors that alter the effects of hormones on the immune response include dose, hormone milieu, and route and timing of administration, leading to divergent results.

Similar to the cell-mediated immune responses, B-cell functions are also modulated by sex hormones. Estrogen as well as prolactin

enhances antibody production by B-cells. Hormone-mediated modulation of antibody production by B-cells might contribute to gender dimorphism of the immune response after injury.

In addition to sex steroids, other hormones also modulate immune responses. After injury, the neuroendocrine response causes release of catecholamines and glucocorticoid hormones that affect both humoral and cellular immune systems. Catecholamines impair T-cell proliferation, IL-2 receptor expression, and immunoglobulin production by B-lymphocytes. Glucocorticoids are known to impair the phagocytic activity of PMNs. Growth hormone improves splenic macrophage immune function and decreases the susceptibility to thermal injury.

Mechanisms of Action of Sex Hormones

The mechanism by which sex hormones modulate the cell-mediated immune response after trauma and hemorrhage is unclear at present and may involve both direct and indirect effects. In addition to the direct effects of sex hormones just described, the presence of estrogen receptors on various immune cells, i.e., thymocytes, macrophages, and leukocytes, also provides additional support for the direct immunomodulatory effects on immune cells. Receptors for male sex steroids have been identified on synovial cells, immature monocytic cells, T- and and B-cells *(37)*. Thus sex steroids may directly modulate the immune response by a specific receptor-mediated process. The MHC also seems to be involved in mediating sex steroid effects on immune cells. Because sex steroids primarily exert their immunomodulating effect after trauma and hemorrhage, it is possible that increased receptor expression, or changes in receptor affinity for these hormones could occur after injury. Expression of the phosphorylated form of p38 mitogen-activated protein (MAP), kinase increased in males after trauma and hemorrhage, whereas it decreased in females. Activation of p38 MAP kinase has been implicated in the modulation of the inflammatory response; the differences in the activation of p38 MAP kinase might contribute to a gender-dimorphic immune response.

Sex steroids may have indirect effects by altering the secondary mediators from immune cells, endothelial cells, and other interactive cells, thereby modulating cytokine release after trauma and hemorrhage. Depletion of testosterone prevents the depression in cardiac function after trauma and hemorrhage. Sex steroids also act through

the thymus; the role of thymus in mediating the effects of sex hormones in immune cells after trauma and hemorrhage remains to be elucidated.

THERAPEUTIC APPROACHES

Therapeutic modulation of the immune system after injury ranges from basic interventions designed to restore homeostasis with aggressive resuscitation and nutritional support to the latest state-of-the-art manipulations of immune cell activity and its products. Fluid resuscitation, hemodynamic monitoring, and judicious use of antibiotics are most important in trauma care. However, despite optimal therapy, sepsis syndrome and the multiple organ dysfunction syndrome remain clinical challenges in the care of the critically injured patient. Numerous pharmacologic agents have been employed in the laboratory to modulate immune function after trauma and hemorrhage (**Table 1**). Following are some approaches currently showing promise for future therapeutic use that may be undertaken to alter the host immune function in the clinical setting.

Nutrition

Acute protein calorie malnutrition is known to depress the cell-mediated immune response. Nutritional repletion can balance some of the abnormalities, as assessed by total lymphocyte count, mitogen responsiveness, and skin-test reactivity. The hypermetabolic state associated with acute injury imposes a significant strain on the patient's nutritional reserves, with a substantial effect on the immune system. Immediate nutritional support after trauma reduces the incidence of septic complications and mortality. The route of administration and the composition of nutritional formulas seem to be important, with enteral feeding apparently being superior to parenteral nutritional support, leading to improved cell-mediated immune function and reduced septic complications. In addition, early rather than delayed, and supplemented rather than standard, diets are associated with better outcomes. Several specific supplemental nutrients have been identified recently as offering specific immunomodulatory properties. Often these nutrients are used in amounts well in excess of normal nutritional requirements, and so they are said to possess a pharmacologic effect, to differentiate the

Table 1
Potential Immunomodulatory Agents for Restoration of Immune Function after Trauma and/or Hemorrhagic Shock

Agent	*Mechanism of Action*	*Cellular Effects*
Nutrition	Amino acid and lipid supplementation	Reduction in eicosanoid release
IL-12	IL-2 and IFN-γ increase	Shift from type 2 to type 1 helper T-cell response
IFN-γ	Macrophage activity stimulation	Reversal of suppression of MHC class II expression
TNF-α antibodies	TNF-α Inhibition	Normalization of antigen presentation and cytokine release
Ibuprofen	Prostaglandin E_2 reduction	Restoration of T-cell proliferation and cytokine release
Pentoxifylline	cAMP phosphodiesterase inhibition	Cardioprotection and inhibition TNF-α production
ATP-$MgCl_2$	Tissue ATP level restoration	Restoration antigen presentation and cytokine release
Chloroquine	Inflammatory response inhibition	Inhibition of TNF-α and restoration antigen presentation
Estradiol	Specific receptor activation	Nuclear binding and gene activation
Flutamide	Androgen receptor antagonist	Inhibition of androgen uptake and or nuclear binding
DHEA	Receptor activation	Estrogenic agonist, glucocorticoid antagonist
Prolactin	Specific receptor activation	Protein kinase C activation gene expression
Metoclopramide	Dopamine antagonist	Increase in prolactin secretion

DHEA, dehydroepiandrosteroue; IFN-γ, interferon-γ; IL-12, interleukin-12; TNF-α, tumor necrosis factor-α.

doses from standard nutritional use. Such nutrients include arginine, glutamine, ω-3 polyunsaturated fatty acids, and nucleotides.

Arginine supplementation improves survival after burns and intra-abdominal sepsis. Lymphocyte blastogenesis and T-helper cell numbers are increased in patients after surgery who receive arginine supplementation. Glutamine is a regulator of protein synthesis and an essential precursor for nucleotide biosynthesis in all cells. It is an important vehicle for nitrogen transfer between tissues and serves as an energy fuel for the gut mucosa and other rapidly dividing cells, such as fibroblasts, lymphocytes, and epithelial cells. Trauma, severe illness, and sepsis are followed by a rapid fall in muscle and plasma glutamine levels, and the use of glutamine has shown some efficacy, with a reduced incidence of infection.

The amount and type of dietary lipids can profoundly influence the immune response and resistance to infection. Lipids become incorporated into the phospholipids of cell membranes and other components within cells, thereby influencing structural integrity, transport systems, membrane fluidity, receptor expression, and cell-cell interactions. Diets high in ω-3 polyunsaturated fatty acids appear to have some beneficial effects on immunosuppression following injury and have been reported to be superior to conventional diets containing mainly ω-6 polyunsaturated fatty acids. Although there is controversy concerning the immunomodulatory mechanisms of such diets, a reduction in Th1 immunosuppressive eicosanoid release and an altered profile of inflammatory cytokines may seem to be responsible for the observed phenomena.

Immunomodulators

Cytokine Modulation

Immune dysfunction in states of profound stress is characterized by impaired balance of immunosuppressive and counter-regulatory influences. Much effort has been expended in interfering with early inflammatory events thought to set the stage for the subsequent generalized inflammatory response. Immunomodulatory therapy has focused on strategies designed to inhibit TNF, IL-1, and IL-6, using cytokine antibodies, receptor antagonists, or soluble receptors. Immune suppression following injury is reflected in polarization of the helper T-cell activity, with a shift in the Th2 cell direction. Impaired Th1 cell activity is reflected by decreased synthesis of IL-2 and IFN-γ. The inability to

produce adequate amounts of IL-2 results in an incomplete proliferative T cell response to antigenic stimuli, whereas the lack of IFN-γ results in inefficient macrophage antigen presentation. Attempts have therefore been made to modulate the cell-mediated immune response by administration of these lymphokines to animals and humans. The administration of IL-2 appears to enhance survival in animals with intra-abdominal sepsis. It is most effective when given at the site of infection and probably acts by stimulating local host defenses. Recently, treatment with IL-12, a cytokine that induces a shift from the type 2 toward the type 1 T-helper lymphocyte phenotype, has been reported to increase survival after major trauma in animals. IL-12 treatment resulted in increased IL-2 and IFN-γ production by splenocytes.

IFN-γ. Exogenous IFN-γ increases resistance to intra-abdominal sepsis and improves survival after trauma and hemorrhagic shock. In humans, IFN-γ reverses the trauma-induced suppression of MHC class II HLA-DR antigens on macrophages. However, no clear benefit in sepsis-related morbidity or mortality has been demonstrated in patients given IFN-γ.

TNF-α *Antibodies*. TNF-α, which is elevated systemically in injury, is believed to play a key role in the pathogenesis of the sepsis syndrome. Studies using anti-TNF monoclonal antibodies have shown them to be protective in rodents administered lethal doses of lipopoly saccharide (LPS) or live Gram-negative bacteria. Anti-TNF antibodies administered prior to blood loss attenuated the suppression of Kupffer cell antigen presentation and Ia expression and restored the ability of cultured Kupffer cells to produce TNF and IL-1. Pretreatment with anti-TNF antibody prior to hemorrhage also normalized peritoneal macrophage antigen presentation, splenocyte proliferation, and cytokine production. However, clinical trials with anti-TNF antibodies in humans showed poor efficacy because of the polymicrobial nature of sepsis in the population studied *(38)*. Accurate modeling of the clinical scenario by using cecal ligation and puncture, which produces a polymicrobial infection, rather than the immune response elicited by LPS alone, is important for studying the effectiveness of, anti-TNF antibody therapy.

Nonsteroidal Antiinflammatory Drugs

Ibuprofen and Indomethacin. PGE_2 production from arachidonic acid by activated monocytes-macrophages is proposed as one of the

most critical immunosuppressive pathways after injury, affecting both humoral and cellular immune function *(39)*. After severe trauma, upregulated PGE_2, synthesis lasts for as long as 21 d. Cyclo-oxygenase-inhibiting nonsteroidal antiinflammatory drugs (NSAIDs) such as ibuprofen or indomethacin restore some of the immunosuppressive effects after trauma. In animals, ibuprofen and indomethacin are effective in reducing PGE_2, preventing depressed T-cell proliferation, improving antigen presentations by macrophage synthesis of IL-1 and TNF, and maintaining lymphocyte IL-2 and IFN-γ production. Their use is associated with improved organ function and survival after septic shock. In patients with major trauma or following operations, indomethacin seems to improve the cellular immune response, including increased IL-2, IL-1, and TNF-α release. Others have reported improvement of hemodynamic and pulmonary functions in septic patients by ibuprofen treatment. The deterioration of renal functions found in some animal studies and the integrity of the gastric mucosa are concerns when NSAIDs are used routinely in the compromised patient. Adequate fluid resuscitation and cytoprotective agents may ameliorate some of these effects, but the use of NSAIDs cannot be endorsed unequivocally without further studies.

Pentoxifylline. Pentoxifylline, which inhibits cAMP phosphodiesterase and increases intracellular cAMP levels, has been shown to restore normally depressed cardiac output and improve tissue perfusion in the posthemorrhagic state *(40)*. Although it is not known whether the improvement in cardiovascular function is responsible for the beneficial effects of pentoxifylline on immune function, in a sepsis model it has been shown that pentoxifylline reduces mortality and improves immune function. Pentoxifylline has been shown to downregulate TNF production following endotoxemia and seems to exhibit both immune-enhancing as well as cardioprotective properties.

Immunoglobulins

The demonstration of impaired humoral immunity in sepsis and following major injury gave rise to numerous studies using immunoglobulins in an attempt to provide the patient with higher titers of antibodies against bacterial endotoxins and exotoxins. In addition, immunoglobulins may synergize with β-lactam antibiotics because they contain anti-lactamase antibodies. Both experimental and clinical trials suggest a protective effect of intravenous immunoglobulin preparations against

various infections, using 5S and 7S immunoglobulin, IgG, IgM, IgG with IgM and IgA, and *Pseudomonas* immunoglobulin. The beneficial effects of immunoglobulin treatment may be seen through a reduction in the number of infectious and septic complications in polytraumatized patients. However, with regard to the ultimate endpoint of all sepsis and trauma trials, reduction in multiple organ dysfunction syndrome and mortality, the results of most controlled clinical trials, have been disappointing.

Thymomimetic Agents

Thymic hormones promote the cytokine-mediated regulation of T-cell development in precursor cells and also the proliferative response of mature T-cells in the periphery. Synthetic thymomimetic purines such as isoprinosine, methylinosine phosphate, and the pentapeptide TP-5 have been used in the past to modulate cellular immune function. Methyl-IMP (ionosine monophosphate) augments lymphocyte responses to mitogens and increases delayed-type hypersensitivity. Isoprinosine reverses in vitro immunosuppressive effects after trauma and burns. The use of TP-5 combined with indomethacin causes improved T-cell reactivity compared with TP-5 alone, indicating that simultaneous cyclo-oxygenase inhibition and T-cell activation can enhance cell-mediated immune mechanisms following trauma.

ATP-$MgCl_2$

The depletion of intracellular energy stores that occurs following trauma and hemorrhage has been well documented. Administration of ATP-magnesium chloride ($MgCl_2$) has been shown to restore tissue ATP levels and improve organ function *(41,42)*. Meldrum et al. *(43)* demonstrated that hemorrhagic shock decreased splenocyte ATP levels and that this depression was correlated with a suppression in IL-2 and IL-3 production by these cells. Treatment with ATP-$MgCl_2$ improved both intracellular ATP levels and cytokine production. Moreover, infusion of ATP-$MgCl_2$ after hemorrhage has been shown to restore the depressed peritoneal macrophage antigen presentation capacity *(44)*.

The pathophysiology of hemorrhagic shock includes depressed microcirculatory flow, and it has been shown that preheparinization of animals prior to hemorrhage preserves blood flow in the circulation. Obviously, preheparinization of trauma patients is not a relevant clinical strategy. However, Zellweger et al. *(45)* demonstrated that the use of

a novel non-anticoagulant heparin (GM1892) after hemorrhage was able to improve both splenocyte IL-2 and IL-3 release as well as IL-6 release. It has been further demonstrated that GM1892 improves cardiovascular and hepatocellular function after hemorrhagic shock and that it decreases susceptibility to subsequent sepsis *(46)*.

Chloroquine

Chloroquine an inexpensive compound used widely in the treatment of malaria and rheumatoid arthritis; it is a potent inhibitor of the inflammatory response. Chloroquine has been shown to inhibit macrophage TNF-α transcription after hemorrhage and to improve Kupffer cell antigen presentation. Mortality from intra-abdominal sepsis after hemorrhage is significantly lessened in animals treated with chloroquine after resuscitation. Finally, chloroquine has been shown to depress the endotoxin-mediated release of proinflammatory cytokines after trauma and hemorrhage, thereby maintaining immune function and decreasing susceptibility to sepsis.

Hormonal Therapy

Dehydroepiandrosterone

Dehydroepiandrosterone (DHEA) is an intermediate for the synthesis of both testosterone and estrogen. In the male hormonal environment, DHEA exhibits estrogen-type effects. Because of the immunoenhancing effect of estrogen, administration of DHEA prevented cell-mediated immunosuppression after trauma and hemorrhage, and the survival rate of animals subjected to subsequent sepsis improved *(47)*. The mechanism of action of DHEA is mediated in part by the estrogen receptor and/or its conversion to estrogen. DHEA also antagonizes the immunosuppressive effects of glucocorticoids and downregulates the activity of glucocorticoid receptors. Systemically, DHEA administration normalizes elevated plasma glucocorticoid levels after trauma and hemorrhage.

Prolactin

Prolactin, a 24-kDa polypeptide hormone secreted by the anterior pituitary, is known to have immunostimulating properties. Prolactin administration restored splenocyte and macrophage function and improved survival in animals subjected to intra-abdominal sepsis after trauma and hemorrhage *(48)*. Treatment with prolactin after resuscitation also inhibits elevated proinflammatory cytokine gene expression,

suggesting that in additon to improving immune function, it may play a role in preventing excess proinflammatory cytokine expression after trauma and hemorrhage. The antinausea drug metaclopramide, which increases prolactin levels, also improves immune function in trauma and hemorrhage *(49)*.

Metoclopramide

The dopamine antagonist metoclopramide induces prolactin secretion and increases plasma prolactin levels. A single dose of metoclopramide after resuscitation prevented the depression of splenocyte IL-2 and IL-3 release and normalized peritoneal macrophage cytokine release even after microbial sepsis. In addition, metoclopramide normalizes the elevated plasma corticosterone levels seen after hemorrhage and antagonizes the immunosuppressive effect of glucocorticoids.

Flutamide

The androgen receptor blocker flutamide has also been shown to be a useful therapeutic agent in improving splenocyte and macrophage functions as well as reducing susceptibility to intra-abdominal sepsis after trauma and hemorrhage *(50,51)*. Administration of flutamide mimicked the immunoenhancing effects of castration on B-cell function. Moreover, flutamide administration has been shown to preserve hepatic and cardiac function after trauma and hemorrhage *(50)*. Flutamide administration on 3 consecutive days after hemorrhage restored depressed splenocyte and splenic macrophage cytokine release even after the induction of sepsis and decreased the mortality associated with septic challenge. Flutamide offers a safe approach for preventing immune dysfunction in male trauma patients.

SUMMARY

There are complex and innumerable alterations in cellular immune function after hemorrhage and trauma (**Fig. 2**). A decrease in the microcirculation and depression in tissue perfusion lead to regional tissue hypoxia and accumulation of metabolic wastes, resulting in acidosis. Concomitant systemic release of stress hormones including catecholamines and glucocorticoids, which are known to be immunosupressive, contributes to the downregulation of immune function after injury. In addition, hemorrhage and trauma lead to the overproduction

of numerous soluble mediators that depress immune function. In response to both tissue hypoxia and soluble mediators released from the gut, Kupffer cells release proinflammatory cytokines including IL-1, IL-6, and TNF-α. Induction of PGE_2 synthesis and inhibition of Kupffer cell antigen presentation, MHC class II receptor expression, and phagocytosis lead to an overall functional depression of both the specific and the nonspecific immune systems.

In response to stress hormones, neutrophils circulate through the gut and are primed. These primed neutrophils accumulate in important end organs such as the myocardium and lungs and, in response to circulating proinflammatory cytokines or infectious challenge, become activated. These activated neutrophils release toxic oxygen species and proteolytic enzymes, inducing organ damage and dysfunction. Tissue macrophages (splenic and peritoneal in particular) undergo a downregulation caused by circulating prostanoids, glucocorticoids, and the effects of hypoxia **(Fig. 2)**. These cells lose their antigen-presenting capabilities and fail to produce cytokines in response to stimulation by bacterial products. T-lymphocytes exposed to hypoxia have depressed intracellular ATP levels and lose their ability to regulate calcium flux. Although there is no change in T-lymphocyte populations as detected by cell surface antibodies, T-helper cells produce decreased amounts of IL-2 and have a diminished capacity to proliferate. It also appears that the helper cell population undergoes a phenotypic selection in which Th2 cells predominate and antiinflammatory cytokines such as IL-4 and IL-10 are released. Again, the effects of glucocorticoids, catecholamines, and prostanoids contribute to the depression in T-cell activity. Finally, fewer antigen-specific B-cells appear to be available to respond to antigenic challenge, lessening the antibody response to newly encountered antigen as well as vaccination.

All the changes described above occur in the context of the overall hormonal milieu of the animal, with testosterone mediating many of the deleterious effects of hemorrhage and prolactin and estradiol protecting against them. Sex hormone receptors have been identified on various immune cells, suggesting direct effects in addition to secondary mediators. Low testosterone and/or high estradiol levels protect the host after trauma and hemorrhage. Several therapeutic strategies of immunomodulation using a wide sprectum of pharmacologic agents (**Table 1**) have been promising in improving immune function.

PERSPECTIVES AND FUTURE STUDIES

Despite more hands-on training and experience in trauma care, trauma continues to be a leading cause of mortality during war. To prepare military doctors better for emergency and combat medicine and to deliver better medical care to wounded soldiers or during natural disasters or terrorist attacks, we need a great deal of understanding of the immunologic consequences of trauma and shock. Civilian communities will also benefit from the increased research in trauma care, and more studies will help the individual cope with stress signals from the traumatized central nervous system and thus play a role in the maintenance of the injured tissue without posing a threat to the host. Despite the enormous progress in clinical immunology and the available data on trauma-induced immune dysfunction, a large number of questions remain to be answered before the immunologic alterations after severe trauma can be beneficially influenced by immunomodulatory therapeutic efforts. It remains difficult to model human trauma and sepsis because of the polymicrobial nature of infections. Patients evaluated and treated in trauma centers typically receive multiple interventions including blood transfusions, ionotropic agents, antiinflammatory drugs, and analgesics, all of which are known to suppress immune function. The effects of many of these interventions have not been fully studied in the period following trauma and hemorrhage, and they may act synergistically and through different pathways to contribute to immune dysfunction.

Other important factors not yet well studied include age, status of the estrus cycle, overall health (i.e., comorbidities), and nutritional status of the injured patient. Differences in immune function in injury caused by various assault weapons, by biologic or chemical warfare, and by types of insults also need to be addressed in military trauma, emergency, and combat medicine. Basal intracellular Ca^{2+} levels have been shown to be elevated after hemorrhage, and studies on the aberrations in T-cell signaling cascade after trauma and hemorrhage may provide new therapeutic interventions in addition to the molecular mechanisms *(52)*. Understanding the molecular mechanism of the complex and multifaceted signals and counter-regulatory signals that are perturbed by trauma and hemorrhagic shock requires further investigation; it is sure that shifts in our current paradigms will result from further in-depth studies of this important problem in emergency medicine.

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6 Complement Inhibitors in Trauma

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INTRODUCTION

Complement is a series of more than 30 proteins in the serum and on cells that are proteolytically cleaved, forming an activated cascade that represents an extremely effective method of pathogen lysis. However, inappropriate or excessive complement activation may become a double-edged sword by causing excessive tissue destruction as well as destroying pathogens. Excess complement activation frequently occurs in autoimmune diseases as a result of immune complex formation. In addition, complement is activated during trauma and increases with the severity of the trauma *(1)*. Multiple complement regulatory molecules exist to prevent nonspecific complement activation. Understanding the role of complement and its natural regulatory molecules will allow the development of therapeutic interventions to prevent excessive damage during trauma.

In this chapter, we briefly review the complement system including the regulatory natural and recombinant engineered proteins that control complement activation, as well as the mechanism of complement activation during trauma. In addition, we discuss current complement activation inhibitors used in animal models of specific traumatic injury. Finally, we discuss clinical applications of current complement activation inhibitors and those that may be used in the future.

COMPLEMENT ACTIVATION

Classical Pathway

The classical complement pathway and the subsequent membrane attack complex (MAC) formation constitute the basis of the comple-

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ment system (reviewed in ref. *2*). This pathway begins with C1q binding to an antigen-IgG or-IgM complex through the Fc region. This induces enzymatic activation of two molecules, C1r and C1s, and the formation of the $C1qC1r_2C1s_2$ complex. The activated complex cleaves both C2 and C4 to form the C3 convertase, C2aC4b. The C3 convertase splits C3-generating C3b, which binds C2aC4b and becomes C2aC4bC3b, the C5-convertase. This complex severs C5-generating C5b, the initiator of the MAC complex. In addition to generating convertases, the classical pathway produces byproducts, C4b, C5a, and C3a. These byproducts are also immunologically active as anaphylatoxins.

The lytic component of the complement system is the MAC. Unlike the other complement pathways that depend on enzymatic cleavage for activation, the MAC is an assembled complex of C5b, C6, C7, C8, and C9. These proteins can attach to cellular membranes or form a soluble complex. When C5 convertase cleaves C5, C5b remains attached to the convertase on the cell surface. C6 and C7 then bind the complex, inducing a conformational change such that the C5b-6-7 complex is released to the fluid phase. If this complex of proteins is not immediately degraded, it will quickly bind tightly to the cell surface without inserting into the membrane. C8 then binds, and the complex is inserted into the membrane, causing the cell to leak. After insertion into the membrane, C9 is recruited and multiple C9 proteins are polymerized, forming a large pore. This results in cellular lysis and tissue destruction.

Alternative Pathway

The complex polysaccharide moieties on bacterial and other surfaces activate the alternative complement pathway (reviewed in ref. *2*). In this initiation pathway, C3 is activated and C3b is produced by low-level hydrolysis of an internal thioester bond. This spontaneous cleavage is termed *tickover*. C3b is fixed on the pathogenic surface and is quickly complexed with factor B. Factor D cleaves factor B, forming the alternative pathway C3 convertase, C3bBb. The C3 convertase is stabilized by the addition of properdin, forming C3bBbP. When this stable C3 convertase enzymatically cleaves additional C3 molecules, another C3b protein is added to the complex, forming C3bC3bBbP—a C5 convertase that cleaves C5-producing C5b and initiating MAC formation.

Lectin Pathway

Although it is called the lectin pathway, only one lectin, mannose binding protein (MBP), is involved in this initiating pathway of complement activation (reviewed in ref. *3*). MBP binds to mannose, *N*-acetylglucosamine, fucose, and glucose but not to galactose residues of carbohydrates on bacteria. Complement is activated when MBP is complexed with MBP-associated serine proteases (MASPs). Recently two MASPs, MASP1 and MASP2, and a related protein, Map19, have been identified *(4)*. These proteins are enzymatically activated like the C1r and C1s proteins. Specifically, MASP2 is similar to C1s, and MASP1 to C1r; Map19 has unknown properties at this time *(4)*. The MBP/MASP-activated complex can then cleave C4 and C2, creating the C3 convertase C4b2a and merging with the classical complement pathway.

Anaphylatoxins

During activation all three initiating pathways of the complement system result in the formation and release of the anaphylatoxins C3a and C5a. These small molecules, 9 and 11.2 kDa, respectively, are potent chemotactic proteins that not only recruit granulocytes but also induce degranulation of phagocytes, basophils, and mast cells, release of hydrolytic enzymes from neutrophils, smooth muscle contraction, and increased vascular permeability *(5,6)*. This proinflammatory response can prevent pathogen invasion but can also induce host tissue injury when inappropriately produced. Therefore, although beneficial, excess C3a and C5a may be potent mediators of injury during trauma.

Complement Regulatory Molecules

Complement is controlled by at least 10 inhibitory proteins found either in the serum or on cell membranes (reviewed in ref. *7*). The inhibitory proteins are designed to prevent lysis of the host cells. Because of the similarities of the three initiation pathways, many of the regulatory molecules can inhibit multiple pathways. In addition, all pathways converge at the formation of the MAC; therefore, inhibitors downstream of C5 regulate all forms of complement activation. The primary function of these natural regulatory proteins is to control the C3 and C5 convertases by degrading C3b and C4b. Control of the C3 and C5 convertases not only prevents MAC formation but also inhibits

release of the anaphylatoxins. The specific regulatory functions of some of the natural inhibitors are detailed below.

C1 inhibitor (C1inh) is found in serum and inhibits both the classical and lectin pathways by covalently binding to soluble C1r and C1s or MASP equivalents. It does not prevent surface complement activation. However, because there is more C1inh in the serum than C1, it quickly deactivates the classical pathway *(8)*. C1inh is also a serine protease inhibitor that prevents MASP activity in the lectin pathway *(4)*.

Complement receptor 1 (CR1; CD35) inhibits the the C3 and C5 convertases of both classical and alternative pathways. C3 cleavage results in C3b and C3f, with C3b undergoing a conformational change that allows it to attach to the surface *(2)*. With CR1 as a cofactor, factor I cleaves C3b, forming iC3b. The same two proteins can split iC3b, forming C3dg and C3c, all of which remain on the cell surface. To inactivate the classical pathway, CR1 binds to C3b, iC3b, and C4b on the surface of other cells only *(2)*. In addition, CR1 also dissociates B and Bb from C3b in the alternative pathway convertases, thus decreasing C3a formation as a result of the alternative initiation pathway. In mice, complement receptor-related protein y (Crry) has activities similar to those of CR1 in humans, inhibiting both C3 and C5 convertases *(9,10)*.

Membrane cofactor protein (MCP; CD46) serves as a cofactor for factor I cleavage of C3b and C4b and, like CR1, inhibits C3 and C5 convertases. However, it does not dissociate the convertase complexes *(11,12)*. MCP is expressed on all cells except red blood cells and is active only on the same cell on which it is expressed. MCP is an alternatively spliced membrane protein with at least four different variants that cleave C4b to various degrees. Most cells express some of each splice variant, although there are some differences in kidney *(11)*.

Decay accelerating factor (DAF; CD55) is a glycosylphosphotyidylinositol (GPI)-linked, recyclable protein that can inactivate C3 and C5 convertases of both classical and alternative pathways. Like MCP, CD55 can only inactivate the complexes that are assembled on the same cell surface on which it exists *(13,14)* and (reviewed in ref. *11*). However, CD55 has no cofactor activity for factor I. When removed from the cell surface by phosphotidylinositol-specific phosphatases, CD55 can also be a soluble complement inhibitor *(15)*.

CD59 (protectin) is a GPI-linked membrane protein that incorporates into the MAC complex after C5b-8 inserts in the cell membrane and inhibits insertion and polymerization of C9. CD59 can be shed

from the cell surface such that the soluble form retains its GPI anchor *(12)*. Thus, the protein can be recycled and inserted into the membrane of other cells.

Clusterin also blocks MAC formation by preventing C5b-6-7 from binding to the membrane. After C5b-6-7 binds the membrane, clusterin can not inhibit the complex. Clusterin is found in high concentration in most body fluids *(2)*.

Carboxypeptidase N is an important soluble enzyme that inactivates the anaphylotoxins by cleaving the terminal arginine from C4a, C3a, and C5a. The resulting proteins, C3adesArg, C4adesArg, and C5adesArg, compete for receptor binding but do not stimulate the cell *(16)*.

Synthetic Small Peptide Complement Inhibitors

With the advent of molecular biology, a number of recombinant engineered inhibitors have been designed. Although some high-molecular weight bivalent molecules exist, the more recent inhibitors are small peptides. The use of peptides in complement inhibition has a number of distinct advantages: they are highly specific, small, and defined by complement components; therefore an antibody response is not expected and they may be delivered orally.

Compstatin, a synthethic peptide that binds to C3 and inhibits both C3a release and MAC formation, has recently been described. *(17,18)*. In vitro and ex vivo animal models have shown that Compstatin is a useful complement inhibitor for transplantation and cardiopulmonary bypass-related pathology *(17,19)*. As an inhibitor that is useful in primates, it is also likely that Compstatin blockade of C3 will prevent excessive complement activation during trauma.

C5a receptor antagonist (C5aRa) is a recently described synthetic small peptide inhibitor that is currently being used in a number of animal models of tissue injury *(20,21)*. The anaphylatoxin C5a mediates its effects by binding the G-protein-coupled cell surface receptor CD88. C5aRa has been shown to inhibit C5a-induced neutrophil chemotaxis in response to sepsis and recently to attenuate both cardiac and mesenteric ischemic and reperfusion local damage in rodents *(21,22)*. In addition, it prevents neutrophil mediated systemic damage in a similar animal model.

MECHANISMS OF COMPLEMENT ACTIVATION IN TRAUMA

Although it is known that complement is activated in trauma patients, the mechanism is not well defined. It is known that complement is activated immediately after injury and that the severity of the trauma is directly proportional to the level of complement activation *(1)*. The complement cascade can be activated by contact with microbes, but during trauma the alternative and classical pathways are both overactivated in the absence of microbial infection. It is well known that the clotting cascade activates complement. Some possible alternative complement activators include reactive oxygen or nitrogen metabolites, exposed collagen, mitochondrial membranes, extracellular adenosine triphosphate (ATP), and exposure of blood to artificial surfaces during treatment of the trauma *(23–25)*. In addition, in vitro data show that damage to the endothelium activates the alternative pathway *(26)*. Although the exact method of complement activation may differ with the traumatic insult, the downstream events of excessive complement activation result in an inflammatory reaction. This inflammation involves anaphylatoxin recruitment and subsequent activation of granulocytes as well as upregulation of endothelial adhesion molecules, the local release of other inflammatory mediators and cytokines. Together, these potent mediators may result in local damage or may activate the inflammatory response (and complement) systemically. Extensive systemic complement activation can lead to a whole-body inflammatory reaction such as adult respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), or multiple organ failure (MOF).

ROLE OF COMPLEMENT IN SPECIFIC TRAUMATIC INJURIES

Ischemia/Reperfusion Injury

The loss of blood flow to a tissue for a limited amount of time results in damage to the tissue. However, reperfusion induces pathologic changes to the tissue that are greatly enhanced compared with that of ischemia alone. These alterations or reperfusion injury include an overexuberant inflammatory response that has deleterious effects on the

organs involved. The role of complement in mediating this injury and the subsequent ability to inhibit complement activation is the focus of much current research.

Myocardial Ischemia

Early studies found evidence of C3, C4, and C5 deposition on ischemic tissue in baboons *(27,28)*. Recently, several groups have shown that all three initiating pathways of complement activation are involved in myocardial reperfusion injury *(29–32)*. In addition, the terminal complement pathway is important in acute myocardial infarction. MAC deposition during the ischemic phase is limited, but additional deposits are seen after beginning reperfusion *(33,34)*. MAC is deposited on the vascular endothelium as well as on the necrotic tissue *(35–37)*. In addition, deposition of MAC on necrotic tissue is associated with decreased tissue expression of CD59 *(36,37)*. Finally, anaphylatoxins alter endothelial adhesion molecule repression *(38)*. Therefore, therapeutics for ischemia/reperfusion-induced myocardial injury have targeted either C3 and C5 convertase activity and the common terminal complement pathway.

Animal studies in which sCR1 was given showed a decrease in the size of the infarcted area and a decrease in neutrophil infiltration *(31,35,36,39)*. In addition, there was less MAC formation and deposition *(37)*. In a clinical trial, myocardial damage was attenuated in patients who received C1inh after coronary surgery *(40)*. Recombinant forms of CD59 that prevent MAC assembly are currently available, and studies are being conducted to determine whether blockade of the MAC is sufficient to decrease or prevent injury *(41)*.

Cerebral Ischemia

Like ischemia/reperfusion-induced myocardial injury, cerebral vascular occlusion and the subsequent reperfusion induces an overreactive and damaging inflammatory response (reviewed in ref. *42*). The components of this inflammation have recently been shown to include complement components synthesized by cells within the central nervous system, including astrocytes, microglia, neurons, and oligodendrocytes *(43)*. In addition, MAC deposition has been found within the infarcted region at autopsy of patients who died after a cerebral ischemia *(44)*. In animal models, there are distinct increases in C1q

deposition in ischemic brain tissue compared with nonischemic control brain tissue, indicating that at least the classical initiating pathway is present in the brain *(45 46)*. Initial attempts to inhibit complement activation within the brain prior to an ischemic event used cobra venom factor (CVF) *(47,48)*. CVF acts as a C3 convertase, but the normal C3 convertase inhibitors do not recognize CVF, leading to total consumption of C3. These studies were contradictory and inconclusive. Additional work showed that administration of sCR1 immediately prior to inducing cerebral ischemia/reperfusion resulted in moderate protection from reperfusion *(45)*. Taken together, these data suggest that complement inhibitors may be therapeutic for reperfused cerebral ischemia.

Intestinal Ischemia

Intestinal ischemia is associated with multiple trauma conditions, such as hemorrhagic shock, burns, myocardial infarction, and MOF *(49)*. These conditions lead to a reduction of blood volume that is believed to cause splanchnic vasoconstriction and functional ischemia of the gut. Mesenteric ischemia results in limited local intestinal inflammation and damage *(9,50–54)*. Reperfusion after mesenteric ischemia causes additional local inflammation characterized by complement activation and deposition, neutrophil infiltration, and eicosanoid generation that coincides with mucosal injury *(9,51,55)*. Gut-derived inflammatory mediators activate systemic polymorphonuclear leukocytes (PMNs) to become the main contributors to the development of the systemic inflammatory response and ultimately MOF *(54–56)*.

Numerous animal models have provided evidence that complement plays an essential role in the induction of intestinal ischemia/reperfusion injury *(52)*. The use of C5-deficient mice showed that either the MAC, the anaphylatoxin C5a, or a combination of both could prevent or substantially attenuate intestinal injury *(53,57)*. Anti-C5 monoclonal antibodies have been administered to mice to prevent C5 activation and subsequent local and remote tissue damage *(57,58)*.

Complement inhibitors are currently being studied to determine their ability to inhibit tissue damage as a result of mesenteric ischemia/reperfusion. Using a rat model of intestinal ischemia/reperfusion, several groups showed that administration of sCR1, a regulator of both classical and alternative pathways, significantly reduced rat local and systemic injury, PMN infiltration, and leukotriene B_4 (LTB_4) production

(51,59). Crry-Ig is a fusion product of the murine equivalent to CR1 and the Fc region of Ig. Despite the presence of a substantial number of neutrophils, in mice treated with Crry-Ig, the ischemia/reperfusion-induced tissue damage was prevented even when the drug was administered 30 min into the reperfusion phase *(9)*. This indicates that although neutrophils may play a role in the damage, complement is required as well. Because the local damage itself is not believed to be life-threatening, other groups have focused on C5a as a cause of the excessive systemic inflammatory response. Using a small-peptide C5a receptor antagonist that binds the human C5a receptor, it has been shown that serum markers of systemic inflammation, neutrophil activation, and remote organ injury can be prevented even when the peptide is given during the ischemic period, prior to beginning reperfusion *(21,60)*. Recently, IVIg [high-doses of immunoglobulins modified for intravenous use *(61)*] has successfully blocked complement-mediated tissue injury in a rat model of mesenteric ischemia/reperfusion *(62,63)*. Therefore, although the exact mechanism of complement activation has not been elucidated, it is apparent that complement plays a substantial role in both local and systemic tissue injury during ischemia of multiple organs.

Hemorrhagic Shock

Patients who have experienced a severe loss of blood or blood volume activate complement. C1 activation and the classical complement pathway are activated by the coagulation pathway and factor XII *(64,65)*. In addition, there are indications that the alternative pathway is also activated; however, the triggering factors are not well defined *(65)*. Recent studies have focused on determining whether complement activation induces damage or whether complement activation is merely a side effect of the tissue damage. Younger et al. *(16)* used a rat model of hemorrhagic shock (HS) to show that induction of complement activation by treatment with CVF increased mortality, whereas complement depletion prior to HS attenuated injury. In addition, they showed that C5a mediated the lethal effects of HS, as indicated by experiments showing that blocking C5a clearance was lethal in at least 80% of the animals—suggesting that C5a is at least one of the critical players in the pathology of HS *(16)*.

During HS the sympathetic nerve response decreases the splanchnic circulation, causing intestinal ischemia. As discussed above, at least

two mechanisms, complement and PMN infiltration, mediate damage to the intestine. In a rat model of HS, inhibition of complement by administering sCR1 prior to resuscitation maintained the splanchnic circulation, thus preventing HS-induced intestinal ischemia *(26)*. Two complement inhibitors, C1inh and soluble CR1, have recently been shown to prevent PMN infiltration *(66)*. Recruitment of PMN into the intestine requires a chemotactic signal followed by increased adhesion molecule expression on the vascular endothelium. This is followed by leukocyte rolling and adhering to the vasculature. C1inh attenuates adhesion and rolling of leukocytes within the mesenteric vascular endothelium *(66,67)*. The PMN chemotactic signal may be either C5a *(16)* or phospholipase A_2 (PLA2) derived eicosanoids *(68)*. Finally, there is evidence that hemorrhage may alter the intestinal barrier such that macrophages in the lamina propria are exposed to bacterial products. The presence of these products may also activate the complement cascade.

Thermal Injury (Burns)

The induction of complement activity as a result of thermal injury is distinct from that of other traumas *(24)*. Although both classical and alternative pathways appear to be activated (serum components are decreased) immediately after a burn injury, there is evidence that the alternative pathway is preferentially activated during the recovery period *(69)*. Additional tissue damage and damage to remote organs is associated with excess complement activation *(70)*. In addition, burn injury has been associated with neutrophil over reactivity to oxidative metabolites and under-reactivity to the C5a, possibly because of C5a receptor internalization *(71)*. Therefore, it is likely that complement inhibitors may be therapeutically useful for thermal injury.

Animal models of thermal injury have tested this possibility. Using a rat model of thermal injury involving approximately 30% of the body surface, sCR1 decreased short term (up to 4 h) inflammation, as indicated by dermal and pulmonary vascular permeability *(40)*. Thermal injury results in decreased C1inh levels and appears to consume C1inh by proteolytic degradation. In animal burn models, treatment with C1inh provided protection against excess inflammation and subsequent remote organ damage *(72)*. Others have used C1inh to reduce edema and inflammatory tissue damage as well as decrease bacterial infections

and increase long-term survival *(40,73)*. C1inh is currently in clinical trials as a treatment for thermal injury *(74)*. Initial results indicate that treatment with C1inh or in combination with sCR1 increases the long-term survival rate of severely burned patients *(40)*. This finding suggests that combination therapy of C1inh and sCR1 may yield even more dramatic improvement.

Systemic Complement Activation

The inflammatory response is necessary after trauma to control or prevent bacterial infection and to aid in healing. However, in cases of severe trauma, the inflammatory response is not confined to a local response; when it is uncontrolled, a systemic inflammatory response occurs. This response includes complement activation and results in damage to organs that were not injured by the initial trauma. In addition to the formation of the MAC, local activation of C3 and C5 results in the systemic release of anaphylatoxins, C3a and C5a, respectively. These small but extremely potent fragments have been implicated in systemic inflammatory conditions, including ARDS and MOF *(75–77)*.

Adult Respiratory Distress Syndrome

ARDS is frequently a sequel to multiple trauma and sepsis. Although the initial injury varies significantly, the pathology of the lung injury is similar, with increased cytokines and protein in the bronchoalveolar lavage (BAL) indicating increased capillary permeability. The mechanism of this pathology is not well defined but is believed to be initiated in part by the anaphylotoxins C3a and C5a *(24)*. This concept is supported by the fact that C3a plasma levels correlate with intensive care unit patient prognosis and outcome *(76)*. In addition to the anaphylotoxins released from remote tissues, the lung itself can produce all the complement components *(78)*. Therefore, the terminal MAC may also play a role in the damage within the lungs *(79)*. Thus, the inhibition of complement activation is currently being studied as a possible therapeutic.

Using animal models of ARDS, a number of complement inhibitors have been studied with similar outcomes. In a lavage-induced rat model of ARDS, inhibition of the classical complement pathway with C1inh was found to decrease the neutrophil infiltration *(80)*. Using an acid aspiration model, others showed that inhibition of C3 but not C4 pre-

vented the pulmonary edema and associated vascular permeability *(81)*. Another group has used multiple rat models to show that C5a inhibition decreases lung injury *(16,57,82)*. Both C3 and C5 convertases are regulated by CR1. Rabinovici et al. found that sCR1 protected against pulmonary injury in another rat model *(83)*. Recently, a phase I clinical trial used recombinant sCR1 to treat patients with acute lung injury *(84)*. At the doses tested, sCR1 did not increase the infections and significantly decreased complement activation. Larger phase II trials are necessary to determine whether there is a significant improvement in the clinical course. Additional studies with complement inhibitors either alone or in conjunction with other factors may lead to improved clinical outcomes.

Multiple Organ Failure

Patients surviving the initial trauma are susceptible to the subsequent complications of MOF that lead to the primary cause of delayed mortality. As with ARDS, the extent of complement activation can be prognostic in recognizing patients who may develop MOF *(85,86)*. Specifically, the sera C3a and C5b-C9 complex concentration on the first day post trauma is significantly increased in the nonsurvivors compared with survivors *(87)*. In a dog model of MOF, Zimmerman et al. *(77)* found that within the first hour after trauma both the alternative and classical complement pathways were activated (as indicated by a decrease in the hemolytic activity of each pathway). In addition, there were significantly elevated plasma C5a levels. Accompanying these biochemical tests was C3c deposition in the kidney, liver, and lung, indicating that complement was activated systemically *(77)*. Combined, these studies show that complement activation may be predictive of the development and outcome of MOF.

FUTURE STUDIES

Complement activation is part of the inflammatory process after all forms of trauma. The complement activation process involves three initiation pathways and a common terminal pathway that is responsible for the infliction of cell and organ injury. Complement activation occurs in a precise cascade and involves a number of naturally occurring inhibitors that safeguard the outright consumption of the complement system. Animal models of trauma have clearly shown, as discussed

above, that inhibition of complement activation can delay, improve, or reverse the pathology and outcome of trauma. A number of important questions need to be addressed to determine the logical candidates for the therapeutic use of complement inhibitors: first, the extent of complement activation; second, the primary pathway involved; and third, the possibility that side effects such as suppression of the innate immunity and the appearance of overwhelming infections may be associated with general complement inhibition.

There has been a logical design of complement inhibitors for therapeutic use in human disease. These include the following:

1. Monoclonal antibodies that eliminate or block the activation of complement factors. These antibodies can be humanized by molecular engineering and used in the treatment of disease. As discussed above, an anti-C5 antibody is in human trials.
2. Natural complement activation inhibitors such as DAF, CD59, and CR1 genetically fused to the Fc portion of IgG to prolong half-life.
3. Complement inhibitors, which act at different stages of the activation cascade, that are genetically or recombinantly engineered or chemically fused. Such compounds have the potential to act at different phases and bring about more specific and more effective complement inhibition.
4. The design of peptide inhibitors that block the interaction of two complement factors or the cleavage of a factor by a protease/activator, such as convertase, at a precise point. Peptide inhibitors such as these have recently emerged as a new promising approach. Compstatin, which was developed by the Lambris lab *(17–19)*, represents such an example as it inhibits complement activation by blocking C3 convertase-mediated cleavage of C3.
5. The fusion of complement inhibitors to molecules that will direct it to the site of inflammation. This type of inhibitor is under consideration, as the use of complement inhibitors may cause systematic inhibition and unwanted side effects (from the complete lack of complement) such as overwhelming infection. Complement inhibitors can be conjugated to selectin ligands that will direct them to sites of increased selectin expression, i.e., inflammation, or delivered via targeted liposomes to a specific location where the inhibitor is released in a concentrated region.
6. Gene therapy to administer complement inhibitors. Recently, in vitro assays showed effective gene transfer of DCF, MCP, and CD59 to aorta endothelial cells *(88)*. Thus, a similarly delivered therapeutic may be possible in the near future.

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7 Infections

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INTRODUCTION

Given the overall pathophysiology of trauma and wounds, it is clear that contamination of wounds with bacteria, either from an environmental source introduced at the time of injury, or from later nosocomial exposure, underlies much of the infectious complications of trauma. Although some of the aspects of wound infections would appear to be somewhat mundane, it is clear that this area of trauma care remains exceedingly important, because infections remain a major cause of complications following traumatic injuries. Continued research into these areas has significant implications for the reduction of morbidity and mortality of the multiply traumatized patient.

HISTORICAL PERSPECTIVE

The history of military medicine is replete with examples demonstrating the terrible toll that medical complications resulting from posttraumatic wound infections have exacted on wounded soldiers. Much of this history predates the modern era of medical and surgical practice, and is necessarily quite grim in terms of the eventual outcome of affected patients. A review of more recent military operations, however, reveals the highly significant impact that wound infections con-

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tinue to have on the military medical system in spite of the availability of broad-spectrum antibiotics and advanced medical and surgical care.

Because of the violent nature of military operations, the history of medical progress associated with trauma wound infections is closely intertwined with contemporary military history. Thus, it is useful to review briefly the development of the current state of prevention and therapy for trauma-associated wound infections in the context of recent military conflicts.

World War I

In World War I, anaerobic wound infections with *Clostridia* spp. and streptococci caused the greatest morbidity and mortality in injured soldiers. Infections with these organisms complicated up to 5% of wounds. As antibiotics were not an option, surgical excision and amputation were necessary and represented the only definitive care possible. It was already accepted at this time that early surgery (ideally within 6 hours (h) of injury) was critical in preventing wound infections. Slow evacuation of patients and overload of surgeons often delayed the time from injury to operation, however, and gas gangrene was already established in as many as 90% of gangrene patients on admission. Gas gangrene was the most frequent cause of amputation and the cause of death in up to 17% of all casualties. *(1)*

World War II

In World War II, hemolytic streptococci, *Clostridia* spp., and *Staphylococcus aureus* accounted for the most infections in wounds of the extremities. Anaerobic infections still accounted for up to 1% of wound infections in some theaters and carried 50% mortality if gas gangrene developed. Systemic penicillin and sulfonamides were employed therapeutically to control impending or established invasive infection. Antibacterial agents for the prophylaxis of infections were used at times in some theaters of operation during the war, but they were not universal and the benefit was contested. Local chemotherapy with topical sulfonamide powder was felt to be of little value, and routine use was abandoned according to War Department Circular Letter No. 160, dated June 1, 1945. Early wound debridement and delayed closure (after initial debridement, wounds were left open to be closed at another date) were considered the primary means of preventing wound infection. *(2–4)*

Korea

During the Korean conflict, medical and surgical interventions were being made closer to the time and point of injury. Often, large wounds were covered with a sterile dressing, and open fractures were splinted right at the point of injury. Evacuation teams were often able to get casualties to a physician within hours of wounding. At the battalion aid station, reapplication of sterile dressings and administration of 300,000–600,000 U of penicillin plus tetanus toxoid was the routine approach prior to further evacuation. Gangrene and hemolytic streptococcal infections, the scourge of previous wars, were dramatically reduced. *(5)*

Vietnam

By the time of the conflict in Vietnam, administration of systemic antibiotics (usually penicillin and/or streptomycin) by medical or paramedical personnel soon after wounding became the standard of care. The average elapsed time from injury to a hospital was 2.6 h, but casualties were often evacuated to a surgeon within 30 min of wounding. Most wounds were in the extremities, and delayed primary closure was used extensively. With this treatment, the organisms associated with wound infections began to change. Early infections, especially with *Clostridia* spp. and streptococci, were significantly reduced. Infections with penicillin-resistant organisms, however, occurred days to weeks after wounding. *Pseudomonas aeruginosa*, *S. aureus*, *Proteus* spp., and *Klebsiella* spp. became the most common organisms isolated from wounds with *P. aeruginosa* and *S. aureus* accounting for up to 70% of the infections. *(6)*

Middle East

Throughout the Arab–Israeli conflict of 1973, it was an Israeli directive that casualties receive early antibiotic prophylaxis and/or therapy on the battlefield or at the field hospital. The goal was administration within 30–60 min of injury. Most casualties were rapidly evacuated from the point of injury, through a field hospital to a civilian medical center, where they could remain throughout the course of recovery. In one review of three large Israeli medical centers that received casualties from this conflict, 9% of the casualties developed wound infections, but only 3% were infected on admission. There were 7.2 nosocomial infections per 100 patient admissions. *P. aeruginosa* was responsible for

36% of burn infections, 25% of fracture infections, and 25% of soft tissue infections (26% of infections overall), with most of these occurring about 10 days (d) after admission. Gram-negative bacilli were responsible for 70% of the infections. Sepsis occurred in 10% of the infected patients. Two percent of the infected patients died.

Notably, *Clostridia* spp. were responsible for only two soft tissue infections, neither of which showed evidence of myonecrosis or gangrene. There were only three localized group A streptococcal infections. This was a very low frequency of clostridial and streptococcal infection compared with the prior experiences of World War II and the Korean War. Whether or not these more favorable statistics would have been sustained with this approach over a longer conflict with higher numbers of casualties is unclear. The early penicillin therapy that lowered the frequency of early clostridial and streptococcal infections almost certainly contributed to the development of late infections with Gram-negative organisms. *(7,8)*

Falkland Islands

Immediate antibiotic therapy, early debridement (within 6 h), and delayed primary closure were accepted as doctrine by the British military and as the keys to decreasing the morbidity and mortality associated with combat-related wounds. Penicillin or an antibiotic with similar spectrum of coverage was to be administered to every casualty as soon after wounding as possible. Rotary wing evacuation, field surgical facilities, and a hospital ship were immediately available to facilitate early surgical wound debridement. Even so, difficult terrain and inclement weather often hampered evacuation and extended the time between injury and surgery. In one series of patients, only 40% underwent surgery within 6 h of injury, and 18% were delayed over 15 h. Antibiotics were used extensively, and early antibiotic use was associated with a dramatic reduction in wound infections, even in those patients with delays in surgery. *(9–11)*

BACKGROUND AND PATHOPHYSIOLOGY

Combat-related wounds carry higher infection rates than do civilian trauma wounds because wounds in combat are universally contaminated. In practically all cases of battle injury, the projectile has to pass through the clothing or equipment of a soldier before entering tissue.

This clothing and equipment is generally covered with soil, food particles, petroleum products, and other substances. Additionally, the wounded soldier may lie in water or, mud, or the injury may be manipulated by dirty hands or instruments. Hence, the many organisms of skin, clothing, and soil are carried deep into the wound and become the organisms of wound contamination. *(12,13)*

The progression from contamination to infection seems obvious. The wound is filled with blood and clot, and the walls of the wound consist of devitalized tissues that have been damaged by the passage of the bullet, so the conditions are right for implanted organisms to flourish and infection to develop. *(12)*

This polymicrobial cesspool has been observed since at least 1915, when Alexander Fleming, in the preantibiotic era, described the natural course of a war wound infection:

> *During the first week after the infliction of the wound the discharge is a dark reddish-brown fluid often foul smelling, and consisting of blood altered to a greater or less extent by the growth of the fecal organisms which have constituted the primal infection. It is in this stage especially that we see the spore bearing anaerobes associated with streptococci and sometimes other organisms.*
>
> *The second phase marks a transition between primary anaerobic infection and the final infection with pyogenic cocci. The discharge loses its bloody character and becomes purulent, the foul smell at the same time becoming less marked or disappearing altogether. This stage lasts from about the seventh to the twentieth day after infection. The bacteriological examination of the wounds in this stage shows that the spore-bearers have tended to disappear, but that there is a gross infection with the non-sporing bacteria of fecal origin—streptococci and* B. proteus. . . .
>
> *The third phase is seen after the first three weeks. In this stage the fecal element of the infection has tended to disappear and we now have a simple infection of pyogenic cocci, staphylococci and streptococci.* (12)

With the introduction of antibiotics and improved surgical techniques, modern day combat wounds will seldom go through all these stages of infection. Infections still occur, however, and Fleming's observations of an evolving or changing flora associated with a wound still hold true. It is clear that different infections occur early after

wounding rather than later in the course of the wound and that the flora will change in untreated or treated wounds following medical intervention or hospitalization. There are two schools of thought as to why this occurs. One holds that all the bacteria in a combat-related wound are introduced at the time of injury—the polymicrobial contamination theory. Over the course of the wound, a certain organism becomes bacteriologically or clinically prominent because conditions in the wound are favorable for its proliferation. Another school maintains that there is more than one opportunity for contamination and that late-appearing organisms are usually introduced after the infliction of the wound. A model for this hypothesis has also been suggested, as follows:

First comes contamination of the injury by organisms from the environment, which are driven into the wound at the time of injury. These flora perish or propagate depending on the organisms, characteristics of the wound (type of debris, amount of tissue damage, blood supply), and the host.

After wounding, self-infection can occur. Organisms from the wounded individual's respiratory tract, skin, or gastrointestinal tract are introduced, by the wounded individual, during manipulation of the wound or bandages.

Finally, added infection may occur. This is representative of typical hospital-acquired nosocomial infections and postoperative infections in which organisms from the initial injury have been eradicated through surgery and antibiotics and the wound is infected anew. This model is very practical as it accounts for infections seen throughout the course of treatment. It explains why antibiotics used early for prophylaxis will not work with infections seen later in the course of the wound and why patients with *S. aureus* colonization have higher rates of *S. aureus* wound infections. Application of the model can assist in developing interventions to prevent infections and guide the choice of antibiotics should infection occur. *(14)*

Classification

Although all combat-related wounds are contaminated, it is clear that not all will become infected. At United States Army Evacuation Hospital No. 8 in World War I, 67% of the contaminated wounds never showed clinical evidence of infection. *(1)* Thus, the classification of wound infection must remain a clinical entity. For the most part, wound infections can be grouped into a number of categories:

Simple Contamination

This occurs when pathogenic and nonpathogenic organisms are proliferating in the dead material of the wound (necrotic tissue, debris, blood clot, and pus) but no invasion of viable tissue is present. Although the organisms are of negligible importance in this state, it is from these organisms that more serious infections arise. Removal of the dead material from within the wound by surgical debridement and drainage removes the contamination.

Cellulitis/Local Infection

This occurs when the organisms that were confined to the necrotic debris of the wound begin to spread to nonmuscular tissues contiguous to the wound. There is no muscle involvement and, as a rule, little systemic toxicity. Locally, one can find surrounding erythema, a dirty wound, a foul odor, and a moderately profuse seropurulent discharge. Inspection of the wound at operation may reveal some necrotic and dying muscle, but this is related to the initial injury rather than subsequent bacterial invasion. The necrotic area can be easily removed surgically, leaving normal muscle tissue.

Myositis/Deep Tissue Infection

This occurs when the organisms have spread into muscles or tissues not adjacent to the wound. Historically, this was caused by *Clostridia* spp. and streptococci, but since the introduction of effective antibiotics, these organisms have all but disappeared. Deep infections with muscle involvement cause the greatest amount of tissue damage and have the greatest association with systemic infections and sepsis. The earliest symptom is the development of pain at the wound site. The pain is accompanied by swelling, edema, and subsequent systemic symptoms. Early examination of the wound may reveal little except edema and a thin watery discharge. Depending on the infecting organism, such infection can proceed to marked edema and a profuse brownish yellow serous or serosanguinous discharge. The skin may appear white and marbled. If untreated, swelling and edema increase, and the patient may develop the clinical syndrome known as sepsis. Only in surgery can the characteristic muscle damage be observed. Early on, there may be few findings except edema and pallor. Later the color changes, the blood supply is lost, and contractility disappears. If clostridial organisms are

present, gas may be obvious. Finally, the muscles become diffusely gangrenous, dark purple or black, and extremely friable. Failure to initiate wide surgical debridement at this stage may doom the patient. Antibiotics alone will not cure this infection.

Streptococcal Infection

Streptococcal infections are a specific subset of wound infection because the course from local infection to systemic infection can be quite accelerated owing to the ability of various streptococcal strains to produce systemically active toxins. The classic progression is local infection or cellulitis, leading rapidly to a sudden onset of severe pain and tenderness out of proportion to the physical findings. Pulse and temperature are elevated, and there are systemic symptoms including delirium or disorientation. Locally, one may find a wet, malodorous, and unhealthy wound with large quantities of thin blood-stained discharge surrounded by rapidly advancing edema. There is a moist edema involving all the tissues around the wound, especially the muscles. The muscles are initially boggy and pale, then bright red, and then dark purple, swollen, pulpy, and friable. The progression from local infection through deep infection and systemic toxicity can occur in hours.

Systemic Inflammatory Response Syndrome and Sepsis

The systemic inflammatory response syndrome (SIRS) is the inflammatory state seen with various forms of clinical insult including tissue destruction, multitrauma, and infection. In the current literature, distinctions are made among SIRS, early sepsis, sepsis syndrome, and various forms of shock including septic shock. Most of these entities, however, have a great deal of overlap and probably represent the same process, albeit with different levels of severity. Here we define sepsis as clinical evidence of infection plus evidence of a systemic response to the infection. Any established infection, if untreated, can progress to sepsis. The clinical response is what marks the degree of sepsis. Early sepsis is manifest by fever, tachycardia, tachypnea, and leukocytosis with bandemia. Decreased organ perfusion can follow, leading to hypoxemia, oliguria, and altered mental status, and finally hypotension and septic shock. Although septic patients are often bacteremic, positive blood cultures are not always found in septic patients. Through the progression to septic shock there is an enormous inflammatory cascade with release of tumor necrosis factor, interleukins, and platelet-

activating factor. This inflammatory response results in capillary leak, bleeding, and/or clotting complications of disseminated intervascular coagulation, kidney, liver, and cardiac failure, and hypoperfusion of the brain. Sepsis carries a high morbidity and mortality and is very manpower-and equipment-intensive to treat. Clearly, in the setting of an austere field hospital environment, patients presenting with septic complications from their wounds will not do well.

Postoperative Synergistic Gangrene

This is an uncommon entity characterized by spreading and highly intractable cutaneous gangrene following surgery on the thoracic and abdominal viscera. It is not part of the usual continuum of wound infection but occurs in the population of wounded soldiers after surgery. The causative organisms are typically *S. aureus* and micro-aerophilic streptococci acting in symbiosis. *(15)*

Causes of Progression to Infection

Many factors impact on progression from contaminated wound to infection including degree of tissue damage, type of soil, climate, bacterial burden, and type of projectile. Tissue damage and foreign bodies in the wound are probably the most significant. Altemeier and Furst were able to demonstrate this by injecting spores of *Clostridium perfringens* into guinea pigs under a variety of conditions. If spores alone were injected, it took 10^6 spores to produce clostridial myonecrosis (gas gangrene). If the muscle was crushed and then spores introduced, it took only 10^3 spores to produce gas gangrene. If sterile dirt and spores were introduced into crushed muscle, it took only 1 spore to obtain the same result. The presence of dead tissue and dirt resulted in a million-fold increase in the susceptibility of a wound to infection. *(16)*

Damaged skin, muscle, and fat all seem to have the same capacity to enhance infection. Not all soils, however, produce the same rate of infection. Matsumoto et al. contaminated experimental wounds in rabbits with soils from different parts of Vietnam. The different soils were associated with different organisms and different mortality rates. Rabbits with wounds contaminated with soils from dry sandy areas had a mortality of 7–67%, whereas wounds contaminated with soils from wet muddy areas had mortality of 83–100%. *(13)* Haury et al. were able to demonstrate several mechanisms to explain this enhancing of infection. The devitalized tissue of the wound acts as a culture medium promoting

bacterial growth. Additionally, the devitalized tissue inhibits leukocyte phagocytosis and killing ability. *(17)* Haury et al. were also able to show these same mechanisms associated with soil contamination of a wound. In addition to aiding in bacterial proliferation, certain types of soil impair the body's ability to fight infection. Montmorillonite clay was shown to impair leukocyte ability to phagocytose and kill bacteria. Additionally, exposure of serum to this clay rapidly inactivated circulating antibodies and eliminated their bactericidal activity as well. Five milligrams of clay reduced the number of bacteria required for infection from 1 million organisms to just 100 organisms. *(18)*

Climate also has an impact on wound infection. Lindberg et al. evaluated the bacterial flora of battle wounds at the time of primary debridement during a summer and winter period of the Korean War and noted that organisms associated with infection changed with the seasons. Forty-four percent of the wounds studied during the summer period contained pathogenic *Clostridia* organisms compared with only 21% of those from the winter period. By contrast, staphylococci and streptococci were significantly higher in winter wounds than summer wounds. The lower rates of clostridial contamination in wounds during the winter months may have resulted from a decrease in the environmental *Clostridia* organisms. In the winter, freezing temperatures and decreased use of organic fertilizer (fields in Korea are commonly fertilized with human excrement) may alter the factors required for clostridial growth. Troop contact with the soil may also be decreased in the winter when they are wearing more clothing and the terrain is frozen or covered with snow and ice. The higher incidence of streptococcal and staphylococcal infections of winter wounds may also result from increased prevalence of upper respiratory infections. *(5)*

High-energy wounds are notably associated with a greater degree of tissue damage, bacterial contamination, and infection. The distinction between a high-energy and a low-energy wound is based on the amount of energy with which a projectile impacts tissue. This is a function of the size of the projectile and its speed of travel. As a projectile slams through tissue, it creates both a temporary and permanent cavity. Pressure from the projectile causes tissue to accelerate away from its path. This explosion of tissue away from the path of the projectile leads to a temporary cavity (which is much larger than the size of the projectile) and causes immediate laceration of muscle and fat, disruption of blood vessels, and fractures beyond the cavity. When the pressure wave

passes, the temporary cavity closes and a smaller permanent cavity remains. Higher energy wounds, like those from modern assault rifles, are associated with greater temporary cavities and greater tissue damage at areas distant from the permanent wound track. Additionally, the temporary cavity creates a low-pressure area behind the projectile that sucks debris into the wound. In higher energy wounds with larger temporary cavities, there is a greater degree of contamination over a larger area. By contrast, low-energy-transfer wounds will have a limited contamination that is much closer to the wound track. *(19)*

PREVENTION AND TREATMENT OF INFECTION

It takes about 1 million (10^6) organisms in a wound before there is clinical evidence of infection. Obviously, a number of factors may modify this number as mentioned just above. In most combat wounds, infection occurs in about 6 h from the time of wounding. It has been understood since the 19th century and affirmed in every conflict of the 20th century that this 6-h window is the critical period in which there must be an intervention to improve casualty outcome. With the introduction of antibiotics came a new intervention that has also had its greatest impact if initiated within this same 6-h window. In an animal model Burke evaluated the period after wounding in which systemic antibiotics are effective in preventing infections with *S. aureus*. As the time interval between contamination and initiation of antibiotics increased, the antibiotic effect decreased. The greatest effect was found if appropriate antibiotics were initiated within 3 h of staphylococcal infection. *(20)* These findings are also supported by observations from military conflicts. In the review by Jackson of 49 British soldiers with soft issue injuries reported from the Falklands Campaign, there were no infections when antibiotics were administered within 3 hours of wounding. The infection rate was 7% in those soldiers receiving antibiotics within 6 h of wounding and 33% if administered 7 or more hours after wounding. *(10,11)* Although antibiotics alone or surgery alone will reduce infections, it is their combined use in most combat wounds that will reduce tissue loss, decrease the extent of excision, and reduce infections.

Debridement

The role of debridement is to remove devitalized tissue and debris from the wound to allow healing and prevent infection. *(21)* Appropri-

ate debridement will reduce the bacterial burden in a wound and remove the debris in which bacteria proliferate. As mentioned in the preceding paragraph, debridement within 6 h of injury is associated with the greatest reduction in infection. In Jackson's review of 49 casualties from the Falklands Campaign, the infection rate approached 25% in casualties treated with surgical intervention after 6 h from wounding. *(11)* Even after this 6-h window, however, longer delays in debridement are associated with increased rates of infection. In the reported cases of gas gangrene at U.S. Army Evacuation Hospital No. 8 in World War I, the average length of time between injury and operation was 41.8 hs. In the similar casualties in which gas gangrene did not develop, the average time between injury and operation was 24.7 h. Gas gangrene developed in 13% of those with less than 12 h between injury and operation and in 50% of those with 36–48 h between injury and operation. *(1)*

In addition to timing of surgery, a number of other factors impact on the need for debridement and extent of excision, including velocity of the projectile causing the injury, location of the injury, size of the injury, presence of fractures, and neurologic complications. Many of these factors do not significantly impact on infection rates and are beyond the scope of this chapter. Velocity of the projectile, however, does seem to impact on infection rates and the degree of debridement required to prevent infection. In high-velocity injuries, devitalized tissue and sources of infection can extend well beyond the obvious wound track. These injuries usually require more extensive debridement to remove devitalized tissue/debris and prevent infection. *(22)*

Low-velocity projectiles from fragmenting munitions such as artillery shells, mortar rounds, and grenades are the most common cause of wounds in modern warfare. These projectiles do not produce the same amount of tissue damage outside the visible wound track and may not require the same degree of excision/debridement to promote healing and prevent infection. Hill et al. support a more conservative approach in some of the multiple small fragment wounds associated with low-velocity fragmenting munitions like artillery and grenades. After reviewing the mounting evidence for nonoperative management of these wounds, they propose criteria for selecting wounds that can be managed with irrigation, antibiotics, and delayed closure. Essentially, these wounds should:

- be small, with entry and exit wounds no more than 1 cm in diameter
- show no evidence of permanent cavitation within the wound
- have no neuromuscular compromise
- have no evidence of compartment syndrome
- have a stable fracture pattern
- have no signs of infection
- be treated early by dressing and antibiotics.

Obviously, if there is any doubt as to the extent of the wound or if there has been a delay between injury and treatment, then surgical exploration is required. *(23)*

Antibiotics

A distinction should be made between prophylaxis of infection in a contaminated, noninfected trauma patient and the empiric treatment of an established infection that often occurs later in the course of the wound. Prophylactic use of antibiotics is the administration of antibiotics before an infection is established. Empiric use of antibiotics is the administration of antibiotics to treat an established infection. Historically, penicillin has been used the most for prophylaxis of infection in combat wounds. In this capacity it can prevent infection and/or delay infection in wounds that cannot get immediate surgical intervention. *(24–26)* Although not considered a powerful antibiotic by most standards today, it still has very good coverage for many of the organisms of contamination, including *Clostridia* and group A streptococci, and may retain a role in the prophylaxis of infection for combat-related wounds. Obviously, penicillin is not adequate for empiric treatment of an established infection later in the course of the wound, given the predominance of Gram-negative organisms associated with these later infections.

Different wounds carry different infection rates and different requirements for prophylaxis and treatment. Data from the 1973 Arab–Israeli conflict showed that penetrating abdominal wounds involving the large bowel, burns of 25% of body surface or more, and fractures of the femur were associated with significantly higher wound infection rates. Penetrating abdominal wounds had an infection rate of 14% if no large bowel area was involved and 58% if associated with large bowel perforation. Burns had an overall infection rate of 39%. If 25% or more of the body surface was burned, there was a 100% infection rate com-

pared with 14% in burns of less than 25% of the body surface. Fractures of the femur had nearly three times the rate of infection of fractures of other bones. Patients with three or more injuries, when one of the above factors was included, had an infection rate of 53%. *(7)* Treatment for these different wounds is addressed below.

Burns

Burns are associated with a very high rate of infection. The incidence increases with severity and percent of body surface burned. Burns and their management are addressed at length in Chapter 11 and are not covered in this section.

Extremity Wounds

Most ballistic injuries in persons who make it to medical care occur in the extremities. Most of the data on the benefits of prophylactic antibiotics have been gathered from this population. Early administration of penicillin in these wounds can extend the 6-h window, prevent infection, decrease the amount of excision, and preserve tissue.

In addition to wound prophylaxis with systemic antibiotics, there is a long history of topical antibiotic use in war wounds. Although it is not entirely accepted, a significant body of literature supports its use. The idea is that initially bacteria proliferate in the dead tissue and debris in a wound—an area not reachable by systemic antibiotics. Additionally, because of local vascular damage and shock, systemic antibiotics may not even reach tissue surrounding a wound. For these reasons the use of topical antibiotics seems attractive. Matsumoto et al. were able to use an antibiotic spray to reduce mortality from 67 to 1% in an animal model of contaminated crush wounds. *(27)*

Noyes et al. evaluated the use of topical antibiotics in the prevention of wound infections in soldiers at a South Vietnamese Army (ARVN) hospital outside Saigon. All patients received the local standard of care for combat-related wounds, which included debridement and systemic antimicrobial therapy. Penicillin and streptomycin were given in triage and continued daily for at least 8 d. Quantitative wound cultures were also taken on admission. Topical antibiotic sprays were started on the day following hospitalization. Various antibiotics in spray form were compared in daily and twice-daily regimens. When added to the parenteral antibiotics, treatment with spray mixtures of neomycin, bacitracin, and polymixin B resulted in significant decreases in quantitative

cultures of *S. aureus*, coliforms, and enterococci compared with controls. By day 8, only 11% of the wounds treated with topical antibiotics contained 10^6 organisms of *S. aureus* compared with 40% of the controls. None of the antimicrobial regimens, however, prevented proliferation of *P. aeruginosa*. (*28)*

Heisterkamp and colleagues investigated the benefit of topical antibiotics in the prevention of wound infections. One hundred twenty-six South Vietnamese soldiers with extremity wounds were treated with a topical antibiotic spray or nothing at the triage site prior to admission to an ARVN field hospital. At the field hospital, cultures of the wounds were taken prior to treatment. Prophylactic use of systemic penicillin or streptomycin was initiated in all casualties at that time. Initial surgical debridement occurred 6–24 h after antibiotic spray and was performed by surgeons who did not know which patients had been treated. In the patients treated with antibiotic spray, 16.4% developed an infection, whereas 39% of the control group developed an infection. The most effective spray was a combination of bacitracin, polymixin, and neomycin; it proved to be effective against all the important pathogens except *Pseudomonas*. All *S. aureus* cultures were sensitive to at least one of the antibiotics in the spray. *(29)*

Abdominal Wounds

In abdominal wounds, as with any combat-related wound, there is great variety in the extent of the wound. Velocity and type of projectile/fragment as well as use of body armor and luck all impact on the extent of the wound, the potential for infection, and the organisms of infection. Abdominal wounds with penetration of the large bowel carry a higher morbidity and mortality. *(30)* Penetration of the bowel leads to contamination of abdominal tissues with bowel contents comprised of microorganisms, food material, and digestive enzymes. This leads to peritonitis, extraperitoneal infection, and sepsis. Additionally, these infections adversely affect the healing of bowel anastomoses and stomas. *(23)* For this reason, the presence, degree, and extent of bowel perforation and contamination must be assessed early. Control and removal of contaminated material within 6 h of injury is required to reduce invasive infection—just as in extremity wounds. Antibiotics with coverage of anaerobes and Gram-negative organisms should be administered to all patients with abdominal wounds until colonic involvement can be determined at laparotomy. *(7)* In forward military

medical facilities with limited antibiotic options, clindamycin and gentamicin would be appropriate. If available or after evacuation to a larger facility, the use of newer combination agents such as ampicillin/sulbactam or piperacillin/tazobactam would be appropriate.

Orthopedic Wounds

A significant number of combat-related wounds are associated with open fractures. Infection is the primary cause of nonunion and bony instability following an open fracture. The severity of the associated soft tissue and vascular damage is the dominant factor determining the risk of infection in open fractures. *(31,32)* Most open fracture infections tend to be caused by *S. aureus*. Higher grade fractures, though, have a higher incidence of Gram-negative infections. Administration of antibiotics within 6 h of wounding has been shown to decrease the incidence of osteomyelitis; however, antibiotic coverage must include staphylococci. Penicillin alone is not adequate coverage, as it will not cover *S. aureus.*

Hill et al. demonstrated in a pig model that prophylactic antibiotics could prevent osteomyelitis in a nondisplaced ballistic fracture if administered within 6 h of inoculation. In the treatment group that received flucloxacillin and penicillin for 5 d, there were no cases of osteomyelitis, whereas all the animals in the nontreatment group developed osteomyelitis. *(23)*

Patzakis et al. also evaluated the use of antibiotics in the treatment of open fractures in a prospective study. Patients received penicillin and streptomycin, or cephalothin, or no antibiotics. The incidence of infection was 13.9% in the control group, 9.7% in the penicillin and streptomycin group, and 2.3% in the group receiving cephalothin *(33)*

Despite the higher incidence of Gram-negative infections in higher grade fractures, there is, as yet, no evidence to support the use of systemic aminoglycosides or other antibiotics with Gram-negative coverage in the prophylaxis of open fracture wounds.

Regardless of antibiotic therapy, the ability to cover an open fracture with soft tissue is paramount in preventing bone infection. Greater rates of infection are seen in higher grade open fractures with extensive soft tissue damage and in open tibial fractures in which soft tissue coverage is minimal at baseline. In most ballistic injuries involving open fractures, there is a delay of days between injury and the closure of the

wound or coverage with plastic surgery. During this period the bone must be kept free of infection and must not be allowed to dry out.

Toward this end, the use of antibiotic-impregnated beads (bead pouch) directly into the wound has shown some success. They have been shown to produce high levels of antibiotic in wound clot and drainage, to inhibit bacterial colonization, to decrease wound infection, and to prevent desiccation of exposed bone. Henry et al. reported a nonrandomized study of 404 open fractures in which patients were treated with tobramycin-impregnated beads and systemic antibiotics versus systemic antibiotics alone. Fracture-associated infections occurred in 2.7% of fractures treated with antibiotic-impregnated beads versus 11% of fractures treated with only systemic antibiotics. In higher grade fractures (grade III), which are associated with higher infection rates, infection occurred in only 8.7% of fractures with beads plus intravenous antibiotics compared with 43.9% of those treated with intravenous antibiotics alone. *(34,35)*

Multiple Trauma Casualties

The course of multitrauma victims is complicated by hypovolemic, traumatic, and septic shock. Hypovolemic (or hemorrhagic) shock is associated with large-volume blood loss. Traumatic shock is associated with severe tissue damage. Septic shock is associated with an infection. Although they are separate entities, they often occur together. *(36)* Sepsis is, unfortunately, a common occurrence in trauma victims. It may arise from an infection associated with the initial trauma or from a nosocomial infection associated with intubation or central lines. Although many new therapeutic interventions have been developed over the past 30 yr, the mortality in sepsis patients remains approximately 50%. Interestingly, the incidence of posttraumatic sepsis and multiple organ dysfunction differs between men and women. In severely injured patients, men are almost twice as likely to develop sepsis and organ dysfunction as women. Based on animal studies demonstrating that androgens are immunosuppressive whereas estrogens are protective against septic challenges, the difference seen in humans may also be related to sexual hormones. *(37)*

In the trauma patient, the clinical presentations of SIRS, sepsis, and early traumatic shock are all marked by temperature elevation, leukocytosis, and hyperdynamic state. Determining whether a patient is

infected and progressing to sepsis/septic shock can be difficult. Much work has been done to elucidate inflammatory and immune markers that can aid in the diagnosis of infection and predict mortality. Recently, elevations of C-reactive protein (CRP) and soluble CD14 have been evaluated and show promise in identifying patients at high risk for infection. *(38–40)* The role of prophylactic antibiotics in multitrauma patients is well established. Extent of antibiotic coverage (broad-spectrum antibiotics versus focused antibiotics as in the types of wounds just described above) and duration of prophylaxis remain controversial. There is mounting evidence that shorter antibiotic prophylaxis in multitrauma patients is as effective as longer therapy. The use of prophylactic antibiotics for 24 h after injury (and certainly 24 h after surgical intervention) seems to be as effective as 3–5 d of prophylactic antibiotics. Additionally, the incidence of resistant organisms significantly increases in patients treated for 3–5 d compared with 24 h. *(41)* Another alternative in preventing infection in multiple trauma patients may be high-dose intravenous immunoglobulins (IVIG). In combination with standard treatment, patients treated with IVIG had fewer pneumonias and non-catheter-related infections. *(40)* Efforts to immunize patients against organisms associated with sepsis also show great promise. In an animal model, a vaccine composed of bacterial membrane proteins significantly improved survival after infection with *P. aeruginosa* and *Klebsiella pneumoniae. (42)*

Primary and Delayed Closure

Decrease in infection with delayed closure of a contaminated wound has been accepted and recommended since at least World War I. *(43)* Since then, it has been supported in a number of studies and applied with success in numerous conflicts.

Edlich et al. evaluated the optimal time for closure of contaminated open wounds using an experimental animal model. Wounds in guinea pig were contaminated with 10^6 organisms of *S. aureus* and closed at varying intervals. Of the wounds closed 24 h after contamination, 73% later became infected. Twenty-two percent of the wounds closed after 48 h became infected, whereas 33% of the wounds closed after 72 h became infected. When closure of the contaminated open wound occurred on or after the fourth postoperative day, only 2% of the wounds demonstrated gross infection. *(21)* In Jackson's evaluation of Falkland casualties, most delayed closures took place between 5 and 7

d after initial surgery in most cases. There was a 15% infection rate after delayed closure in this group. Infections developed in 75% of the cases closed within 4 d of injury. No infections developed in those wounds closed after 8 d. Jackson suggests that the second operation in the treatment of a battle wound provides an opportunity to inspect it and re-excise it where necessary and not just to close it. Indeed, altering the emphasis of the second operation from closure to inspection may permit a more conservative initial excision. *(11)*

Other authors suggest that primary closure is an acceptable option in some casualties. In 1993, Nuzumlali and colleagues reported on their treatment of 48 patients with 60 gunshot wounds related to terrorist acts or regional conflicts. They used the Sisk method for classifying type of injury:

Type 1. Small wounds caused by low-velocity trauma such as the protrusion of a fragment of bone out from within or by a low-velocity bullet passing from without, with minimal soft tissue damage.
Type 2. Wounds extensive in width and length but with little or no avascular or devitalized soft tissue and relatively little foreign material.
Type 3. Wounds of moderate or massive size with considerable devitalized soft tissue or foreign material or both.

A combination of third-generation cephalosporin and metronidazole or penicillin G and an aminoglycoside were administered in "full doses" parenterally for 1 wk in all patients. Eleven type 1 injuries were treated with just irrigation and sterile dressing. No infections occurred. Thirty-nine type 2 injuries were treated with extensive debridement and primary closure. The infection rate was 2.6%. Ten type 3 injuries were treated with extensive debridement and primary closure. The infection rate was 20%. Although the idea is controversial, what is suggested in this report and described by Hill and colleagues *(26)* is that in a certain group of injuries the classic approach of debridement and/or delayed closure may not be required to reduce infection. In some wounds, equal and acceptable infection rates may be achievable with extensive debridement and primary closure or minimal debridement and delayed closure. Apart from infections, though, what will have to be decided by the surgeons is whether the benefits of primary closure warrant extensive debridement and the additional loss of tissue. *(44)*

Additionally, a number of published series from civilian trauma centers have demonstrated the safety of primary closure in gunshot wounds

of the colon. There are also a number of papers from recent armed conflicts around the world supporting primary closure of colon wounds. Parameters that take into account extent of injury, interval between injury and surgery, and level of contamination have been developed to help determine patient populations in which primary closure is more appropriate. In any patient, however, assessment of the degree of contamination is always required, and this remains subjective. Although primary closure versus exteriorization, excision, and colostomy remains controversial, what is agreed on is that management of bowel injuries must include evaluation of the bowel for perforation, halting of spillage of bowel contents, removal of as much contaminated material as possible, and appropriate antibiotic coverage. *(23,30)*

Quantitative wound cultures may be of value in determining when to close wounds. As discussed earlier, contamination seems to progress to infection when 10^6 organisms are present. Robson and Heggers evaluated the role of quantitative bacterial cultures in wounds of patients about to undergo skin graft. Clinically, these patients were not infected. By quantitative culture, however, organisms were present in the wound. Wounds with less than 10^5 organisms per gram of tissue had an average graft success rate of 96%. If there were more than 10^5 organisms per gram of tissue, average graft survival was less than 20%. A similar study showed that in decubiti, skin graft survival was 92% when fewer than 10^5 organisms were present. This held true for both Gram-positive and Gram-negative organisms. *(45)*

In postoperative wound infections of combat-related wounds, it is sometimes difficult to determine whether the infection is a result of the initial contamination or is a nosocomial infection related to the surgery. According to the Centers for Disease Control and Prevention National Nosocomial Infections Surveillance, infections of surgical sites are the third most common nosocomial infection and complicate up to 3% of surgeries. *S. aureus* is implicated in up to 25% of hospital-acquired infections. At any given time, 25–30% of the population is colonized with *S. aureus* in the anterior nares, and carriers of *S. aureus* are two to nine times more likely to have surgical site infections than noncarriers. This finding would suggest that *S. aureus* carriers might be an important subset of patients requiring additional monitoring or intervention. Perl et al. evaluated the role of mupirocin in reducing postoperative infections with *S. aureus*. The preoperative application of mupirocin

calcium ointment to the anterior nares of patients with *S. aureus* colonization led to a significant reduction in the postoperative risk of nosocomial infection with *S. aureus*. Although more evaluation is required, this may become an easy, inexpensive, well-tolerated way to reduce postoperative infections. *(46)*

CURRENT QUESTIONS

Despite significant improvements in the management of combat-related wound infections, many areas that impact on our ability to predict, prevent, and treat these infections require further evaluation.

Prediction

Can quantitative cultures of wounds be used to determine which wounds will progress to infection?

Do specific early markers of infection exist that can assist in the determination of which SIRS patients are truly infected and progressing to sepsis? Are CRP and CD14 among these markers? Do we have the means to make these markers practical for deployed medical units?

Prevention

Should early use of prophylactic antibiotics be the standard of care in combat-related infections?

Is penicillin still the best antibiotic for prophylaxis of combat-related wounds? What about newer combination antibiotics?

What should be the optimal duration of prophylactic antibiotics? Does duration of prophylaxis differ between types of wounds or patient populations?

What role can topical antibiotics play in the prevention of wound infection, osteomyelitis, and postoperative infections with *S. aureus*? What is the best delivery mechanism for topical antibiotics—spray, antibiotic-impregnated beads, or one of the newer delivery mechanisms? Are multiple applications more effective than one application?

Is there any value to further development of immunization approaches to prevent or ameliorate the sepsis syndromes?

Treatment

Is debridement required in all combat-related wounds? Can uncomplicated low-energy-transfer wounds be treated with irrigation and

antibiotics alone without increasing infection rates? How good are our criteria for determining uncomplicated low-energy wounds?

Is there a role or need for primary closure of combat-related wounds? What wounds should be considered for primary closure? Do the benefits of primary closure outweigh the potential for increased infection rates?

FUTURE DIRECTIONS

Many of these issues are being evaluated by the civilian sector and will be applicable to the military, for example, advances in predicting and treating sepsis. What will be critical is the rapid integration of these advances into the military medical system, which may require development of military-hardened applications of civilian equipment or simple field-friendly laboratory assays.

A number of areas are specific to combat-related casualties and will require military-directed evaluations. The role of prophylactic antibiotics in combat-related wounds is one such area. Controlled trials evaluating various antibiotics versus penicillin are needed. Also, evaluation of where these antibiotics should be administered is required. Should casualties self-administer antibiotics or should they be given by medics at the point of injury? Additionally, is there a role for topical antibiotics in place of or in addition to systemic antibiotics, and what is the best means of delivery?

Developing criteria for wounds or patients that will do well with limited surgical intervention is also paramount. Evacuation assets, surgeons, and operating areas are always limited in combat. The ability to focus these limited assets on the casualties with the greatest need is important. Collecting the experience of past conflicts and continuing to evaluate the management of combat wounds in animal models will ultimately save human lives.

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II Current Concepts

8 Hemorrhagic Shock and Resuscitation

Trauma Research at the Trauma Research and Readiness Institute for Surgery

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INTRODUCTION

Hemorrhagic shock is the leading cause of death and complications in combat casualties as well as in civilian trauma. Analysis of the historical data demonstrates that the mortality rates from World War II, the Korean War, and the Vietnam conflict are not only very similar but have also not shown any improvement over this period. Additionally, the rates of soldiers who died of wounds (DOW; death after reaching treatment facility) during the Vietnam conflict did not improve in spite of the rapid evacuation times (**Table 1**) *(1)*.

The published data also demonstrate that approximately half of those killed in action died of hemorrhagic shock. Thus the focus of research undertaken in the Trauma Readiness and Research Institute for Surgery (TRRI-Surg) has been on improving the outcome after traumatic hemorrhagic shock. TRRI-Surg was developed in 1997 at the Uniformed Services University of the Health Sciences, Bethesda, Maryland. The purpose of this institute was to coordinate combat casualty research and to aid in training military personnel to treat the injured combatant. We have been able to gather a group of qualified researchers, visiting surgeons and surgical residents to perform research in this area.

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Table 1
Killed in Action (KIA) and Died of Wounds (DOW)

War	*KIA (%)*	*DOW (%)*
World War I	19.6	8.1
World War II	19.8	3.0
Korean War	19.5	2.4
Vietnam Conflict	20.2	3.5

The approach undertaken by TRRI-Surg has been to investigate the entire spectrum of care dealing with hemorrhagic shock, including basic science (to understand the immunology of hemorrhagic shock, small and large animal work (to research resuscitation and treatment of injuries) and exploration of new ideas to save the lives of the severely injured.

The first section chronicles our search for an optimal method of resuscitation and also discusses the direction of future research in this area. It explains how we discovered that the current methods of resuscitation might be harmful to the bleeding combatant. It then demonstrates our work and progress to identify the optimal resuscitation method. Finally, it discusses our current recommendations for resuscitation as well as future plans for developing the optimal resuscitation fluid.

The second section focuses on new methodologies to treat the combat casualty including testing of titanium vascular clip staplers to repair injured vessels. As the vast majority of surgeons who are expected to treat the casualties will be newly trained general surgeons, this type of device will facilitate the repair of injured vessels and other structures. We are also testing novel methods to diagnose and treat hemo/pneumothorax, as this was felt to be a treatable cause of many deaths in combat. Finally, the last aspect in this arena is the testing of hemostatic agents to aid in the control of bleeding tissues.

The third section chronicles our work on future methods to treat the exsanguinated combatant. This area will require the reader to think out of the box, as we demonstrate the feasibility and usefulness of inducing hypothermic suspended animation for the repair of lethal injuries.

SEARCH FOR THE IDEAL RESUSCITATION METHOD

Currently, the major cause of death in potentially salvageable battlefield casualties is hemorrhage *(1)*. About 20% of these deaths are preventable if the bleeding can be quickly controlled or minimized *(2,3)*. In addition to the control of hemorrhage, the combat casualty is often treated with resuscitation fluids. Since the Vietnam War era, there has been little change in how we resuscitate. Our current resuscitation protocols are adopted mostly from civilian trauma literature in spite of recent propositions designed for the treatment of traumatic hemorrhage in the battlefield *(4)*.

At present, there is no clear consensus regarding the optimal resuscitation strategy for combat casualties. It is now being recognized that resuscitation fluids are not completely innocuous and that they may actually potentiate the cellular injury caused by hemorrhagic shock *(5)*. This concept of resuscitation injury has steadily gained attention since the Vietnam conflict. It was during this period that the appearance of shock lung/Da Nang lung [later termed acute respiratory distress syndrome (ARDS)] was first described in soldiers who received massive crystalloid resuscitation. Today, ARDS and multiple organ dysfunction syndrome are the leading causes of delayed mortality in trauma patients. This raises an important question: Is the resuscitation injury purely a reperfusion phenomenon or does the type of fluid infused alter the degree of subsequent cellular damage? During shock, tissue beds have low flow, but they rarely reach a state of no flow. Thus the classical paradigm of ischemia-reperfusion may not explain all the facets of cellular injury under these conditions. It is entirely possible that the type of fluid we use for resuscitation contributes to this injury. It is generally accepted that the cellular damage sustained during resuscitation is multifactorial in etiology. Its intensity depends on the severity and duration of hemorrhagic shock, the presence of associated injuries and comorbid diseases, second hit insult, and the resuscitation approach, to name just a few. However, compared with most of the other variables, the resuscitative strategy is entirely under our control. All these issues assume even more importance in the setting of limited resources and long delays to definitive care, as expected in the combat zones of the future.

Immunologic Response to Resuscitation Fluids

Aberrant overactivation of neutrophils and altered interactions between neutrophils and endothelial cells play a critical role in the postresuscitation organ injury *(6–8)*. One of our first experiments explored the activation of neutrophils following hemorrhagic shock and resuscitation. Using a swine model of volume-controlled (40% total blood volume) hemorrhagic shock, we found that resuscitation with lactated Ringer's solution (LR) resulted in increased neutrophil activation *(9)*. Activation was defined as increased neutrophil oxidative burst activity, which was measured using flow cytometery. The unique aspect of this experiment was that we did not isolate neutrophils, as the isolation procedure itself can activate the neutrophils; instead, we used a whole blood assay to study the circulating neutrophils. This experiment was also performed in awake, unanesthetized animals, to eliminate the possible effects of anesthesia. We detected no significant neutrophil activation during the hemorrhage or the shock period of 60 mins. However, a significant increase in neutrophil oxidative burst activity was noted following resuscitation. This observation was not surprising, as it was supported by the traditional concept that reperfusion of ischemic tissues sets into motion numerous cascades of adverse events. The unexpected result that challenged this simple explanation was the fact that infusion of LR without hemorrhage also caused neutrophil activation (**Fig. 1**). Furthermore, neutrophil activation was not observed following resuscitation with fresh whole blood or hypertonic solution (7.5% HTS). In these two later resuscitation methods, the animals had been subjected to the same degree of hemorrhage and resuscitation without causing activation of the circulating neutrophils. Taken together, these findings suggested that it is not merely restoration of flow that is important but that an equally critical variable is the type of fluid used.

With this surprising information, the next study examined whether the observed effects of LR on neutrophil activation were dose- and rate-dependent. Again, a swine model of volume-controlled hemorrhagic shock was used. Animals were resuscitated with three times the volume of LR over either 1 h (high volume/fast rate) or 3 h (high volume/slow rate). Another group was resuscitated over 1 hr with a volume of LR equal to lost blood (low volume/slow rate). The highest degree of neutrophil activation was seen in the high volume/high rate group, fol-

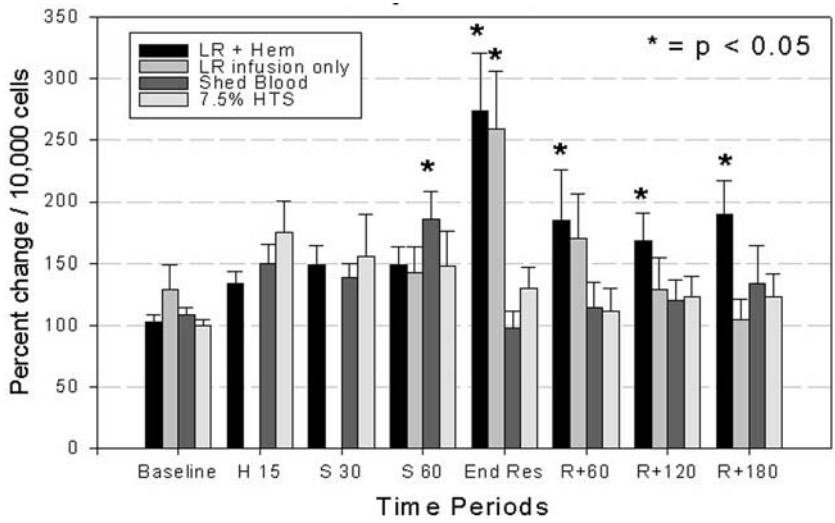

Fig. 1. Neutrophil oxidative burst activity. Neutrophil fluorescence (measured by flow cytometery) reported as percent change from baseline values per 10,000 cells ± SEM. * $p < 0.05$, compared to baseline values, ANOVA with Tukey's *b* multiple comparison test. Baseline, before hemorrhage; H 15 end of 15-min hemorrhage (28 mL/kg); S 30, 30 min into shock period; S60 60 min into shock period; End Res, end of 60 min resuscitation period; R + 60, 60 min after end of resuscitation; R + 120, 120 min after end of resuscitation; R + 180, 180 min after end of resuscitation. LR + Hem, hemorrhagic shock and LR resuscitation; Shed Blood, hemorrhagic shock and shed blood resuscitation; 7.5% HTS, hemorrhagic shock and 7.5% hypertonic saline resuscitation.

lowed by the low volume/slow rate and high volume/slow rate groups (**Fig. 2**). However, all three LR infusion protocols were associated with significantly increased neutrophil activation compared with anesthesia alone or hemorrhage and sham resuscitation *(10)*. Resuscitation with dextran and Hespan® (artificial colloids) was even more stimulating then LR (**Fig. 3**). Fresh whole blood and natural colloid (5 and 25% albumin) resuscitation did not cause neutrophil activation in this experiment *(11)*.

Human Neutrophil Studies

To determine whether human cells would react in a similar fashion, we tested the effect of various resuscitation fluids on human neutrophils from

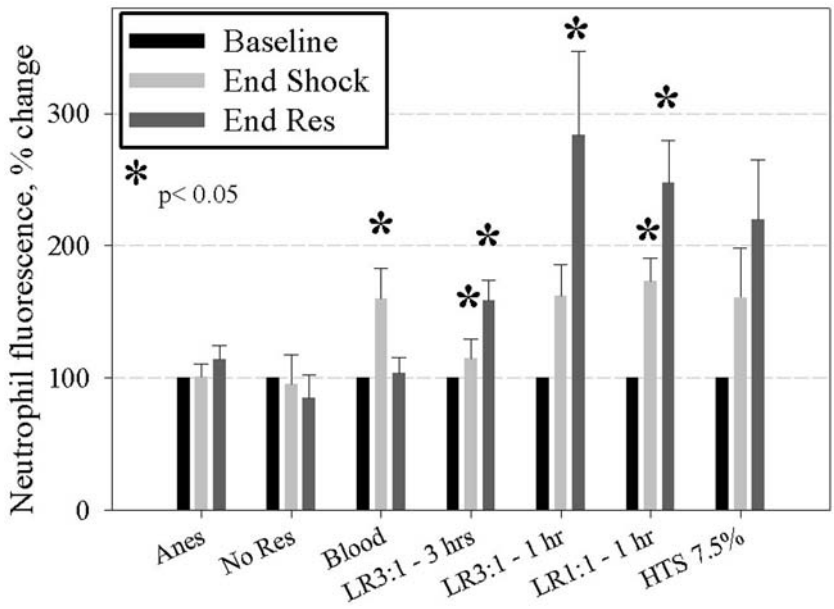

Fig. 2. Neutrophil oxidative burst activity. Neutrophil fluorescence measured by flow cytometery and reported as percent change from baseline values ± SEM. *, $p < 0.05$, using t-test compared to baseline values. Baseline, before hemorrhage; End Shock, end of 60 min of shock period; End Res, end of resuscitation period; Anes, Anesthesia and sham hemorrhage; No Res, hemorrhage and sham resuscitation; Blood, hemorrhage and resuscitation with shed blood; LR3: 1–3hrs, hemorrhagic shock and 3:1-volume lactated Ringer's resuscitation over 3 h; LR3:1–1hr, hemorrhagic shock and 3:1-volume lactated Ringer's resuscitation over 1 h; LR1:1–1hr, hemorrhagic shock and 1:1-volume lactated Ringer's resuscitation over 1 h; HTS 7.5%, hemorrhagic shock and 0.3:1-volume 7.5% hypertonic saline resuscitation over 1 h.

healthy volunteers. Again, we utilized the whole blood assay to avoid neutrophil activation during the isolation process. Our findings revealed that exposure of human neutrophils to isotonic crystalloids and artificial colloids caused a significant increase in oxidative burst activity in a dose-dependent fashion, whereas albumin (5 and 25%) and hypertonic saline did not activate the neutrophils (**Fig. 4A**). Using the same method, we also studied the expression of neutrophil adhesion molecule (CD18). Artificial colloids (dextran and Hespan) caused the highest expression of CD18 (**Fig. 4B**). Once again, natural colloids (albumin) and hypertonic fluids did not cause any increase in CD18 expression *(12)*.

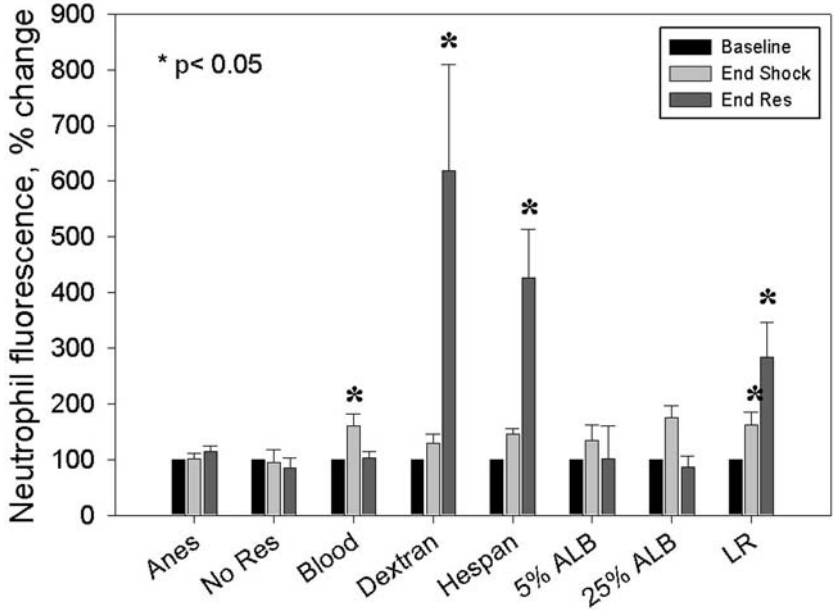

Fig. 3. Neutrophil oxidative burst activity. Neutrophil fluorescence measured by flow cytometery and reported as percent change from baseline values SEM. *, $p < 0.05$, using t-test compared to baseline values. Baseline, before hemorrhage; End Shock, end of 60 min of shock period; End Res, end of resuscitation period; Anes, anesthesia and sham hemorrhage; No Res, hemorrhage and a sham resuscitation; Blood, hemorrhage and shed blood resuscitation; Dextran, shock and 1:1-volume Dextran 40 resuscitation; Hespan, shock and 1:1-volume 6% hetastarch resuscitation; 5% ALB, shock and 1:1-volume 5% human albumin resuscitation; 25% ALB, shock and 0.2:1-volume 25% albumin resuscitation; LR, shock and 3:1-volume lactated Ringer's resuscitation.

A combination solution of dextran and 7.5% saline is now being used in Europe for volume expansion. As dextran stimulates and hypertonic saline suppresses neutrophil oxidative burst activity, we were interested in evaluating the effect of this combination solution on neutrophil function. This was done, again using flow cytometeric analysis of human blood; our findings demonstrated that the hypertonic saline component of the solution exerts the dominant suppressive effect. The combination solution decreased neutrophil oxidative burst activity similar to hypertonic saline alone, and this effect was even more pronounced when the neutrophils were additionally stimu-

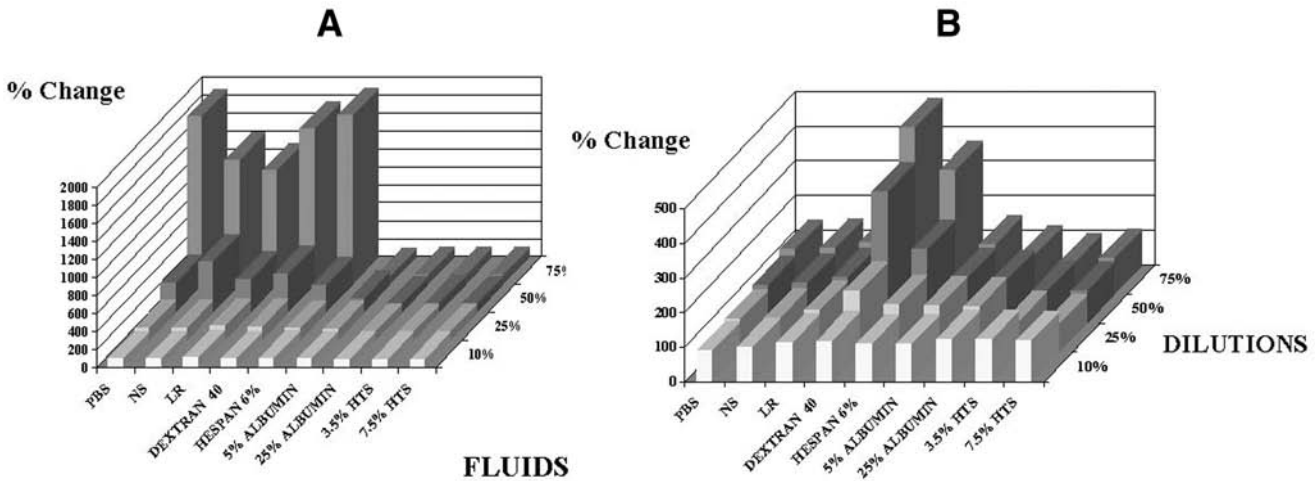

Fig. 4. (**A**) Human neutrophil oxidative burst. Intracellular fluorescence following 30-min incubation with various fluids at 10%, 25%, 50%, and 75% dilutions. (**B**) Human neutrophil CD18 expression. Immunofluorescence following 30 min incubation with various resuscitation fluids at 10%, 25%, 50%, and 75% dilutions. Data presented as percent change in fluorescence compared to normal saline at 10% dilution. PBS, phosphate buffered solution; NS, 0.9% saline; LR, lactated Ringer's; HTS, hypertonic saline.

lated with *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP) or *E. coli (13)*.

We now have emerging data to suggest that changing the isomer of lactate in LR can alter its effect on the circulating cells *(14)*. The commercially available LR contains an equal amount of the L-and D-isomers of lactate (14 mmol of each isomer/L). Using a human whole blood preparation, we showed that L-LR (containing 28 mmol/L of the L-isomer only) caused significantly less neutrophil activation than the standard LR solution. A similar attenuation of neutrophil oxidative burst was also seen when the D-L-lactate in LR solution was completely removed and replaced with ketone bodies (B-hydroxybutyrate). These findings suggest that the D-isomer of lactate may have been partially responsible for some of the findings noted in our previous studies.

Markers of Cellular Injury in Various Organs of Rats

Expression of Adhesion Molecules

Once activated, neutrophils bind to the endothelial cells, establish firm adhesions, and finally migrate into the surrounding tissues (**Fig. 5**). This process involves numerous adhesion molecules. For example, the early rolling phase depends on the selectin group (L-, P-, and E-selectin), whereas the firm adhesion phase involves the B_2 integrins: vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Our group studied the effect of resuscitation fluids on the expression of a number of these adhesion molecules. Since examining circulating neutrophils only pinpoints one aspect of a complicated inter-related event, we wanted to determine the effects on the endothelium. The findings once again demonstrated significant differences between commonly used fluids. LR resuscitation and even LR infusion (without prior hemorrhage) caused increased expression of these adhesion molecules in the lung and spleen. This increased expression was not seen in the nonresuscitated animals, nor in the animals resuscitated with fresh blood. When LR infusion was preceded by hemorrhagic shock, the increased expression of adhesion molecules was accompanied by histologic evidence of pulmonary edema and inflammation *(15,16)*. The effect of hypertonic saline on adhesion molecules was better than LR, but not as good as fresh whole blood.

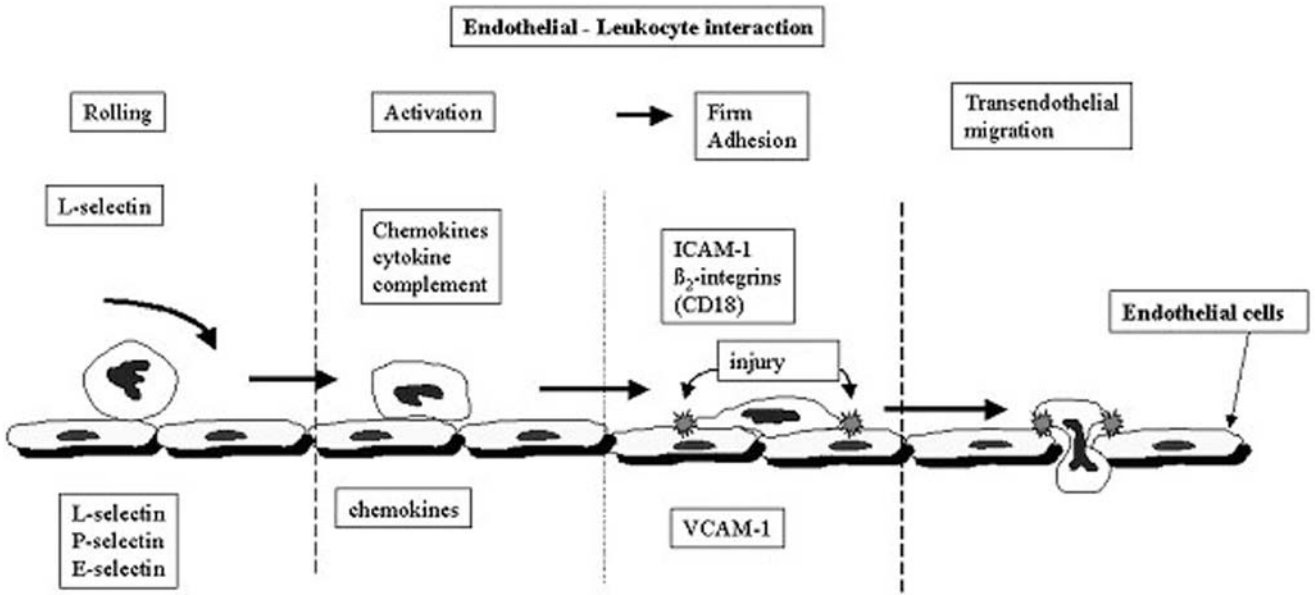

Fig. 5. Various stages of neutrophil and leukocyte interaction. ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

Cellular Apoptosis

Apoptosis is a highly specialized and well-regulated physiologic process for elimination of cells that represent a threat to the integrity of the organism. As apoptosis is a homeostatic mechanism for the removal of damaged cells, we used increased apoptotic cell death in various organs as a marker of cellular injury. In a rat model of hemorrhagic shock, it was demonstrated that LR resuscitation causes increased apoptosis in intestinal mucosa, smooth muscle, liver cells *(17)*, and lung *(18)*. On the other hand, sham resuscitation and resuscitation with plasma, fresh blood, and hypertonic saline did not induce significant apoptosis.

cDNA Array Analysis

With the recent availability of the high-density cDNA array technology, we now have the ability to perform rapid, systematic, global evaluation of cellular functions and regulations at the genomic level. We therefore decided to study the acute impact of hemorrhagic shock and resuscitation on gene expression in major organs after application of different resuscitation strategies in rats. Expression of 1176 genes in four different organs (spleen, lung, liver, and muscle) was determined after resuscitation with LR, plasma, and 7.5% HTS and compared with a control group (sham hemorrhage). Following resuscitation, 82 of the genes studied (7%) displayed an altered expression of at least twofold compared with the sham hemorrhage group. In these 82 genes, a total of 167 alterations (114 increased and 53 decreased expression) were noted (**Table 2**). The largest number of altered expressions was noted in the liver (63/167), followed by the lung (57), muscle (25), and spleen (22). The largest number of alterations was caused by plasma resuscitation (68/167), followed by LR (51) and HTS (48). For every organ studied, alterations in genetic expression were dependent on the fluid used for resuscitation *(19)*. **Figure 6** shows the differential expression of two such genes, c-jun and heat shock protein-70, in the control and resuscitation groups.

Development and Testing of Potential Resuscitation Fluids and Methods

In 1999, the United States Navy, through the Office of Naval Research, requested the Institute of Medicine (IOM) to examine the

Table 2
Summary of Gene Alterations According to Tissue Studied and Fluid Used for Resuscitation[a]

	Liver	*Lung*	*Spleen*	*Muscle*	*Total*
L R	25 (18 up, 7 down)	17(12 up, 5 down)	2 (2 up, 0 down)	7 (4 up, 3 down)	51 (36 up,15 down)
Plasma	22 (13 up, 9 down)	30 (23 up, 7 down)	6 (5 up, 1 down)	10 (3 up, 7 down)	68 (44 up, 24 down)
7.5% HTS	16 (11 up, 5 down)	10 (7 up, 3 down)	14 (13 up, 1 down)	8 (3 up, 5 down)	48 (34 up, 14 down)
Total	63 (42 up, 21 down)	57 (42 up, 15 down)	22 (20 up, 2 down)	25 (10 up, 15 down)	**167 (114 up, 53 down**

[a]Data are number of significant gene alterations. Data in parentheses show direction of alteration, with up for upregulation and down for downregulation. Total of 167 alterations noted in 82 genes (some genes represented in more than one cell). HTS, hypertonic saline; LR, lactated Ringer's solution.

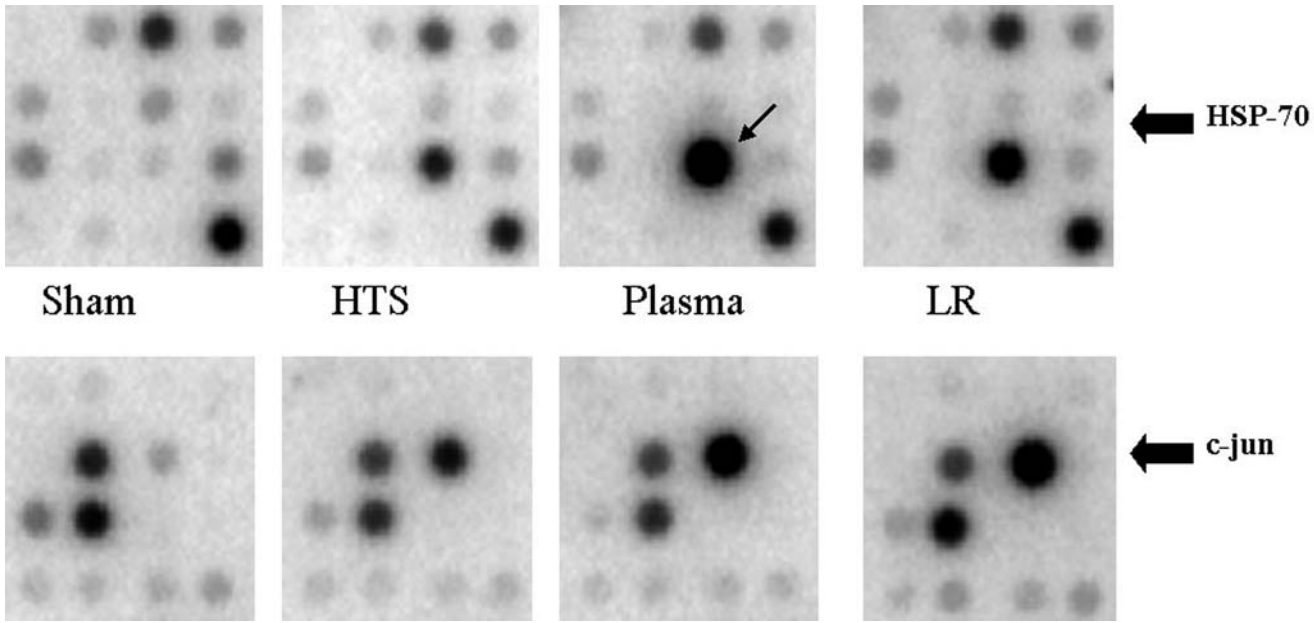

Fig. 6. Sections of cDNA array showing differential expression of heat shock protein-70 (**upper row**) and c-jun (**lower row**) in different experimental groups. Sham, sham hemorrhage; HTS, hemorrhage and 7.5% hypertonic saline resuscitation; Plasma, hemorrhage and plasma resuscitation; LR, hemorrhage and lactated Ringer's resuscitation. Small arrows point to the genes of interest on the cDNA array.

information available on resuscitation fluids. As a result of this process, the distinguished committee gathered by the IOM made a number of recommendations *(5)*. One of the recommendations was to modify the existing LR solution by eliminating D-lactate, reducing total L-lactate, and adding ketone bodies as an energy source. Following these recommendations, we have formulated and produced a product that we call ketone Ringer's (KR) solution.

During hemorrhagic shock, administration of exogenous ketone bodies has clearly been shown to improve the metabolic profile *(20)* and inhibit protein catabolism *(21)* in animal models. Similarly, in severely injured patients, resuscitation with a ketone body (β-hydroxybutyrate)-containing solution for the first 3 h significantly decreases posttraumatic protein catabolism *(22)*. β-hydroxybutyrate is also a membrane stabilizer and free radical scavenger. When tested in a severe model of hemorrhagic shock, KR solution significantly attenuated pulmonary apoptosis and decreased the expression of adhesion molecule (ICAM-1) in rats (**Fig. 7**) *(23)*. As seen in our previous models, plasma resuscitation did not cause any significant increase in cellular apoptosis. We are currently studying whether it is the elimination of lactate or addition of ketone bodies that confers these beneficial effects. The efficacy of other energy substrates (such as pyruvate) is also being tested in the in the setting of hemorrhagic shock.

In all our experiments we have included HTS as a potential resuscitation fluid. The rational for testing HTS in our experiments was the logistical advantage (weight and cube) it offers to a soldier who is already burdened with heavy gear. Our findings, similar to other studies in the literature, showed that HTS resuscitation in general suppressed inflammation, while providing vascular volume restoration equal to that of other fluids. Other researchers have previously reported similar findings, but the subtle difference has been in the interpretation of the results. It has been assumed that the cellular injury and immune activation seen with standard crystalloid resuscitation were an expected consequence of the preceding shock period. Almost all the studies have used crystalloid resuscitation as the control (gold standard) to which other resuscitation strategies were compared. Our data emphasize an often overlooked fact, that although crystalloids may be the standard of care, they are certainly not free of side effects. Hence, the observed cellular damage may not be purely owing to reperfusion of ischemic tissues, but the manifestation of a much more complex resuscitation injury.

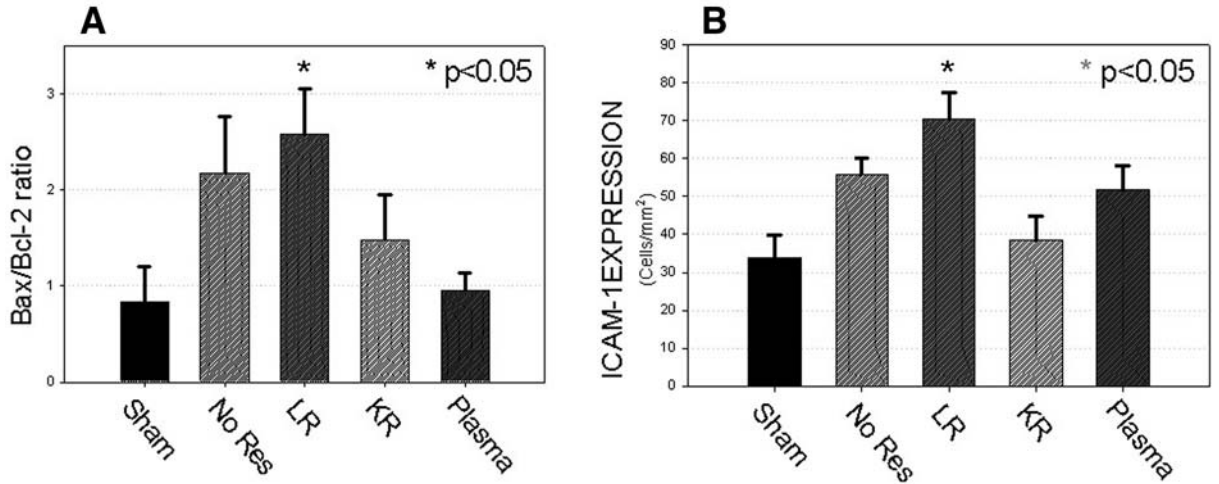

Fig. 7. (**A**) Pulmonary apoptosis. Ratio of Bax and Bcl-2 proteins in lung tissue of different groups as measured by Western blot analysis. (**B**) Intracellular adhesion molecule-1 expression as measured by immunostaining. Data presented as positive cells per mm^2 at 40× magnification. All data shown as group means ± SEM. *$p < 0.05$ ANOVA and Dunnett's test for multiple comparisons. Sham, sham hemorrhage; No Res, hemorrhage and sham resuscitation; LR, hemorrhage and lactated Ringer's resuscitation; KR, hemorrhage and ketone Ringer's resuscitation; Plasma, hemorrhage and plasma resuscitation.

As a result of the favorable data in the literature, one of the final recommendations made by the 1999 IOM committee was to use HTS as the initial fluid for resuscitation of combat casualty *(5)*. The report states that the initial fluid resuscitation of the hemorrhaging battlefield casualty should be a 250-mL bolus of 7.5% saline delivered by a rapid infusion system. Obtaining reliable intravenous access in the battle field can be difficult; therefore the committee proposed that the fluid could be infused through an intraosseous (IO) needle placed in the anterior tibia and that the total volume of infusion should be kept under 500 mL. There were two factors in this recommendation that we felt deserved further investigation. The first was whether, in the setting of uncontrolled hemorrhage, the bolus of 250 mL of 7.5% HTS (which is roughly equivalent to 2 L of 0.9% normal saline) would cause more bleeding in contrast to smaller more frequent volumes of infusion. The second concern was the method of infusion. Anterior tibial IO had not been adequately tested in a dehydrated, long-term, survival model of hemorrhagic shock.

To resolve these questions, a 12-G needle was placed in the right anterior tibia of 14 dehydrated Yorkshire swine under isoflurane anesthesia. Uncontrolled hemorrhage was induced via left iliac artery and vein injury. Animals were kept in shock for 2 h and then resuscitated over 2 h with 5 mL/kg of 7.5% HTS given either as 10 small boluses (group I) or 2 large boluses (group II) to compare the physiologic response and blood loss. The control animals (group III) received an equal volume of 0.9% saline IO and additional saline intravenously to equalize the salt load in all groups. Our results showed that HTS resuscitation was effective in dehydrated animals and did not increase the bleeding from the uncontrolled vascular injury. However, IO infusion of HTS in this model carried a very high rate of local soft tissue complications. Between the second and fifth postresuscitation days, the 7.5% HTS-resuscitated animals developed soft tissue necrosis (as a result of compartment syndrome) or bone marrow necrosis of right hind leg (group I, 100%; group II, 66.6%, group III, 0%). We feel that until further investigations, the use of 7.5% HTS in humans should be limited to intravenous administration *(24)*.

Although HTS provides both the logistic advantage and possible immunosuppressive effects to help counterbalance the postresuscitation inflammatory response, it is still not the ideal fluid. This has led us to investigate other fluids, including freeze-dried plasma. This product has a

very long shelf life and can theoretically be made in advance for soldiers using their own plasma. The powder can also be reconstituted quickly and dissolved in a small volume of solvent, which would yield a hyperoncotic/hypernatremic fluid. The hemodynamic response to an infusion of this small volume of fluid is equal to the much larger volumes of isotonic crystalloids that are currently in use. We have also noted on a consistent basis that natural products (such as whole blood or plasma) had the most favorable effects on the cells and did not cause immune overactivation. Although this seems intuitive, historically our goal has primarily been the restoration of hemodynamic parameters. The makeup of the solution was of no particular importance to the clinicians as long as the desired hemodynamic results were achieved. For example, during the Korean War, blood and plasma were used for resuscitation, resulting in numerous saved lives, but during the Vietnam conflict, the use of blood products fell out of favor, and the less expensive and easier to use crystalloids gained popularity. However, plasma has many beneficial properties in addition to its ability to restore blood volume. Thus, we are currently investigating the potential benefits of products such as the hyperoncotic/hypernatremic freeze-dried plasma for military application.

Recommendations on Fluid Resuscitation for Battlefield Casualties

Based on our own research and review of contemporary data on combat casualty resuscitation, we propose a simple treatment algorithm (**Fig. 8**). These recommendations are specifically for the initial field resuscitation; once the injured patient has been transported to a more stable environment, conventional methods may be used. These recommendations will change as more information becomes available in the future, but for now the recommended algorithm has been based on the following information.

1. *Most combat casualties do not require fluid resuscitation*. The vast majority of those with combat casualties are not in shock. Examination of the casualty data from the last several military conflicts has shown that the vast majority of battlefield wounds are to the extremities and soft tissues as a result of firearms or fragments *(25)*. Most of these injuries can be treated initially without any fluid resuscitation. Although in civilian trauma practice the placement of an intravenous line is convenient, and often required, the same is not true for the initial care of an

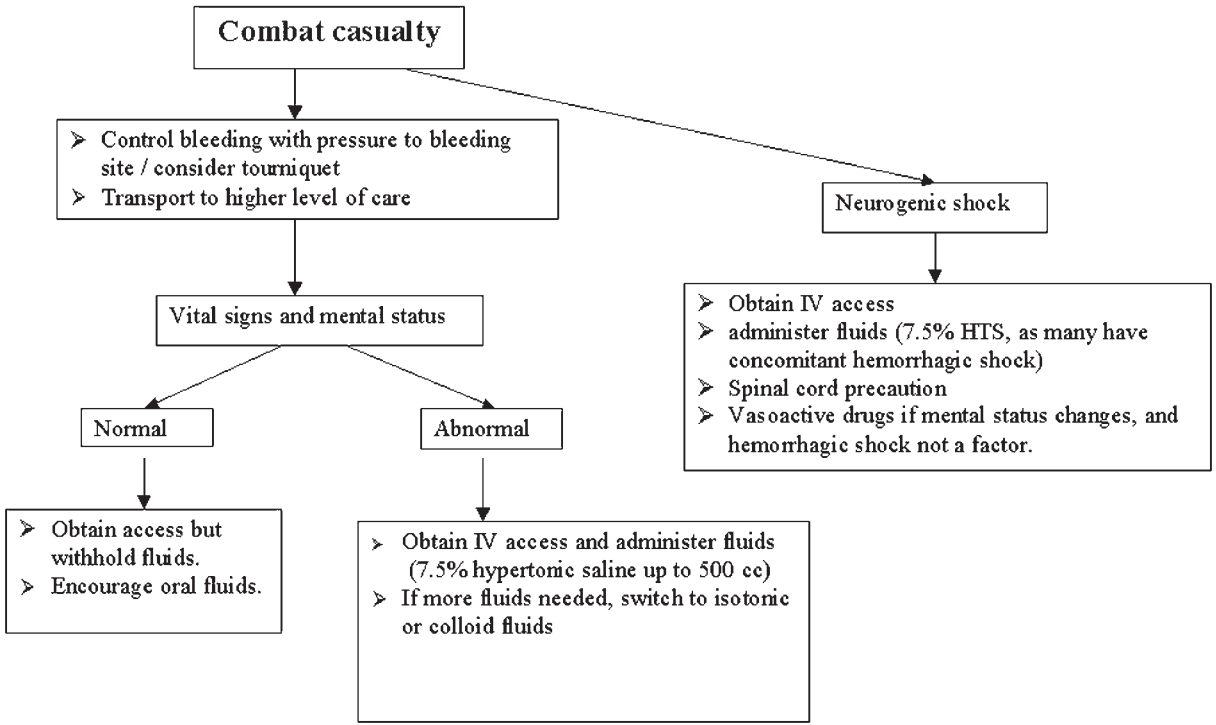

Fig. 8. Algorithm for the initial resuscitation of combat casualties.

injured soldier. This can wait until the soldier has been removed from the line of fire and has arrived at the level of echelon II care.

2. *Oral hydration is an underutilized option*. In a civilian trauma setting, typically all oral intake is withheld during the early postinjury phase. This simplifies the evaluation process and decreases the chance for aspiration of gastric contents. This is sound practice, as most of the patients who need surgery are quickly taken to the operating room. However, because of the changing environment in the military (with transport to higher echelons of care possibly delayed more than in previous battles), the use of oral hydration in soldiers who have injuries to the extremities makes sense. During the Korean War, typical transport times were approximately 4 h. The Vietnam conflict saw that time reduced to approximately 30 mins. Future military conflicts are expected to be similar to the 1993 battle in Somalia *(24,26–28)*, where the delay in evacuation was several hours, and many of the casualties could not be evacuated until the next day. The types of operations anticipated by the United States Marines will be over larger distances with a rapid nonlinear battlefront, as exemplified in the Desert Storm experience.

   The doctrine of the U.S. Marines engaging in operational maneuvers from the sea dictates that transport to casualty-receiving facilities will be hampered by longer distances and by the priorities of rapid and violent engagements. Longer transport times and the fact that the vast majority of the wounded will not have life-threatening injuries make, the oral route for hydration logical and convenient. Once out of the line of fire, obtaining vascular access in a stable environment is more appropriate. Currently, the natural instinct of providers is to start an intravenous line as soon as possible on all casualties and to withhold oral intake. Although oral fluids are absorbed at a decreased rate in animal models of hemorrhagic shock, most of the injured soldiers are not in shock and do not need immediate intravenous fluid resuscitation. Finally, the possibility of aspirating gastric contents is a concern. In civilian trauma care, the chances of aspiration during induction of anesthesia are very low when rapid-sequence intubation is carried out. In fact, the vast majority of civilian trauma patients have a full stomach when urgent surgical care is provided. When the injured soldier finally receives surgical intervention under general anesthesia, it will be delivered at a higher echelon of care and in a controlled environment. Rapid-sequence intubation in this setting can minimize the chances of aspiration.

3. *Aggressive resuscitation has not been shown to be beneficial in civilian victims of penetrating trauma.* The test of this hypothesis in humans was published in the *New England Journal of Medicine* in 1994 *(29)*. In this study, hypotensive patients with penetrating injury to the torso were randomized to routine fluid resuscitation, or the start of an intravenous line but no fluid resuscitation until surgical control of bleeding had been achieved. The rationale was that early fluid resuscitation would increase the blood pressure and thus make the patients bleed more from the uncontrolled source of hemorrhage. The results of the study demonstrated a survival advantage for the patients whose initial crystalloid resuscitation was withheld. This study has generated vigorous debate and has been extensively scrutinized for its faults. One of the major criticisms has been that the study did not include the patients who died in the field in an intent to treat analysis. When all these patients were included in the analysis, it was discovered that withholding of fluids provided no survival benefit. The surprising aspect of this subsequent reanalysis is the fact that the withholding of fluids showed no increase in mortality.
4. *Moderate resuscitation in animal models of uncontrolled hemorrhage offers the best outcome*. Some researchers have focused on the detrimental aspects of aggressive fluid resuscitation during the initial treatment of uncontrolled hemorrhage. It was speculated that the preservation systems of the body have a built-in set of compensatory mechanisms that would allow it to withstand moderate levels of shock. Animal models have actually demonstrated that aggressive resuscitation in the setting of uncontrolled hemorrhage could cause increased bleeding and thus worsen outcome *(30)*. In this study, Burris et al. *(30)*, using a rat model of uncontrolled hemorrhagic shock via an aortic laceration, showed that the group that received no fluids had the lowest survival rate. The groups resuscitated with LR to a mean blood pressure (mbp) of 100 mmHg also had low extremely survival rates. The highest survival rates were seen in the animals that were resuscitated to an mbp of 80 mmHg with LR, or 40 mmHg with 7.5% HTS and 6% hetastarch solution.
5. *Large volumes of LR may not be totally innocuous*. Since the inception of LR (Ringer, 1883), it has been widely used for the treatment of hemorrhagic shock, burns, and sepsis. However, its use has not been without controversy. Cushing recognized the cytotoxic effects of LR as early as 1901 in isolated nerve and muscle preparations. LR is a racemic mixture of two stereoisomers of lactate: D(−)-lactate and L(+)-lactate. The

metabolism of these two lactates occurs via different pathways and produces distinct metabolic consequences. A rise in serum D-lactate alters neurologic function and produces encephalopathy *(31)*. D-lactate has also been shown to produce various degrees of cardiac arrhythmogenicity, premature ventricular contractions, ventricular tachycardia, sinus bradycardia, ventricular fibrillation, third-degree heart block, and asystole *(32)*. With recent advances in our ability to examine the immune response closely, there is accumulating evidence that the type of fluid used for resuscitation makes a difference. This chapter has summarized some of the data from our laboratory to substantiate this claim. We feel that it may be more prudent to develop and test a fluid that does not promote increased cellular damage rather than modulating the response after the process has already been set into motion.

6. *Hypertonic saline administered intravenously has been shown to be safe in dehydrated animal models of hemorrhagic shock*. Numerous studies have tested the efficacy of hypertonic saline resuscitation in dehydrated animals subjected to hemorrhagic shock. Review of this data reveals that dehydration (20% weight loss) does not compromise the efficacy of hypertonic resuscitation *(33)*. When compared with LR in a conscious, dehydrated (for 48 h) swine model of hemorrhagic shock, 4 ml/kg of hypertonic Saline dextran (HSD) was found to be equally effective and safe *(34)*. Although dehydration increased the mortality (compared with the euhydrated group) from hemorrhagic shock, it did not effect the early hemodynamic response to HSD treatment *(35)*.
7. *Hypertonic saline has been found to be safe in eight prospective randomized clinical trials of trauma patients*. The use of HTS for resuscitation from hemorrhage was first described in 1980, when Velesco et al. *(36)* and DeFelippe et al. *(37)* reported in separate studies that hyperosmotic sodium chloride rapidly expands plasma volume after major blood loss. These early studies generated a storm of experimental and clinical research examining the use of HTS for the early restoration of blood pressure and cardiac output in the field. Since then, HTS has been used in a variety of circumstances, and over 300 papers have appeared in the literature over the last 10 yr.

   Recently, there have been eight double-blinded randomized trials evaluating HTS or HTS with dextran (HSD) for prehospital or emergency department treatment traumatic hypotension. Improved rates of survival after discharge were reported with HSD in seven of eight trials, although statistically significant improvement in overall survival was seen in only

one trial. A meta-analysis *(38)* for the evaluation of HSD as the initial treatment for hypovolemic shock reviewed the original records from these trials. Overall discharge survival rates were better with HSD resuscitation compared with conventional resuscitation. HSD resuscitation was particularly effective for the subgroup of patients who had sustained head injury, with a discharge survival rate of 38%, compared with a rate of 27% for the control group receiving saline. In the clinical literature, there has been a remarkable absence of deleterious effects with HTS administration in more than 1000 trauma and surgical patients. No increase in the incidence of hypernatremic seizures, increased bleeding or blood transfusion requirement, coagulopathies, renal failure, cardiac arrhythmias, or central pontine myelinolysis has been attributed to hypertonic resuscitation in trauma patients.

8. *The IOM has recommended 250 cc of 7.5% HTS infusion for initial use in resuscitating combat casualties and a second bolus of 250 cc if necessary*. As previously mentioned, the IOM report has recommended use of 7.5%HTS (up to 500 cc) for the treatment of battlefield casualties in shock *(5)*.
9. *HTS offers significant advantage in terms of weight and cube for the military medic or corpsman.* Carrying fluids in the field is difficult because of its weight (1 L of fluid weighs 1 kg). However, the use of 7.5% HTS can provide the same hemodynamic response as isotonic fluids with only 1/8–1/10th the volume (and weight). Our recommendation is not to use this fluid as the only resuscitation fluid. As HTS may not be the best fluid to use over long periods, a combination of HTS and LR should be available in field hospitals. However, use of HTS as the first fluid in the far forward area would markedly improve the logistics involved.

METHODOLOGIES TO TREAT THE COMBAT CASUALTY

The development of novel techniques to treat injuries efficiently is of high value. Blood loss from vascular injury has always been a major cause of death and morbidity in military conflicts, because highly trained vascular surgeons and sophisticated techniques/equipment are not always available to the general surgeon. Tools and techniques to assist the surgeon in the repair of tissues including injured vessels would be of benefit during times of combat. This area of research is therefore designed to develop and test products that can optimize surgical therapy for combat casualty.

Titanium Vascular Staples

Since Carrel *(39)* first described the triangulation technique of vessel anastomosis in 1902, the field of vascular surgery has made significant progress. A variety of surgeons in addition to vascular surgeons (such as cardiothoracic, neurologic, plastic, and transplant surgeons) make use of vascular anastomosis. Many novel methods of vascular anastomosis have emerged over time, such as stapling and clipping devices, gluing, and laser welding. The application of tissue adhesives such as fibrin glue and cyanoacrylic glues has been disappointing. Fibrin glue is thrombogenic, and cyanoacrylic products are toxic. Various lasers have been utilized in creating vascular anastomosis including the Nd:YAG, argon, and CO2 lasers; however, the reported incidence of pseudoaneurysm and rupture at the site of anastomosis in experimental models has been high, and further refinements need to be worked out. The nonpenetrating arcuate legged titanium vascular closure staples (VCS) reported by Kirsch et al. *(40)* in 1992 have shown the most promise in vascular surgery. This device offers the benefits of being nonpenetrating, having better healing properties than traditional sutures, and being easy to use, which allows for faster anastamosis. These properties may ultimately translate into less bleeding and faster treatment of combat casualties.

The VCS (Autosuture, United States Surgical, Norwalk, CT) are currently available in four sizes based on the clip span between the legs at the tip (**Fig. 9**). The four sizes are extra large (3.0 mm), large (2.0 mm), medium (1.4 mm), and small (0.9 mm). The extra large clips were used and placed with a clip applier that contains 25 clips and comes individually wrapped in sterilized packs. Specially designed forceps for aligning the vessel edge with intimal eversion and clip removers were also used (**Fig. 10**).

We have tested titanium VSC as an alternative to suture repair of vessels as well as other nonvascular tissues. When tested in a surviving model utilizing swine, we found that VCS clips were quicker and easier to use and resulted in improved tissue healing because of their nonpenetrating design *(41)*. The tissues healed well because they were gently approximated by the staples without undue pressure or trauma. Nonvascular tissues in which the VCS clips were tested included the gallbladder, common bile duct *(42)*, and ureter *(43)*. These tissues are notorious for causing strictures, and penetrating suture can also act as a nidus for stone formations.

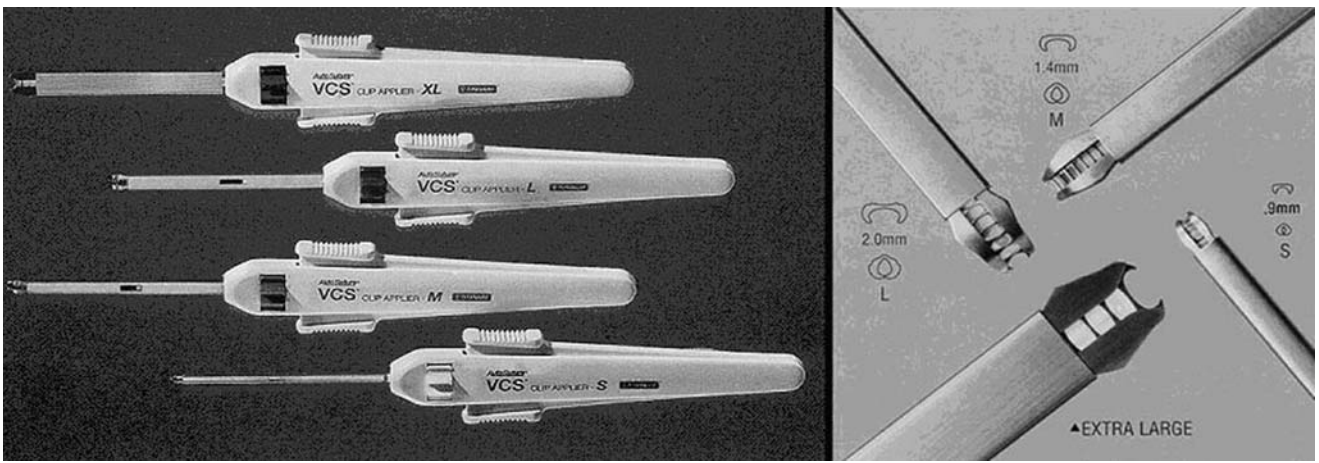

Fig. 9. Titanium VCS clip applier in four different sizes.

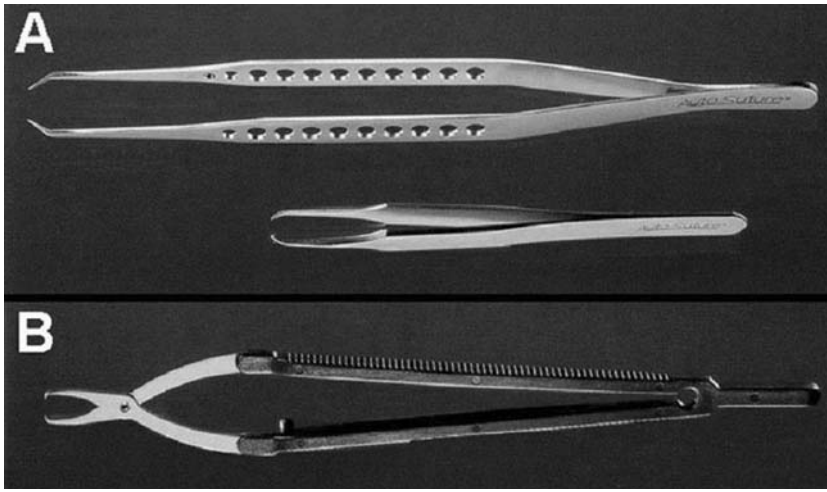

Fig. 10. (**A**) VCS forceps, (**B**) VCS clip remover.

We have tested the VCS clips for vascular repair in a variety of tissues such as the iliac arteries *(44)* and the aorta *(45)*. Leppaniemi and Wherry have shown that arteriotomies and veniotomies of the iliac vessels repaired with VCS clips or standard sutures were identical in terms of patency, leakage, and intimal reaction, with a marked reduction in time required for clip closure. In addition to merely testing it on native arterial tissues, we have applied the clips on other artificial and autologous material as well, by performing vein and polytetraflouroethelene (GOR-TEX) patch repairs of aortic injuries. We have recently tested the extra large VCS clips on the thoracic aorta and found it to be useful *(46)*. This study demonstrated that the anastamosis time was reduced as well as the blood loss during the repair. The advantages of the titanium VCS clips over conventional repair are that they can repair tissues faster, they produce leak-free repair, and healing is equal to or better than that with sutures (**Fig. 11**). Finally, because of these promising laboratory findings, the VSC clips were tested in human trauma patients and were found to be useful and effective (**Fig. 12**) *(47)*.

Portable Microwave Detector

Researchers from TRRI-Surg and others who are collaborating are also investigating a new technology using a microwave-based detector

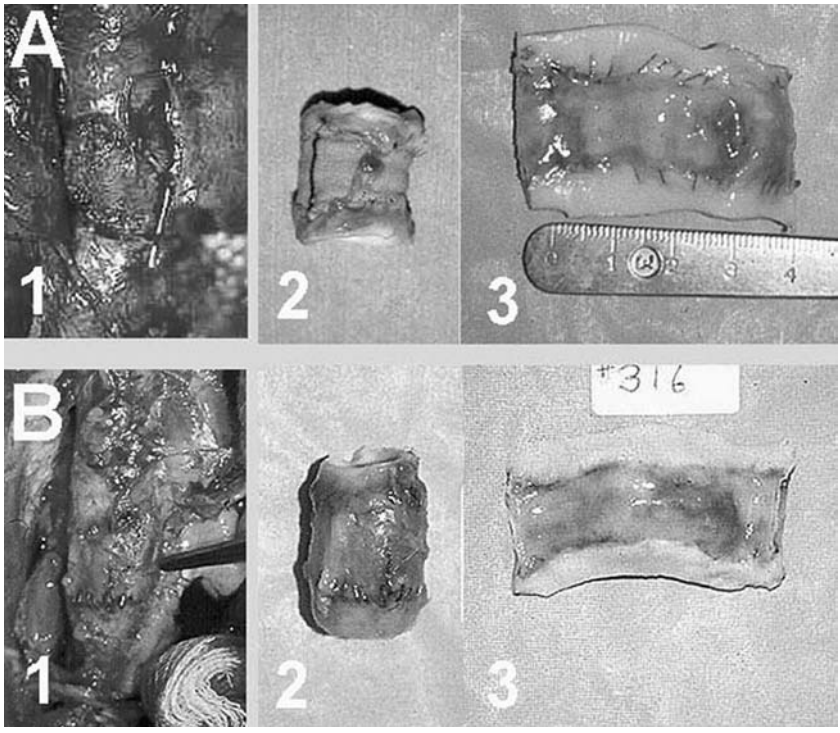

Fig. 11. (**A**) *1*. Healed suture-repaired aorta *in situ* at 8 wk. *2*. Suture-repaired graft excised and dissected out. *3*. Inside the suture-repaired graft with native aortic edges, suture visible. (**B**) *1*. Healed VCS-repaired aorta *in situ* at 8 wk. *2*. VCS-repaired graft excised and dissected out. *3*. Inside the VCS-repaired graft with native aortic edges, clips not penetrated. VCS, vascular closure staples.

to identify air and blood in various cavities within the body. For those treating combat casualties, this technology offers portability and is thus suitable for use in the field as well as treatment facilities.

Pneumothorax is a common complication of thoracic injuries. It has been reported in a significant number of combat related wounds and confirmed in many patients autopsied during the Vietnam War. It is also a common injury in civilian trauma. This potentially lethal condition can be readily treated if detected early. The diagnosis of pneumothorax is especially important because the military often transports the injured with the aid of a helicopter. When heights of 1000 feet are obtained, the decrease in atmospheric pressure can worsen the condition. Previous

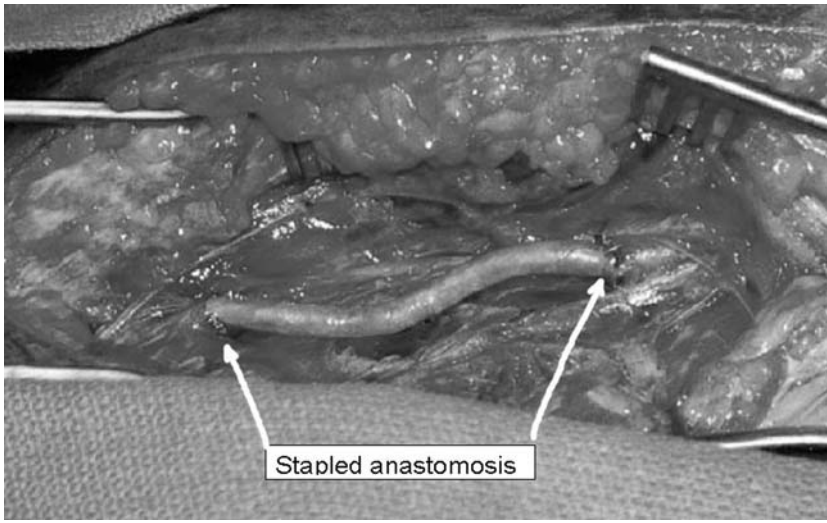

Fig. 12. Femoral artery anastamosis with end-to-end reverse saphenous vein graft using large size VCS clips on the right (proximal) and medium on the left (distal).

experience has shown that up to 30% of these patients with pneumothorax can die if not treated promptly.

The key to a favorable outcome is rapid diagnosis and treatment of the pneumothorax. The physical exam is notoriously inaccurate, with a sensitivity of 58%. Therefore, chest X-ray is commonly used for the detection of a pneumothorax, but this technology is not available in the battlefield. Even if the technology could be advanced to make the X-ray machine portable, the development of the X-ray images makes the process difficult. Attempting to auscultate the chest in the field or during transportation to diagnose this condition is especially difficult in the military setting owing to the high noise level in the surroundings.

A noninvasive easy-to-use detector system has been developed that utilizes harmless low-energy electromagnetic waves in the microwave radio frequency region. This device uses a modified algorithm for the estimation of the angle of arrival of radar signals, which can potentially detect changes in signal caused by various tissues as it is reflected. We have termed this technology the radiofrequency triage tool (RAFT).

We tested this new technology to determine whether it could be used to detect conditions such as pneumothoraces in anesthetized swine. We found that in comparison with chest X-rays, RAFT could detect a 20%

pneumothorax at a rate equal to that of the chest X-ray *(48)*. This new system has also been tested in other battlefield relevant conditions such as accumulation of intracranial blood. We found that volumes of blood as small as 2 cc could be detected within the cranium, including blood in the epidural, subdural, intraventricular, and intraparenchymal spaces *(49–51)*.

We are also assisting the Office of Naval Research in determining the usefulness of a marine polymer dressing for control of hemorrhage in the battlefield. It is well known that hemorrhage is the leading cause of battlefield deaths. In approximately 80% of soldiers who die owing to hemorrhage, the source of bleeding is internal and hence extremely difficult to control. However, about 20% have extremity bleeding that can potentially be controlled in the field if treated promptly and effectively. A hemostatic dressing that can be applied to the bleeding wound by minimally trained personnel may slow or stop the bleeding long enough to allow evacuation of the injured to a higher level of care. A number of dressings have been tested in various animal models, but only one dressing has received approval from the Food and Drug Administration (FDA) for human use so far. This dressing, manufactured by Marine Polymer Technologies, (Danvers, MA) uses a polymer derived from sea algae (poly-*N*-acetylglucosamine) and has shown promising results in animal and human testing. This dressing, however, has not been studied in a complex model of the combined arterial, venous, and soft tissue injuries we are likely to encounter in the modern battlefield. We are therefore testing the efficacy of this dressing (and other promising materials that are pending FDA approval) in a highly lethal complex extremity wound in swine.

FUTURE RESEARCH WITH INDUCED HYPOTHERMIC SUSPENDED ANIMATION

The Concept

The goal of this project was to determine whether profound hypothermia could be induced in a model of exsanguinating uncontrolled hemorrhage. This would create a state of suspended animation in order to allow time to perform surgical repairs *(52,53)*. In this study, we set out to develop a technique for inducing hypothermic arrest in a clinically relevant model. The method should utilize currently available techniques and resources. We also wanted to test the concept of infus-

ing cold acellular fluid to arrest metabolism in order to create a state of suspended animation. Therefore, the aim was to induce hypothermia through an emergency department thoracotomy (EDT) incision following uncontrolled exsanguinating arrest in a swine model.

Currently, penetrating trauma patients who do not respond to resuscitation may undergo EDT. The survival rates after this procedure are generally low and depend on many factors, including type and location of injury as well as physiologic status at the time of the procedure *(54, 55)*. Although those who undergo EDT often have injuries that are repairable, the survival rates are not optimal and the procedure can be costly in terms of the resources used. Despite many advances in medicine, little progress has been made in improving outcome after this procedure over the last several decades. If these patients *in extremis* could be put into a state of suspended animation, surgeons may be able to repair their injuries in a bloodless field.

The concept that low temperatures may be protective at the cellular level is not new. We know that cold temperature slows down biologic activity, and the use of refrigeration, based on this concept, has made a major impact on modern life. Hypothermia can slow down the metabolism of living organisms by slowing active ion transport, homeostasis, and enzyme activity. Because hypothermia suppresses metabolism, it minimizes the demand for oxygen and extends tissue tolerance for ischemia *(56)*. Controlling the biologic regulators that are used during hibernation can also decrease metabolism. However, the control of metabolism through pharmacologic means is not yet available. These concepts have led to the development and use of techniques to induce hypothermic arrest in humans *(57)*. Hypothermia is currently employed in neurologic, pediatric, and cardiothoracic surgery *(58–60)*. In humans, the limit of hypothermic arrest has been found to be approximately 1 h at 15°C *(61–64)*. The question then becomes: can the advantages of hypothermic arrest be used in the setting of trauma?

Recent research in this field has been led by Dr. Peter Safar, who demonstrated that hypothermia can be induced with complete recovery after normothermic controlled hemorrhagic shock and profound circulatory arrest of 60 min in dogs *(65)*. The development of acellular fluids to protect and preserve the whole body has further advanced this field. Taylor et al. *(66)* have demonstrated that 3 h of profound hypothermic cardiac arrest can be achieved in dogs with normal neurologic preservation by using blood replacement with acellular fluids. The use of acellular flu-

Table 3
Components of Hyperthermosol M and Hyperthermosol P Solutions (mmol/L)

Components	*Hyperthermosol M (Maintenance solution)*	*Hyperthermosol P (Purge solution)*
Ionic		
Sodium	100	141.2
Potassium	42.5	3.0
Calcium	0.05	1.5
Magnesium	5.0	1.0
Chloride	17.1	111.0
Sulfates	—	1.0
pH buffers		
H_2PO_4	10.0	1.2
HCO_3	5.0	25.0
HEPES	25.0	25.0
Impermeants		
Lactobionates	100.0	—
Sucrose	20.0	—
Glucose	5.0	5.0
Manitol	20.0	—
Colloid		
Dextran-40	6%	6%
Metabolites		
Adenosine	2.0	1.0
Glutathione	3.0	3.0
Osmolality	350	305
pH	7.6	7.6

ids also addresses some of the technical problems associated with hypothermia, such as increased plasma viscosity and cellular sludging through the microvasculature. The fluids developed by Taylor were Hypothermosol P, which contains normal levels of electrolytes, and Hypothermosol M, which contains impermeants and 42.5 mmol/L of potassium (**Table 3**). Experiments by Safar and Taylor established the circumstances and feasibility of hypothermic arrest, but they were performed under reasonably controlled conditions with closed chest and

peripheral cannulation. We wanted to determine whether asanguinous hypothermic circulatory arrest could be induced through an EDT incision following exsanguination with the use of currently available equipment.

Animal Models of Exsanguination and Induced Suspended Animation

Through a left anterior thoracotomy, a laceration of the descending thoracic aorta was produced (26 swine, 45–70 kg), resulting in exsanguinating uncontrolled hemorrhage. After 5 min of severe hypotension (systolic BP < 20 mmHg), a 22-F Foley catheter was directed cephalad through the large aortic wound. A solution (containing 42.5 mmol/L K+ precooled to 1°C) was infused to arrest/preserve the heart and brain. A second 24-F Foley catheter was then directed caudally through the same wound. The right atrium was opened to drain the venous system. The animal was cooled with a cardiopulmonary bypass pump (>5 L/min) through the Foley catheters. Once 10°C was reached, a cannula was placed in the aortic root, and the descending aortic laceration was repaired (**Fig. 13**). The animal was maintained at 10°C for a total of 90 min. Prior to the rewarming process, the circulation was rinsed with a solution containing normal levels of electrolytes followed by infusion of whole blood.

Rewarming was performed by maintaining a 10-degree gradient between the heat exchanger and core body temperature. The first 16 animals were used in nonsurvival experiments to develop the technique and to record intracranial temperatures and electroencephalographic tracings. The last 10 animals were used to determine long term survival and neurologic outcome. In group I, seven animals were kept at less than 10°C with flows of less than 2 L/min. In group II, three animals underwent 20, 30, and 40 min of no flow once they were cooled to 10°C. After 6 wks of survival and neurologic examinations, the brains were fixed for histologic evaluation.

The average times to cool the head to 18°C and 10°C were 6 and 12 min, respectively. The hematocrit fell below 2% by the end of the cooling period. Seven of the 10 animals from the long-term study survived. In group I, five of seven animals survived; four of these survivors had no appreciable neurologic deficits, were fully functional at 6 wks, and had no evidence of histologic injury. One of the five survivors in this group had moderate neurologic disability. Of the two that died, one died owing to air embolism from the intravenous IV line. The second

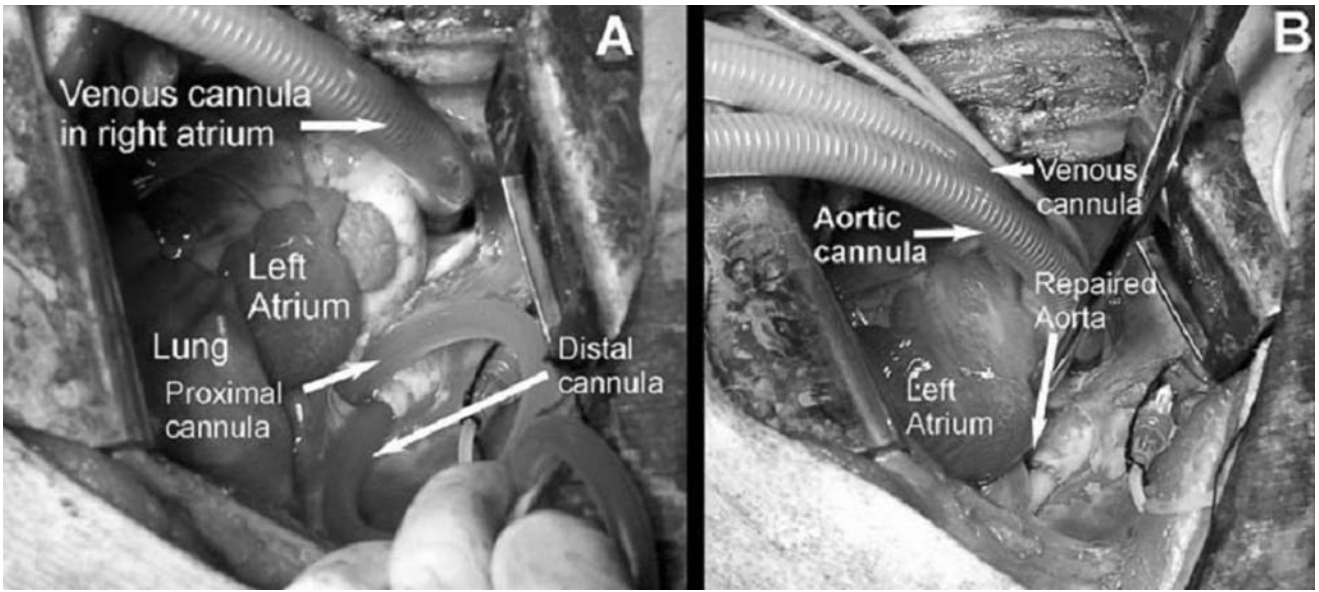

Fig. 13. (**A**) Silastic foley catheter in aorta, venous cannula in right atrium. (**B**) Descending aorta repaired, 7-mm aortic cannula in root of aorta, and venous cannula in right atrium.

death was in the animal that was maximally cooled to 2.7°C. In group II, the first two animals that had no flow for 20 and 30 min were fully functional and had normal neurologic exams. However, the second animal was found to have brain injury on histologic examination. The last animal in this group died of accidental extubation during recovery.

The main goal of this study was to determine whether profound acellular hypothermic arrest could be induced through the chest following exsanguinating hemorrhage in a swine model. We purposely chose techniques and equipment that are available in most trauma centers. This model has shown that it is possible to induce acellular profound hypothermic arrest following exsanguinating hemorrhage and then resuscitate the animal without detectable neurologic injury.

The recovery appeared to be complete in four of the five animals that underwent 90 min of low-flow acellular deep hypothermia (10°C). No neurologic deficits were identified on clinical or histologic examinations. One animal in this group survived with residual neurologic deficits. All animals that survived were extubated within 1–6 h of closing the chest. They were able to take water within 12 h and usually resumed their diet within 24 h. Within 36 h, they were fully ambulatory, with return of preoperative behavior. There was a mild elevation in the total bilirubin, ALT, and GGT during the 6-wk postoperative period. A similar rise in these enzymes has also been reported in the canine model *(67)*. One animal had persistent fever and leukocytosis that resolved with administration of antibiotics for 1 wk.

The last animal in group I was cooled as much as possible to a nadir temperature of 2.7°C and was kept at this temperature for 90 min. This was attempted because Tisherman et al. *(68)* have shown that cooling to 10°C results in better neurologic outcome than cooling to 15°C in dogs. Thus we wanted to see whether a further decrease in temperature (<10°C) offered additional protection to the brain. However, upon resuscitation, the animal demonstrated neurologic injury within 3 h of extubation. It has been previously shown that cooling below 5°C results in neurologic injury, pulmonary dysfunction, and systemic edema in dogs *(69)*. Since we performed only one animal experiment with maximal cooling, it is not known whether this injury was owing to the nadir temperature or other technical problems.

The three animals in group II underwent complete circulatory arrest for various time periods (20, 30, and 40 min). This experiment represented a clinical scenario in which total cessation of flow might be

helpful during surgical repairs. The second animal was not as responsive on postoperative 1 and 2, which was reflected in the neurologic scoring. This animal recovered completely by the third postoperative day, with no clinical evidence of neurologic injury. However, the histologic examinations showed injury to the brain. This emphasized the need for both the clinical and histologic correlation. The third animal in this set of experiments, which was left in arrest for 40 min, recovered for 20 h but demonstrated obvious neurologic deficits immediately postoperatively. The animal was found dead at 24 h after surgery.

Since hypothermia protects against ischemia, it is key to induce hypothermia rapidly for the purpose of protecting the brain. This allows additional time for surgical repair. We chose to induce hypothermia through the chest because EDT is currently the standard approach in an exsanguinating patient not responsive to resuscitation. Induction of hypothermia through the femoral approach has been used successfully in a canine model, but since the chest is opened during the EDT and the descending thoracic aorta is readily available, it was felt that this makes the thoracic approach more clinically appealing. It can be expected that half of exsanguinating patients would require exploration of the chest cavity anyway.

The highest survival rate from EDT is when the source of hemorrhage is in the chest and is easily treatable. In these cases, it may be more logical to try primary repair without the induction of hypothermic arrest. There may be situations in which induction of hypothermia would be more appropriate through a femoral approach or in combination. If rapid high flows can be achieved selectively by the development of specially designed catheters, it would help to advance this field of research. Woods et al. *(70)* have shown that a 500 cc 4°C saline flush using a catheter with an occluding balloon designed to flush the heart and brain can provide protection after prolonged exsanguination and 30 min of complete arrest. This technique may also provide additional time to access the chest. However, obtaining femoral vessel access in a hypovolemic patient may be difficult.

Ordinary Silastic urinary Foley catheters were used to induce hypothermia because our intention was to use techniques and materials that are available in most trauma centers. Cardiopulmonary bypass (CPB) roller pumps with a membrane oxygenator and heat exchanger are also available in most Level I trauma centers. Because rapid

infusers and warmers are primed and ready for use in most hospitals, it was envisioned that, with modification, a refrigerator unit with a reservoir could be manufactured with pumps that could stand ready for use to induce hypothermia. We are currently developing small pumps the size of a grapefruit that can replace the conventional CPB pumps used for elective cardiovascular surgery.

The use of the precooled Hypothermosol M solution was another novel approach tested in these experiments. The solution contains 42.5 mmol/L of K+, which helps to arrest metabolism and passive ionic exchange in cells when metabolic pumps are switched off during hypothermia. However, in our model, the serum K+ rose only to a maximum level of 10.9 mmol/L. The Hypothermosol M solution was specifically designed as an aqueous blood substitute for total body hypothermic perfusion. It embodies many of the principles now identified as contributory and important for success in organ preservation *(71–73)*. In contrast to Taylor's experiment, we utilized the Hypothermosol M solution initially to arrest metabolism, whereas he induced hypothermia with Hypothermosol P. The use of Hypothermosol P solution alone had resulted in motor and sensory deficits.

The use of acellular solutions may offer an additional advantage in that precious blood is not lost while the surgical repair of the injury is being performed. The resources of banked blood are thus not being used until the source of bleeding has been controlled. The use of manufactured acellular fluid is less costly than banked blood. We purchased the Hypothermosol M solution for these experiments at a cost of $60 per L. The cost of packed red blood cells can be more than $250 for one 250-cc U.

The model presented in this study did have several drawbacks. The exsanguinating hemorrhage was acute and it may be more clinically representative if the exsanguination period were slower and the of hemorrhage from an abdominal vascular source. In this model, subtle neurologic deficits such as memory were also not assessed.

We therefore set out to train the animals to determine learning and memory capabilities following induced hypothermic suspended animation. The main focus of this study was to determine cognitive function in survivors by testing for learning ability and memory. Swine (100–140 lb.) were subjected to uncontrolled lethal hemorrhage before they were rapidly infused with a hypothermic (1°C), hyperkalemic (70

mEq/L), acellular solution. We had made several modifications in this model. The key ones were that in this model the initial source of hemorrhage was in the abdomen. The second was that we used a specially designed catheter that replaced the Foley catheters previously used. This was a double-lumen catheter with a balloon that allowed us to control cephalad and caudal flows with only one catheter. This catheter could also be used to rewarm and reperfuse the animal without having to place a separate catheter in the aorta. Finally, the last modification was that the hypothermic induction solution had several alterations in its composition, specifically a higher potassium content. Cooling proceeded in the same manner until the core temperature dropped below 10°C. Once at this temperature, hypothermia was maintained for 60 min, during which time the vascular injuries were repaired. Hyperkalemia was then reversed by flushing with a hypokalemic solution. Finally, whole blood was infused, and the animal was resuscitated and warmed to normal temperatures as in the previous experiment. All animals were closely monitored for 6 wks.

Cognitive function was tested by monitoring the capacity to learn new skills and by the ability to recall this learned skill during the postoperative period. The task these animals were expected to accomplish was to identify and open a color-coded box to retrieve food. Food in three identical boxes (blue, red, yellow) was placed in various locations. Only the blue box allowed access to food. Time and attempts taken to learn that only the blue box allowed access to the food were recorded to generate a composite score. Postoperatively, the ability to remember this task as well as a 75-point objective neurologic scale was used to determine neurologic function.

In the first part of this experiment, five untrained animals were tested for ability to learn the skill postoperatively, and four pretrained animals were tested for their memory of the skill following the hypothermic arrest for 60 mins. Their performance was compared with that of control animals that did not undergo the procedure ($n = 15$). In the second part of this experiment, the uncontrolled lethal hemorrhage was induced by creating an iliac artery and vein laceration ($n = 15$). Animals were kept in shock (BP < 40 mmHg) for various periods (15, 30, and 60 min), before aortic laceration and induction of suspended animation in order to determine the length of shock that effected cognitive ability.

In the first part of the experiment, the animals that survived (7/9) were neurologically intact, and their capacity to learn new skills was no

different from that of controls. All pretrained animals demonstrated complete memory retention during the postoperative period. In the second part of the experiment, survival rates with 15, 30, and 60 min of shock were 80%, 60%, and 80%, respectively. Learning and memory were not impaired in survivors. This study demonstrates that rapid induction of asanguinous, hyperkalemic, hypothermic metabolic arrest following uncontrolled lethal hemorrhage, to provide additional time to repair complex vascular injuries, is possible, whether the injury is above or below the diaphragm. Survival with normal neurologic function is obtainable even after various periods of shock.

The induction of hypothermic arrest should not be compared with spontaneous hypothermia in trauma patients. It has been shown that spontaneous hypothermia following trauma is associated with worse outcome *(74,75)* and that active rewarming may be beneficial *(76)*. If the perfusion to tissues falls below some threshold, metabolism cannot keep up with the heat loss. Therefore ischemia from hemorrhagic shock can contribute to hypothermia. Rewarming in these situations may decrease the volume of blood and fluid needed, as coagulopathy becomes an important consideration during resuscitation. Thus, the concept of rewarming spontaneously cold patients is different from the induction of deep hypothermic arrest in normothermic patients to help establish surgical control of hemorrhage.

Future Applications and Visions

The limits of induced hypothermia have been tested over decades *(77–79)*. Recently this field has made marked progress principally because of the work done by Safar, Taylor, and their colleagues. We have taken what has already been learned and developed a method in a clinically applicable model to induce hypothermic, hypokalemic arrest that can be later reversed. Surgeons facing the exsanguinating patient with potentially repairable injuries often find themselves needing more time. Induced hypothermic arrest can provide this time. The question is whether hypothermia can be applied in the trauma setting, as well as in the battlefield. Historically, the idea of putting someone in a state of unconsciousness, in which surgery could be performed and the patient awakened after surgery, was thought of as ludicrous. However, the field of anesthesia has made surgery commonplace. The same can be said of open-heart surgery: the heart is stopped and arrested in order to allow surgical repairs. The field of cardiothoracic surgery again is well estab-

lished today. We have to be able to look into the future, where we can create a state of suspended animation quickly and efficiently so that otherwise lethal injuries can be repaired. It is difficult to state when this will take place, but it will certainly occur. It is just a matter of developing the materials and technique to do so safely and efficiently.

We have tested the concept in a simulated combat scenario. We have taken an animal in a field operating room (comprised of a operating room in a tent) and induced hypothermic, hypokalemic suspended animation using a CPB pump. Once in the state of suspended animation, the animal was transported into our operating room at another location to repair the injury and resuscitate the animal. Field application of induced suspended animation is not yet fully ready for use; however, with the availability of air transportation, 30 min can mean transportation of over 50 miles. If methods can be developed to sustain the suspended animation for several hours, then transportation to hospital ships or casualty receiving ships is entirely within the realm of possibility.

SUMMARY

We have demonstrated the advances that can be made when bright minds are allowed to work in the proper environment with ample resources. Our main concern is to better the care given to the injured combatant. We have focused on the study of hemorrhagic shock, resuscitation of hemorrhagic shock, better methods to treat the injured, and exploration of futurie treatment of exsanguination. We hope that the efforts by the many people who have been a part of TRRI-Surg will contribute to improved care of the injured in the future.

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9 Ischemia/Reperfusion Injury

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INTRODUCTION

Survival of traumatic abdominal injuries is essentially a 20th century development. Historically, abdominal wounds were left untreated because of a lack of anesthesia, and repairs were performed only in cases of evisceration. Even after the discovery of ether anesthesia in the mid-19th century, surgery remained controversial because of the documented poor survival. Marian Sims, the American military surgeon, was a proponent of the interventionists, those who advocated laparotomy in an attempt to treat abdominal injuries, but an equally vocal group opposed this strategy. Statistically, most abdominal injuries were fatal in World War I, with the greatest loss among soldiers who developed acute pneumonias. The initial cause of the mortality was thought to be a toxic factor; however, the beneficial effects of whole blood in shock were noted. Of interest is that a woman surgeon, Princess Gedroitz of Russia, observed that prompt treatment greatly reduced mortality during the Russo-Japanese War of 1904–1905 *(1)*. It is now appreciated that laparotomy is necessary in the treatment of abdominal trauma and that damage to the large bowel is far more often associated with significant morbidity and mortality because of the increased incidence of sepsis.

The Vietnam War heralded a major improvement in evacuation of critically wounded soldiers to field hospitals, and the problems of treat-

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ment became more sophisticated. Indeed, in 1967, Col. Hardaway *(1)* stated that

> *A new type of surgical patient has appeared in Vietnam which is of great importance to surgical research. This is the critically wounded patient who is suffering rapid blood loss from vascular or organ injury, who under any other circumstances would have died shortly thereafter. However, he is now delivered to a hospital frequently within 15 minutes of injury.*

Nevertheless, many patients developed pulmonary complications reminiscent of wet lung in World War I, later described as shock lung or Da Nang lung in Vietnam. This became a defining feature of forward instillations and was a complication of many severe nonthoracic traumas.

Shock is thought to induce injury, in part by its effects on local organ blood flow. The maintenance of adequate perfusion is the basis of autoregulation of blood flow in many organ systems including the small intestine. The mesenteric circulation can receive up to 25–30% of the cardiac output, more than any other organ system. Thus, the abrupt changes in mesenteric blood flow that may accompany severe hemorrhage or trauma-induced shock by are particularly devastating to the intestinal mucosa because of its susceptibility to oxidative stress *(2)*. Although hypoxia alone is detrimental, it is generally considered that reperfusion of the affected area is associated with a more profound damage. Indeed, reperfusion of an area previously deprived of blood is common to both hemorrhagic and ischemia/reperfusion (I/R)-induced injury. Finally, it should be noted that I/R includes both low-flow and no-flow conditions; however, with the extensive collateral circulation in the gut, the latter state is more difficult to achieve. Most experimental models involve occlusion of the superior mesenteric artery, resulting in low-flow conditions. To date, models of I/R have been established in a number of species including pigs, cats, rats, and mice.

PATHOLOGY OF MESENTERIC ISCHEMIA/REPERFUSION

Local Changes

Reduction of blood flow to the gut induces mucosal injury that varies by region. Some generalities include the following: (1) the gut can withstand brief periods of ischemia without injury upon reperfusion;

(2) the small intestine is more vulnerable to oxidative stress than the colon, such that for a given period of ischemia, damage in the small intestine is greater than that in the colon *(2)*; (3) after some interval, increasing the duration of ischemia does not induce further damage in any region; and (4) at a certain point, longer periods of ischemia are lethal, with death occurring in the reperfusion period. These time points vary by species and by the extent of the reduction in blood flow. A major difference is that, in contrast to the small intestine, the colon is reported to show injury in response to ischemia, but there is no further damage in response to reperfusion *(2)*. In the rat, occlusion of the superior mesenteric artery for less than 10 min does not cause mucosal injury after reperfusion. Intervals between 20 and 60 min, however, induce progressively worse mucosal reperfusion injury; longer durations are relatively refractory to intervention, and the damage extends beyond the mucosa and may even be transmural. Venous congestion is an exacerbating factor in the development of damage. Thus, there appears to be a window of reperfusion injury, as described by Park et al. *(3)*, and the clinical correlate can be exploited as an investigative target for the development of therapeutic interventions.

Splanchnic ischemia can be defined as a sequence of events that is initiated upon generation of reactive oxygen species. From this point forward, a number of pathways are activated during the postischemic period, the importance of which have been demonstrated by the attenuation, interruption, or prevention of intestinal mucosal injury and/or inflammation after their inhibition or inactivation. The following description is derived from studies in our laboratory using a moderate period of intestinal ischemia (30 min) in rats (**Fig. 1**); however, the literature reveals that I/R produced similar changes in a number of species and that the major differences are the duration of ischemia required to induce injury and the time-course of the development of reperfusion injury.

Within the first hour of reperfusion in the rat model, the macroscopic appearance of the affected area is dusky and edematous, with numerous petechiae. The lumen often contains a greater amount of fluid than is present in the respective controls, the mucosa can be striated, and there is often a sloughing of surface cells and exudation of mucus that worsens with longer periods of ischemia. Microscopically, changes are confined to the mucosa and submucosa. There is denuding of surface epithelial cells, which may be more or less continuous along the villi, exposed lamina propria, submucosal edema, neutrophilic infiltration,

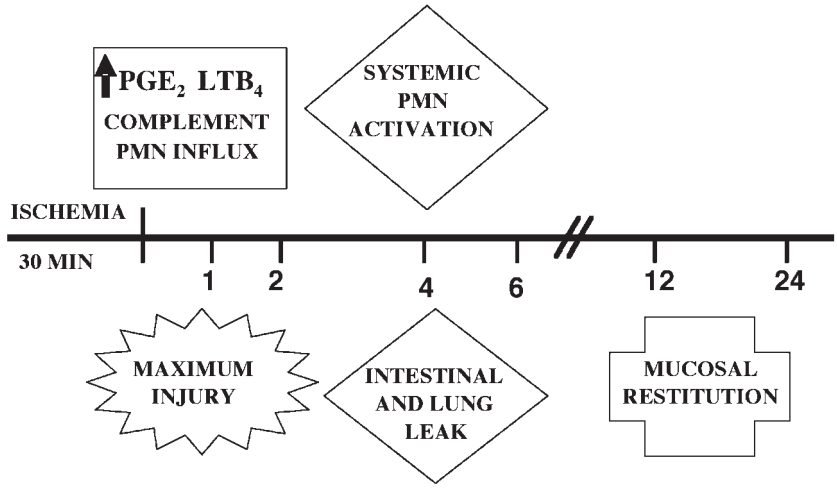

Fig. 1. A number of events are initiated during the reperfusion following sublethal mesentric ischemia. Following the development of maximal injury and inflammation there is an increase in endotheial permeability associated with the activation of circulating leukocytes. Mucosal restitution is complete by 24 h after sublethal ischemia. LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; PMN, polymorphonuclear leukocytes.

and hemorrhage, all of which range in severity from mild to moderate. In rodents, there is a prominent increase in Paneth cell size and granulation *(4)*. Mucosal injury and inflammation are maximal by 2 h into the reperfusion period. By 4 h into the reperfusion period, there is evidence of reannealing of the surface epithelial cells, but villi remained stunted. Six hours into the postischemic period, submucosal edema is resolving, and the mucosa is intact. Villi approximate normal height at 12 h, and by 24 h the mucosal restitution is complete; however, neutrophils are still present in the tissue, and there is an increased number of goblet cells *(5)*.

The alterations in histology are associated with functional changes as well. In the first 2 h of the postischemic period, there is an increase in the generation of oxygen free radicals, lipid peroxidation, blood flow to the affected area, mucosal permeability *(6)*, generation of the inflammatory mediators leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), and complement activation *(4,7,8)*. In addition, there is a decreased production of nitric oxide (NO) by vascular endothelial cells and by enteric nerves in the affected area *(8,9)*. Each of these factors plays a contributing role in the deleterious effects of reperfusion, as the inhibi-

tion of one factor alone attenuates or prevents microscopic injury and inflammation and illustrates the complex nature of reperfusion injury.

After 30 min of intestinal ischemia in the rat, the period between 2 and 4 h in the postischemic period is characterized by mucosal restitution; however, a second series of events is initiated through exposure of circulating neutrophils to the local area of intestinal inflammation. Thus, inactive neutrophils become activated after passing through the microvasculature of the affected region *(10)*. This caused emergence of the concept that the postischemic gut serves as a priming bed for the development of the systemic inflammation that precedes a more generalized multisystem dysfunction.

Systemic Changes

The primary feature of shock is regional and systemic hypoperfusion; implementation of resuscitation produces changes in intestinal morphology that bear some resemblance to those induced by mesenteric I/R. This is because reduction of the circulating blood volume in severe hemorrhage renders the gut ischemic. The gut is prone to disproportionate splanchnic vasoconstriction, and the resulting injury is proposed to be a major factor in multisystem dysfunction associated with abdominal trauma. Even today, multiple organ failure remains the most common cause of morbidity/mortality after trauma and shock.

In the repertoire of responses to I/R available in each organ system, acute inflammation of the affected area is the common denominator. Intestinal I/R induces local microvascular changes, mucosal injury, and generation of inflammatory mediators that are linked to the development of a systemic inflammatory response (SIRS). Thus, the postischemic gut is considered to be the motor in the development of acute lung injury *(11)*, the earliest manifestation of acute respiratory distress syndrome (ARDS). First identified by Asbaugh et al. *(12)* in 1967 in a group of patients suffering respiratory failure unrelated to direct thoracic injury, ARDS is a spectrum of responses ranging from mild congestion to severe pulmonary edema. Of interest is that despite differences in the initiating factors, the pulmonary pathology is surprisingly similar, with plasma fluid and protein exudation as major features.

ARDS can be induced by a single massive ischemic insult to the gut in which mortality approaches 100%, leaving little room for intervention. A dominant model in the literature employs two sublethal insults *(13)*, based on the assumption that the initial exposure to a sublethal

cellular insult primes destructive pathways of cellular response to subsequent injury. Thus, the postischemic intestine serves as a priming bed for circulating neutrophils that act as the fuel to produce injury in distant organs such as the lung. The contribution of the postischemic gut to acute lung injury, the earliest manifestation of ARDS, initially focused on endotoxemia/bacteriemia as the triggering event *(13)*; however, the development of ARDS in the absence of infection led to consideration of the neutrophil as the critical mediator *(12–15)*. This is supported by studies showing that pulmonary vascular responses to intestinal I/R were attenuated by agents directed against leukocyte or endothelial adhesion molecules. In rodent models of I/R, 30 min of ischemia induced an activation of circulating neutrophils at 4 h post reperfusion and coincided with increased macromolecular leak in both the gut and lung *(16,17)*. In addition, enhanced neutrophil production of superoxide is evident in patients after multiple trauma *(15)*. Conner et al. *(17)* introduced the concept of the neutrophil priming state, which emphasized the importance of both the number and the activity of circulating neutrophils in the development of postischemic microvascular leak in the lung. In these studies, neutrophil activation reached a maximum between 6 and 12 h post trauma, but after 24 h, neutrophils were unresponsive to further stimulation.

In a two-hit model of multiple organ failure, the first hit is the initial severe, survivable injury or physiologic insult, followed by a second hit, which provokes multiple organ failure by taking advantage of a patient's overall primed condition. More importantly, it has been proposed that the second hit can be a subclinical physiologic insult causing little or no injury by itself. In an animal model developed to investigate the effects of sequential episodes of sublethal mesenteric I/R, 10 min of mesenteric ischemia alone produced no intestinal injury *(18)*. If the noninjurious 10 min of mesenteric ischemia, however, was given within 6 h of a previous episode of mesenteric ischemia, then significant acute lung injury was produced. Conversely, if the second episode of mesenteric ischemia, severe enough to cause acute gut and lung injury, was delivered within 24 h into the reperfusion period, then acute lung injury was not produced *(5)*.

These data demonstrate that acute lung injury measured by macromolecular capillary leak could be provoked by innocuous episodes of mesenteric ischemia. Furthermore, these findings support the concept that a subclinical insult (such as a hypotensive event in a critically ill

patient, sustained within a certain window of time) can provoke multiple organ dysfunction. Moreover, not only was there a vulnerable period in the development of multiple dysfunction after a patient sustained a noninjurious episode of mesenteric ischemia, but there was also a period of relative protection against a second injurious mesenteric ischemic insult. This protection occurred despite significant neutrophil infiltration in the small intestine and continued activation of systemic neutrophils. These data also suggest that the neutrophil plays a supportive rather than a central role in the development of ARDS after mesenteric I/R. The important clinical implication is that in a two-hit model of intestinal I/R injury, the timing, not the severity, of a second episode of mesenteric ischemia was a more important factor in producing distant organ injury. This two-hit model may be highly relevant to clinical or combat situations that involve exposure of the intestine to multiple incidences of transient hypoperfusion that occur in patients who may be considered, clinically, to be adequately resuscitated.

MECHANISMS

The mechanisms underlying I/R injury and inflammation are an intricate network of local and systemic effects (**Fig. 2**). The benefit of a better understanding of these mechanisms lies in the ability to design improved or novel therapeutic interventions that interfere with, or interrupt, critical processes. There are common mechanisms in the response of every organ system to I/R; the activity of reactive oxygen species, nitric oxide, and neutrophils has dominated the field over the years. With improved technology, however, more recent studies have shown the importance of other factors including cytokines and adhesion molecules. It should be emphasized, however, that the multifaceted nature of reperfusion injury precludes an ability to establish a rigid hierarchy among these major parameters.

Xanthine Oxidase

The precise mechanism(s) of I/R-induced injury has not been fully elucidated; however, the xanthine-oxidase generation of reactive oxygen intermediates was an early contender for a major role in organ damage during reperfusion *(18–20)*. Antioxidants, free radical scavengers, or inhibitors of xanthine oxidase, such as allopurinol, attenuate I/R-induced injury. During hypoxia or ischemia, the generation of

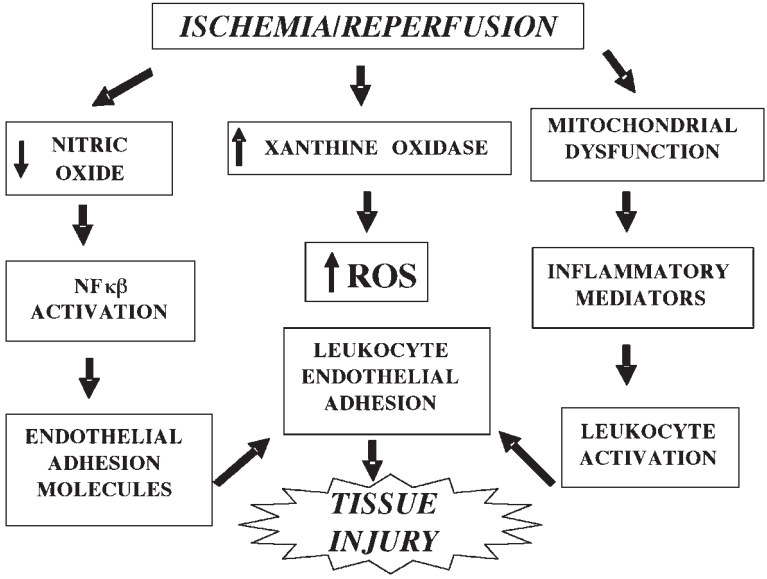

Fig. 2. There are several mechanisms that contribute to the development of local and systemic inflammation and injury in response to msenteric ischemia/reperfusion. Generation of xanthine oxidase results in elevated production of reactive oxygen species (ROS). ROS contribute to reduced levels of nitric oxide and lead to increased activation of NFκB, upregulation of endothelial adhesion molecules, and infiltration of leukocytes. Impaired mitochondrial function and enhanced formation of inflammatory mediators activate circulating neutrophils that contribute to leukocyte infiltration and later to the development systemic inflammation. NFκB, nuclear factor-κB.

adenosine triphosphate (ATP) is reduced in the face of a continued demand for ATP. This leads to the production of adenosine as a result of the degradation of energy-rich phosphates from ATP to adenosine monophosphate (AMP) (**Fig. 3**). Upon leaving the cell, adenosine is broken down further to hypoxanthine and inosine. Accumulation of these purine metabolites provides a ready substrate for the enzyme. The time it takes to deplete all the ATP varies by species as well as by the severity of the hypoxia but is reported to be within 20 min after complete mesenteric ischemia in rats *(21)*.

The source of the cytotoxic radicals is xanthine oxidase. Under normal conditions, xanthine dehydrogenase metabolizes hypoxanthine in two steps to uric acid with oxidized nicotinamide adenine dinucleotide

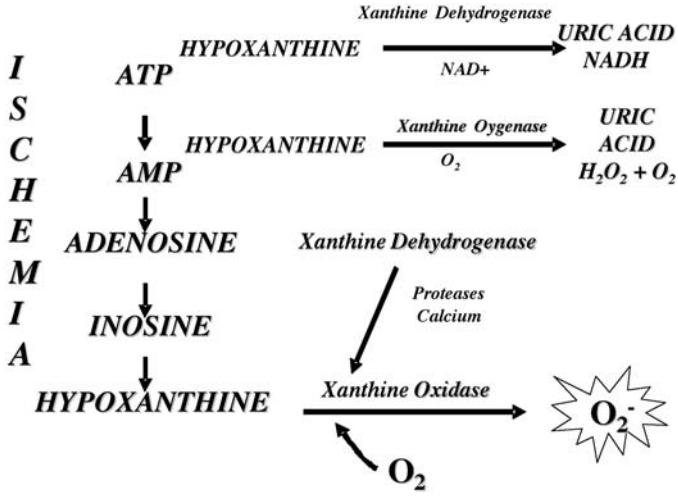

Fig. 3. A major source of the reactive oxygen metabolites in hypoxia or ischemia is xanthine oxidase. Hypoxanthine is metabolized normally to uric acid by xanthine dehydrogenase. During hypoxia or ischemia, xanthine dehydrogenase is converted to xanthine oxidase. In addition, the generation of ATP is reduced but the demand for ATP continues resulting in the production of adenosine as a result of the degradation of energy rich phosphates from ATP to AMP. Adenosine is broken down further to hypoxanthine and inosine. Accumulation of these purine metabolites provides a substrate for xanthine oxidase generation of reactive oxygen metabolites. AMP, adenosine monophosphate; ATP, adenosine triphosphate; NAD+, oxidized nicotinamide adenine dinucleotide.

(NAD^+) acting as the electron acceptor. Under conditions of ischemia or anoxia, xanthine dehydrogenase is converted to xanthine oxidase because the decreased energy state results in a influx of calcium and limited activation of proteases *(22)*. Upon reperfusion of the affected organ, xanthine oxidase reacts with molecular oxygen and the accumulated hypoxanthine to produce the reactive oxygen metabolites superoxide (O_2^-), hydrogen peroxide, and hydroxyl radical. Compared with other organs, the intestine and the liver have abundant levels of xanthine oxidase, and the contribution of this enzyme to free radical production in other organs may not be as significant. Xanthine oxidase is localized to the capillary vascular endothelial cells *(23)*, and endothelial surface-associated xanthine oxidase is proposed to metabolize circulating xanthine and hypoxanthine to form reactive oxygen metabolites *(24)*.

There are regional differences in the susceptibility of the gastrointestinal tract to I/R damage that can be linked partially to levels of xanthine oxidase. Thus, areas with high enzyme content, such as the gastric antrum and small intestine, exhibit a marked susceptibility to I/R injury relative to the colon, which has significantly less epithelial xanthine oxidase *(2,25,26)*. It is important to note that levels of circulating xanthine oxidase increase during clinical oxidative stress *(27)*, and thus the ability of the enzyme to bind to vascular endothelial cells increases, rendering it a ubiquitous factor in I/R-induced injury *(23)*.

Considerable data link xanthine oxidase with neutrophil adhesion. Neutrophilic infiltration is a prominent feature of I/R and early on was considered a critical factor in the development of reperfusion-induced mucosal injury *(28)*. In support of this hypothesis, rendering an animal neutropenic or administration of monoclonal antibodies to the leukocyte adhesion molecule CD18 *(29)* attenuated ischemic-induced injury and microvascular dysfunction. Superoxide radicals increase plasma levels of a neutrophil chemoattractant, a response attenuated by superoxide dismutase *(29)*.

Leukocytes

The recruitment of leukocytes into an area of inflammation begins with the activation of neutrophils and/or endothelial cells, followed by their binding to the endothelium and transmigration into tissues. This may be considered the rate-limiting step in the initiation of acute inflammation, and I/R-induced damage is reduced significantly if neutrophil infiltration is prevented. This is supported by studies showing diminution of effects in animals that are deficient in neutrophils *(29)* or in one of the critical adhesion factors *(30,31)* and in animals treated with antibodies to the factors *(29,32,33)*. The neutrophil plays a promiscuous, but vital, role in both the local and systemic effects of intestinal I/R including generation of oxygen free radicals, synthesis of proteases, elaboration of chemoattractants, and expression of adhesion molecules.

Once it has arrived at the site of I/R, the activated neutrophil elaborates a number of factors that provide an effective, but indiscriminant, arsenal for the destruction of undesirable or damaged cells. An oxidative or respiratory burst results in the release of the oxygen radical O_2^-, by the membrane-bound enzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase *(34)*, as well as myeloperoxidase, which converts hydrogen peroxide into the more deleterious

hypochlorous acid (HOCl). A second weapon used by the neutrophil is the cytotoxic proteases, which fall into five major categories, each designed to destroy a key structure or effector protein, resulting in the death of the targeted cell. These categories are cationic proteases, neutral proteases, the metaloproteases (collagenase and gelatinase), elastase, and heparinase. Release of the proteases from activated granulocytes is a key component in the subsequent tissue damage and dysfunction.

The recruitment of neutrophils to an area of inflammation also appears to be responsible for some unintended damage. Vascular occlusion by neutrophils has been implicated in a no-flow phenomenon following reperfusion. There are also data supporting the stimulation of platelet aggregation and elaboration of a locally active vasoconstricting agent by activated neutrophils. These effects may worsen the ischemic insult and delay or impede mucosal healing. At one time, it was considered that activation of neutrophils, sequestrated in small vessels in areas remote from the site of inflammation, was critical to the development of multisystem dysfunction following mesenteric I/R *(35)*.

Neutrophil interactions with endothelial cells are governed by three groups of adhesion glycoproteins that act in a programmed and sequential manner to control entry of neutrophils into areas of inflammation. Inflammation can be amplified by infiltrating cells through the production of chemotaxins like LTB_4 *(36)*. Emigration occurs primarily in postcapillary venules and proceeds from slowing or margination along the vessel wall, loose tethering, and rolling, followed by firm adhesion accompanied by a change in configuration from spherical to flat to facilitate entry into the tissue *(37)*.

Adhesion Molecules

Adhesion molecules are integral to the localization of the neutrophil on the damaged tissue and are found on neutrophils (L-selectins) and on vascular endothelial cells (E- and P-selectins). When expressed, these adhesion molecules facilitate rolling of the neutrophil along the vascular endothelium (**Fig. 4**). This interaction is influenced by activation of the integrins, which are transmembrane-spanning adhesion molecules expressed on neutrophils. Each functional unit consists of one α- and one β-chain. The β_2-integrins, leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18) and membrane attack complex 1 (MAC-1) (CD11b/CD18), are constitutively expressed on all leukocytes. Follow-

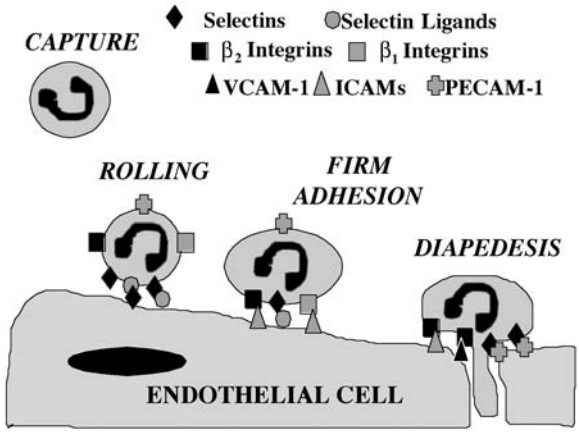

Fig. 4. Adhesion molecules play a critical role in the recruitment and transmigration of leukocytes into damaged tissue. Selectins preferentially expressed by leukocytes (L-selectin), platelets (P-selectin), and the vascular endothelium (E- and P-selectin) mediate the first events of the adhesion cascade, slowing and rolling of the circulating leukocytes. Interaction of integrins with intracellular (ICAM) and vascular (VCAM) adhesion molecules is required for firm adhesion to the endothelial cell surface. Transmigration of adherence cells is facilitated by a presence of a chemotactic gradient and platelet cell adhesion molecule (PECAM).

ing activation or priming, these integrins interact with intercellular adhesion molecule (ICAM) on the vascular endothelium to facilitate firm adhesion of leukocytes. ICAM-1 has been demonstrated to be critical to the neutrophil's ability to stop rolling, stick to the vascular endothelium, and migrate across the basement membrane to address the I/R damage within the tissues *(37)*. LFA-1 and MAC-1 are integral to this process because blockade of these complexes attenuates the local inflammatory injury in response to I/R *(38)*.

The importance of the β_2-integrins is demonstrated by the lack of neutrophil infiltration in response to infection in patients who have leukocyte adhesion deficiency type I (LAD I) *(39)*. Blockade of ICAM blunts the local inflammation, despite the presence of neutrophils *(37)*, and there is a lack of neutrophil-mediated inflammation in response to I/R in ICAM-1 knockout mice *(40)*. Knowledge of these interactions progressed from bench to bedside, with phase I trials showing increased survival of donor kidneys if the donor was treated with antibody to ICAM *(41)*. This is presumably because of the inability of host neu-

trophils to stick to the vascular endothelium and migrate into the graft tissue. More detailed information on adhesion molecules and the microcirculation can be found in a Chapter 3.

Nitric Oxide

In the past decade there has been a plethora of articles dealing with NO. It is a diverse molecule produced by endothelial cells. NO is a gas at normal temperature and pressure and is quickly metabolized; therefore its effects are predominantly local, within a distance of 200–600 microns *(42)*. A complication in assigning a role for NO is its schizophrenic nature as a good/bad molecule. Much of this anomaly can be linked to the amount of NO, which in turn is related to the activity of the individual isoforms of nitric oxide synthase (NOS). The constitutive form of NOS, cNOS, is calcium-dependent and produces low levels of NO that have a functional importance in the regulation of vascular tone, in the maintenance of epithelial permeability, and as a major inhibitory neurotransmitter in the enteric nervous system. NOS has two major tissue-specific isoforms, endothelial NOS (eNOS, NOS-III) and neuronal NOS (nNOS, NOS-I). The inducible form of NOS, iNOS (NOS-II), is calcium-independent, generates large quantities of NO, and is transcriptionally upregulated in response to a number of stimuli including infection and inflammation. In the large quantities produced by iNOS, NO is cytotoxic, interfering with iron hemostasis.

Regardless of the isoform, L-arginine is the precursor molecule for NO production by NOS *(43)*. L-arginine is combined with molecular oxygen in the presence of the flavin cofactors, NADPH, heme-producing NO, and L-citrulline in a 1:1 ratio. L-citrulline can be resynthesized to L-arginine via two cytosolic enzymes in the citric acid cycle *(43)*. Cellular signaling with NO involves activation of soluble guanylate cyclase and synthesis of cyclic guanosine monophosphate (cGMP).

Both forms of NOS contribute to the local and systemic effects of mesenteric I/R. Generation of oxygen free radicals in response to mesenteric I/R precipitates the rapid reaction of NO with superoxide anion to form peroxynitrite ($\mathbf{ONOO^-}$), which breaks down into additional products including the hydroxyl radical ($\mathbf{OH^-}$) *(44)*.

$$\mathbf{O^-_2} + \mathbf{NO} \rightarrow \mathbf{ONOO^-} \leftrightarrow \mathrm{ONOOH} \rightarrow \mathbf{OH^-} + \mathrm{NO_2} \rightarrow \mathrm{NO_3^-} + \mathrm{H^+}$$

Peroxynitrite is an extremely reactive oxidizing substance capable of inducing significant tissue injury, whereas the hydroxyl radical is

responsible for the fragmentation of DNA and other proteins in the initiation of lipid peroxidation, which is considered to be one of the earliest events in reperfusion *(4)*. The enhanced production of reactive oxygen species leads to an increase in NO metabolism and a decrease in cNOS activity *(45,46)*. Depletion of endothelial NO leads to increased endothelial permeability. In addition to its effect on the microvasculature, NO exhibits a number of important antiinflammatory functions. NO opposes the conversion of xanthine dehydrogenase to xanthine oxidase *(47)*, inhibits leukocyte rolling induced by P-selectin *(48,49)* or tumor necrosis factor-β (TNF-β) *(50)*, and suppresses lipid peroxidation *(51)*. These data demonstrate that NO is a major inhibitor of leukocyte-endothelial cell interactions and, therefore, that reduced NO production in mesenteric I/R removes the endogenous brake on this interaction and contributes to the influx of neutrophils into the affected area. These conclusions are supported by the observation that supplementation of the precursor, L-arginine, maintains NOS activity and NO production in the intestine, indicating that a limited availability of substrate is a critical factor under conditions of oxidative stress *(46,49,52)*.

NO also contributes to the development of the SIRS that may following intestinal I/R. Inflammation in the intestine serves to prime circulating neutrophils exposed to the affected area. This effect is evident at 4 h into the reperfusion period and is not temporally correlated with the development of maximal local intestinal injury *(5,45,46)*. The involvement of NO is supported by the observations that maintenance of substrate availability prevents the development of capillary leak *(46)* and, conversely, that leak occurs much earlier in the postischemic period following inhibition of NOS *(45)*. We proposed that the development of maximal microvascular permeability at 4 h represents an interval characterized by a reduction in cNOS activity prior to upregulation of iNOS activity later in the reperfusion period *(53)*. The inducible isoform appears to be involved in the systemic adaptive response by a mechanism that is independent of circulating leukocytes *(52)*. Vascular changes are normalized between 6 and 8 h, coinciding with the generation of NO by iNOS. This is supported by reports demonstrating that NO downregulates iNOS gene transcription in the hepatocyte by inhibiting NF-κβ activity *(54)*. These data suggest that the reduction in cNOS activity during the first 4 h of the reperfusion period precipitates, in part, the upregulation of iNOS activity.

For many years it was thought that translocation of bacterial products through a defective mucosal barrier played a significant role in the multiple organ failure associated with mesenteric ischemia. In this model, the production of large quantities of NO by iNOS benefits the host because of the antibacteriocidal effect of NO. More recent studies, however, proposed that following gut ischemia, SIRS is related to a more generalized inflammation that involves the upregulation of endogenous gene expression of iNOS and other cytokines independent of endotoxin *(55)*. Thus, during the course of reperfusion, iNOS is likely to exert a greater role later in the reperfusion period. This is supported by data showing that inhibition of iNOS prior to intestinal ischemia failed to alter the severity or development of mucosal injury, neutrophil infiltration, or microvascular leak *(18)*. In contrast, inflammation and capillary leak were exacerbated and mucosal injury was attenuated following inhibition of cNOS *(45)*. It should be noted that inhibition of iNOS severely impaired mucosal healing, an observation consistent with the delay in healing of colonic inflammation reported in iNOS deficient mice *(56)*.

Soluble Inflammatory Mediators

The local and systemic manifestations of intestinal I/R are beholden to a number of mediators that initiate, amplify, and/or maintain tissue injury and inflammation. Many are activated as a consequence of oxygen free radical production, whereas others are products of the infiltrating inflammatory cells. In this manner, reactive oxygen intermediates generated during tissue injury activate resident inflammatory cells that then produce chemotactic substances to recruit additional inflammatory cells to the injured area. Of the many chemoattractants, C5a, LTB_4, platelet-activating factor (PAF), and (IL-8) interleukin-8 are those that mediate the localization of neutrophils to the site of injury. C5 is a pivotal protein in the complement cascade; when it is cleaved, a potent anaphylotoxin, C5a, is released along with C5b that initiates formation of the membrane attack-induced injury *(57)*. Reactive oxygen species also activate phospholipase A_2 (PLA_2) *(58)*, the enzyme responsible for cleaving arachidonic acid, the precursor for the synthesis of both prostaglandins and leukotrienes as well as PAF. PGE_2, a product of cyclo-oxygenase (COX-I and-II), is also increased during I/R and serves as a cytoprotective mediator *(4)*, since inhibition of PGE_2 generation often exacerbates I/R-induced mucosal injury. LTB_4, a potent promoter of polymorphonuclear leukocyte (PMN) adherence, activation,

and extravasation, is a product of the 5-lipoxygenase (5-LO) pathway and is increased in the small intestine in response to mesenteric I/R *(4,7,8,36)*. PAF synthesis is elevated during I/R and acts to aggregate platelets and inflammatory cells to produce a local vascular obstruction and vasoconstriction *(59)*. Both LTB_4 and PAF increase granulocyte adherence by increasing the expression of CD11/CD18 *(60)*. IL-8 is a potent chemoattractant and proinflammatory cytokine *(61)*.

It is important to note that in addition to interventions that alter generation of reactive oxygen species or endothelial cell/neutrophil interactions, inhibition of complement or PLA_2 activation *(7,8,16,57,62–64)*, IL-8 expression *(65)*, or PAF or LTB_4 generation *(66,67)* significantly limits I/R-induced injury and inflammation (**Fig. 5**). The corollary to this observation is that without the development of intestinal inflammation to serve as a priming bed for circulating neutrophils, there is also an inhibition of the development of SIRS, which precedes microvascular leak and multiple organ failure.

CLINICAL PRACTICES

I/R is a common scenario in the clinical practice of medicine and occurs in virtually all organ systems. Its hallmarks are relatively consistent across patient populations and organ systems. I/R-induced injury is particularly relevant to combat medicine and the care of the injured patient. The austere environment may lack common diagnostic and therapeutic modalities; therefore, to be successful, administering care in such an environment requires an understanding of the pathophysiology behind I/R injuries. This understanding is paramount to the management of I/R and the prevention of morbid sequelae.

Of all the organ systems, there are a few specific I/R syndromes that are particularly relevant to the combat trauma patient. Mesenteric I/R, myocardial ischemia. and limb compartment syndrome are the most pertinent. The guiding principles of pathology, diagnosis, and treatment of I/R injury are the same regardless of the affected organ. Successful outcomes depend on accurate and timely diagnosis, early intervention, and minimizing the damaging effects of reperfusion while maximizing its benefits.

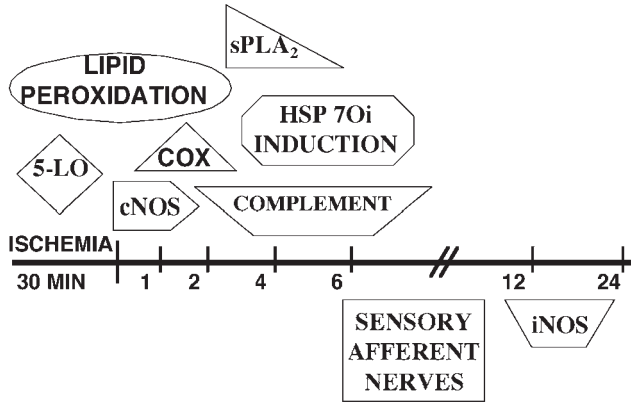

Fig. 5. Intervention at a number of sites has beneficial effects on the inflammation and injury induced by mesenteric ischemia/reperfusion. Pretreatment with agents that interrupt or limit membrane damage (inhibitors of lipid peroxidation); development of a chemotactic gradient (inhibitors of LTB_4); activation of complement; or cyclooxygenase (COX) or phospholipase A2 (PLA2), or 5-lipoxygenase (5-LO) generation of inflammatory eicosanoids have therapeutic potential. Some of these strategies are also effective (e.g., inhibition of complement activation) when administered post ischemia. Alternately, induction of heat shock proteins (HSP), maintenance of constitutive nitric oxide (cNOS) activity (L-arginine) provide advanced protection against ischemia/reperfusion-induced injury. Finally, inhibitors of inducible NOS (iNOS) or agents that desensitize mucosal sensory afferents do no alter the development of injury and inflammation but significantly impair mucosal restitution and repair.

Mesenteric Ischemia

Mesenteric ischemia in the trauma patient typically results from a low-flow state, as occurs during hypotensive shock and abdominal compartment syndrome. The diagnosis of shock-induced mesenteric ischemia requires a high index of suspicion, and treatment requires an understanding of the pathophysiologic processes that ensue. During a global low blood flow state, as seen in shock, the splanchnic bed constricts in an effort to conserve blood flow to the vital organs. Vasoconstrictive agents, often used to provide a temporary increase in systemic perfusion pressure, exacerbate the diminished splanchnic blood flow and may precipitate bowel ischemia. This ischemia can progress along a clinical course from mucosal injury to transmural damage, perforation, peritonitis, and frank sepsis.

Conventional treatment of this clinical spectrum focuses on limiting the ischemic insult and preventing its complications. Aggressive fluid administration and blood product resuscitation are the cornerstones of treatment. Discontinuing pressors at the earliest possible time is also paramount. Bowel rest, antibiotic treatment, and prevention of concomitant infections can limit the progression of the systemic effects. Angiography can be diagnostic, demonstrating vasospastic mesenteric vessels. It can also be used therapeutically with direct instillation of vasodilatory agents that can augment return of blood flow and limit ischemic time.

Abdominal compartment syndrome can occur following many injuries, including trauma, electrical injury, massive resuscitation, and hemorrhage. The diagnosis is suspected on exam by observing a tensely distended abdomen. Regardless of the underlying cause, the common denominator is an increase in the intra-abominal compartment pressure. This impedes venous return through the compartment by collapsing the thin-walled veins. In addition to obstructing venous flow, arterial inflow is affected as well as pulmonary function. These effects combine to produce a local low-flow state to the abdominal organs, resulting in impaired renal blood flow, ischemia to the bowel, hepatic insufficiency, and poor venous return affecting cardiac output *(68)*.

Once the diagnosis of abdominal compartment syndrome is suspected, an indirect measurement of the compartment pressure can confirm its presence. A Foley catheter attached to a transducer can be used for this purpose. A transmitted pressure of greater than 30 cm H_2O is associated with imminent cardiopulmonary collapse. This makes abdominal compartment syndrome a surgical emergency necessitating an emergent decompression celiotomy *(69)*. The operation requires open packing or a temporary closure with synthetic material. Regardless of the underlying cause, once mesenteric ischemia occurs, there is a common cascade of events leading to local and systemic damage. The details of these events have been covered previously in this chapter. The clinical scenario of mesenteric ischemia requires early detection, a high index of suspicion, and an understanding of the causes and the consequences to ensure maximal care of these patients *(70)*. Interventions to minimize the clinical morbidity of this disease are targeted at each step it its development. Understanding the phases and the interventions is paramount in the successful treatment of mesenteric I/R injury.

Myocardial Ischemia

Myocardial ischemia occurs when the demand for oxygen by the myocyte is greater than the supply, causing a conversion to anaerobic metabolism to the detriment of function. Ultimately, if the demand for oxygen exceeds the need for oxygen to perform vital cell functions, cell death occurs. Many physiologic changes may contribute to myocardial ischemia in the injured patient. It can be relevant to trauma as a primary insult during increased myocardial metabolic demand in a person with predisposing coronary artery disease, or a secondary insult from decreased oxygen delivery associated with hypotension or diminished oxygen-carrying capacity. Trauma is associated with increased circulating cathecholamines, changes in circulating intravascular volume, hypoxia, labile blood pressure, platelet activation, and hypercoagulability. These changes contribute to the oxygen supply/demand mismatch, and the diagnosis must be made expeditiously to limit irreversible ischemic damage. Myocardial I/R has been an intense area of research in the last decade because of its high incidence, morbidity, and mortality.

In trauma patients, a history of myocardial ischemia or infarction, congestive heart failure, or previous cardiac interventions, such as percutaneous cardiac angioplasty (PTCA) or coronary artery bypass graft (CABG) surgery, should be ascertained. Additional risk factors, such as cocaine use, should be part of a history as they may cause myocardial ischemia in patients without known risk factors. Complaints such as chest pain may be absent in the trauma patient because of analgesic use, depressed mental status, altered pain perception, or a painful distracting injury. On exam, symptoms of dyspnea may indicate heart failure, as can bibasilar lung rales or jugular venous hypertension. Vital signs can be variable, with tachycardia, bradycardia, hypertension, or hypotension. The physical exam of a patient undergoing cardiac ischemia may uncover new murmurs from mitral regurgitation or pathologic heart sounds such as an S3 or, less commonly, an S4.

Diagnostic modalities should include an electrocardiogram *(71)*. This may, in the face of myocardial ischemia, reveal peaked T waves, ST-segment elevation, a Q wave, or inverted T waves. Quantitative assessment of cardiac enzymes such as the MB fraction of creatine kinase (CK-MB) or troponin T or I can indicate cardiac myocyte injury. If the history is convincing but the diagnostic workup is negative, a car-

diac stress test or catheterization may be necessary to evaluate thoroughly for at-risk myocardium. Adjuncts such as chest radiograph, echocardiogram, or radionucleotide studies may aid in identifying and delineating the extent of cardiac ischemia. Many of these advanced diagnostic modalities, however, are unavailable in a combat medical environment; therefore, successful management entails a high index of suspicion and early evacuation to a higher level of care.

Interventions for those patients with cardiac ischemia are aimed at re-establishing adequate blood flow and limiting the myocardial damage following reperfusion. The current mainstay of treatment involves nitrates, which are effective in producing coronary vasodilation. Nitrates are also beneficial in congestive heart failure and the associated pulmonary edema through preload reduction. When nitrates, are used, continuous cardiac monitoring is warranted because the preload reduction can lead to significant hypotension, thereby exacerbating the cardiac ischemia.

Adrenergic blockade, through the use of β-blockers, has been shown to improve survival and reduce both the duration of ischemic symptoms and the incidence of ventricular arrhythmias. The goal of β-blockade is to limit the double product (an indication of cardiac work calculated by the product of the systolic blood pressure and the heart rate), decrease metabolic demand, and thus reduce the ischemic burden. β-blockers also decrease sympathetic tone, platelet aggregation, and the incidence of arrhythmias. These medications should be strongly considered unless contraindicated. The contraindications include bradycardia, hypotension, severe left ventricular dysfunction, conductive heart block, and severe chronic obstructive pulmonary disease.

Other medications that are useful in the face of cardiac ischemia include angiotensin convertase inhibitors, aspirin, heparin, analgesics, and thrombolytics *(72,73)*. Angiotensin convertase inhibitors have been shown to improve short-term survival when used early in the course of myocardial ischemia. Aspirin inhibits platelet aggregation, prevents thrombus propagation, and limits ongoing ischemia, thereby decreasing the mortality of a cardiac event. Heparin may also prevent further thrombus formation and has been shown to inhibit complement activation. Analgesics such as morphine serve to reduce anxiety and pain, which limits myocardial oxygen demand. Thrombolytics directed at clot dissolution are indicated for most patients with acute myocardial

infarction; however, they are contraindicated in most patients who have concurrent head injury, or major trauma or who have had major surgery, making them less attractive in the trauma patient population.

In addition to medications, acute operative intervention may be employed to limit cardiac ischemia. Urgent cardiac catheterization and percutaneous angioplasty can be used to restore blood flow quickly to the ischemic myocardium *(74)*. Emergent coronary artery bypass grafting is also an option in the setting of acute ischemia. These options, however, require advanced technology and supportive care, making their use limited in the care of combat casualties until evacuation to an appropriate facility is accomplished.

Supportive care during the myocardial reperfusion period can prevent reversibly injured cells from progressing to cell death. Current research revolves around limiting the reperfusion injury and subsequent inflammation, thereby minimizing collateral cell injury. Reperfusion is necessary for ultimate cell survival; however, it can induce inflammatory cascades that can damage surrounding cells and expand the injured area. The cellular and humoral mechanisms of the reperfusion injury have been specifically addressed previously in this chapter. The understanding of these mechanisms has led to novel therapies that interrupt the inflammatory cascade and limit its damaging effects. These include free radical scavengers, inhibition of neutrophil function and adhesion, monoclonal antibodies directed at interrupting the complement cascade, and promotion of cellular defense mechanisms. These exciting areas promise a new understanding and control of the I/R pathway, which will hopefully translate into diminished damage and increased survival.

Compartment Syndrome

Compartment syndrome is a clinical entity defined by the elevation of pressure within a closed anatomic space that compromises the viability of the tissues within that space. Increased fluid within a fascial compartment, whether blood or edema, leads to a significant rise in pressure. This is classically described as occurring in the fascial compartment in the upper and lower extremities. The volar or dorsal compartments in the forearm, the intrinsic muscles of the hand and buttocks, and the fascial compartments of the lower leg are particularly vulnerable areas. This syndrome can develop after fractures, crush

injuries, thermal or electrical burns, or surgical restoration of blood flow, or with the placement of a constricting cast or dressing.

The diagnosis must be made expeditiously to limit irreversible damage. Early symptoms include pain that is out of proportion to the clinical exam and varying degrees of parasthesias. Significant swelling, palpable tenseness, pain on passive stretching, limb pallor, and muscle weakness all aid in the clinical diagnosis, but the presence or absence of these findings cannot rule the diagnosis in or out *(76)*. The loss of palpable or Doppler pulses is typically a late finding. The diagnosis of compartment syndrome should be made prior to exam findings of neurologic deficits. The most commonly affected muscular compartment is the anterior compartment of the lower leg. Symptoms referable to the deep peroneal nerve, which traverses the anterior compartment, include weak toe dorsiflexion and diminished sensation between the first and second toes and the dorsum of the foot.

To confirm a clinical suspicion of limb compartment syndrome, a needle catheter manometry can be performed. This can be done with a needle and a pressure transducer or a number of commercially available kits. The concept is to measure the pressure within the compartment. Normally, soft tissue compartment pressures remain less than 10 mmHg. Above 30 mmHg, tissue ischemia ensues. The pathologic cascade in the limbs is identical to that in the abdomen. Initially venous congestion occurs, followed by the disruption of arterial inflow. This leads to neural tissue ischemia, death, and myonecrosis. This damage further increases local edema and compounds the problem.

The goals for treating an I/R injury of the limb are the same as in other I/R injuries: limit the ischemic time, restore perfusion, and manage the complications of reperfusion. Simple maneuvers such as removing a compressive dressing or cast or limb elevation may decrease the compartment pressure; however, decompression of the affected compartments through fasciotomy incisions is the mainstay of therapy. This surgical therapy involves long skin incisions and deep fascial incisions to allow full decompression. In the lower leg, a fibulectomy can be used to relieve the compartment pressures in all four compartments; however, this is rarely necessary in the face of an adequate standard four-compartment fasciotomy. Necrotic tissue must be removed at the time of fasciotomy to minimize further inflammation and prevent infection. Fasciotomy wounds are then left open, covered with a moist dressing;

they can undergo delayed primary closure or split-thickness skin grafting to close the defects once the clinical scenario has run its course. After revascularization procedures for acute or chronic arterial insufficiency, a prophylactic fasciotomy may be used in anticipation of tissue edema owing to the reperfusion *(75)*. This may be closed primarily or secondarily after the limb has become accustomed to the return of adequate blood flow.

The systemic complications of limb compartment syndrome are relative to the quantity and duration of ischemic tissue. The complications include myoglobinuria, systemic hyperkalemia, and the systemic release of inflammatory mediators. Myoglobin and potassium, both rich in skeletal muscle cells, are released locally following cell death. Upon reperfusion, these are both washed into the systemic circulation. The myoglobin levels in the serum peak 3 h after reperfusion. Myoglobinuria can block renal tubules and precipitate renal failure. Close urine monitoring is necessary, and forced diuresis with judicious isotonic saline administration and an osmotic diuretic may be necessary to ovoid the renal toxic effects of the myoglobin. Both sodium bicarbonate and acetozolamide may decrease the precipitation of myoglobin in the renal tubule through alkalinization and diuresis.

The systemic hyperkalemia that follows the reperfusion can have deleterious affects on cardiac function. The effects of hyperkalemia are exacerbated in the face of possible renal tubule injury from myoglobinemia. Treatment of hyperkalemia involves protecting the myocardium and promoting normokalemia. The myocardial membranes can be stabilized against the effects of hyperkalemia through the administration of calcium. Acute and chronic means of controlling the hyperkalemia include concomitant glucose and insulin administration, albuterol nebulizer therapy, promotion of diuresis, and oral or rectal potassium binders.

The systemic inflammatory cascade that is activated following reperfusion of an ischemic limb is similar to that found following all other I/R syndromes. Care must be taken to support the patient's hemodynamic and immune systems in response to this transient upregulation. Limb compartment syndrome, a sequela of many battlefield injuries, again highlights the concepts of early diagnosis and treatment. An understanding of the pathologic processes is paramount to accurate diagnosis and timely treatment.

QUESTIONS AND FUTURE DIRECTIONS

The mechanism(s) that orchestrates I/R-induced damage and inflammation is under active investigation. It is known that interruption of local inflammatory events abrogates the translation of these events to SIRS; however, there is still a lack of consensus on the mechanism. Most of the information on mesenteric I/R has been derived from studies in rodents, and a second area of interest is establishing the time-course of mesenteric I/R injury in larger species. Preliminary data suggest that significant intestinal I/R in non-human primates requires occlusion of coelic and superior mesenteric artery for up to 2.5 h to achieve a similar degree of reperfusion injury observed in rats after 30 min (Shea-Donohue, unpublished data).

A better understanding of the mechanism of I/R-induced local and systemic events will lead to the development of improved therapies that can be divided, arbitrarily, into those that are relevant to clinical or combat trauma and those that are relevant to the prevention of potential or expected trauma. Experimental models have already demonstrated the efficacy of a number of interventions suitable to the treatment of mesenteric I/R in the clinical or combat trauma setting (**Fig. 5**). Such therapies must be amenable to administration prior to, or early in, the resuscitative (reperfusion) phase Several exciting new avenues of research are (1) the role of neutrophil-derived proteases in the spatially mediated disruption of epithelial cell apical junctions *(77)* and in lung injury after mesenteric I/R *(78)*; (2) immunologic control of epithelial cell detachment *(79)*; (3) the contribution of apoptosis to I/R-induced injury and inflammation *(80)*; (4) the role of innate immunity complement-mediated effects in mesenteric I/R *(16)*; and (5) the protection against I/R injury afforded by the peroxisome proliferator-activated receptor-γ (PPAR-γ) *(81)*.

One of the most effective inhibitors of I/R-induced damage is heat stress-induced upregulation of heat shock protein (HSP)-72 *(36)*. Unfortunately, administration of heat stress prior to the event is not feasible in either the clinical or combat setting; however, recent studies show that supplementation with L-glutamine upregulates HSP-72 *(82)*. In addition, pretreatment with L-arginine *(46)*, the NO substrate, L-glycine *(83)*, or total enteral nutrition *(84)*, alleviated or prevented I/R-induced damage. These data indicate that therapies based on nutritional supplementation may enhance resistance to I/R damage in the general population, and

this knowledge may be exploited for future use in development of adjunctive or preventive therapy in combat and clinical trauma.

In conclusion, clinically evident I/R syndromes are common in all fields of medicine. Combat casualty care, however, necessitates a complete understanding of the precipitating events, the at-risk organ systems, and the pathophysiology of the injuries. In addition, providers must be facile in the timely diagnosis of these life- and limb-threatening conditions. A prompt and accurate diagnosis will lead to early intervention and improved outcomes. These knowledge and skill sets are paramount to providing quality care to the combat casualty.

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10 Acute Respiratory Distress Syndrome

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INTRODUCTION

In 1967, Ashbaugh and colleagues *(1)* described a cohort of 12 patients who had acute onset of tachypnea, hypoxemia, panlobular infiltrates on chest radiograph, and decreased lung compliance. It was noted that this syndrome was similar to the infant respiratory distress syndrome, and in 1971 these same investigators coined the term adult respiratory distress syndrome (ARDS) *(2)*. Since that time, it has been noted that this same condition also occurs in children, and consequently it was renamed acute respiratory distress syndrome. In 1988, Murray and colleagues *(3)* defined ARDS via the lung injury score (LIS) based on the chest radiographic findings, the degree of hypoxemia (PaO_2/FiO_2 ratio), the level of positive end-expiratory pressure (PEEP), and the lung compliance (**Table 1**). The American-European Consensus Committee (A-ECC) was formed in 1994 to develop a universal definition of ARDS and acute lung injury (ALI). The definition, outlined in **Table 2**, included the acute nature of the disease process, oxygenation abnormalities, radiographic findings, and the exclusion of left atrial hypertension when measured, but did not include PEEP, as in the LIS *(4)*. This definition recognizes ARDS as the most severe manifestation of ALI. Although highly useful in stratifying and identifying patients for clinical studies, the definition is currently being considered for revision.

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Table 1
Murray Lung Injury Score[a]

Parameter	*Score*
Chest radiograph	
No consolidation	0
1 quadrant	1
2 quadrants	2
3 quadrants	3
4 quadrants	4
Hypoxemia (PaO_2/FiO_2)	
≥300	0
225–299	1
175–224	2
100–174	3
<100	4
PEEP (cm H_2O)	
≤5	0
6–8	1
9–11	2
12–14	3
≥15	4
Compliance (mL/cm H_2O)	
≥80	0
60–79	1
40–59	2
30–39	3
≤29	4

[a]The final value is obtained by dividing the sum of the individual component scores by 4. Scores: 0 = no injury; 0.1–2.5 = mild to moderate injury; >2.5 = severe injury (acute respiratory distress syndrome).
PEEP, positive end-expiratory pressure.

The exact incidence of ARDS has been relatively difficult to establish. A 1972 population study in New York by the National Heart and Lung Institute reported the incidence of ARDS in adults to be 150,000 cases/yr *(5)*. Other investigators have reported an incidence ranging from 1.5 to 75 patients/100,000 inhabitants/yr *(6–10)*. In children, the exact incidence has also been difficult to establish *(11)*. A prospective epidemiologic study is currently under way that makes use of the A-ECC definition of ARDS and will hopefully provide more definitive data on incidence.

Table 2
American-European Consensus Committee Definition of ARDS and ALI

	Timing	*Oxygenation*	*Chest radiograph*	*Pulmonary artery wedge pressure*
ALI	Acute onset	PaO_2/FiO_2 ratio ≤ 300mmHg (regardless of PEEP level)	Bilateral infiltrates seen on frontal chest radiograph	≤18 mmHg when measured or no clinical evidence of left atrial hypertension.
ARDS	Acute onset	PaO_2/FiO_2 ratio ≤200 mmHg (regardless of PEEP level)	Bilateral infiltrates seen on frontal chest radiograph	≤18 mmHg when measured or no clinical evidence of left atrial hypertension.

ALI, acute lung injury; ARDS, acute respiratory distress syndrome; PEEP, positive end-expiratory pressure.

CLINICAL COURSE AND HISTOPATHOLOGY

The initial phase of ARDS (acute/exudative phase) manifestes clinically by progressively refractory hypoxemia. The chest radiograph demonstrates bilateral patchy pulmonary infiltrates (similar to those seen during cardiogenic pulmonary edema, **Fig. 1**), whereas computed tomography of the chest reveals that alveolar filling, consolidation, and atelectasis occur predominantly in the dependent lung zones. Histologic examination reveals diffuse alveolar damage, hyaline membranes, protein-rich alveolar fluid, parenchymal infiltration by neutrophils and macrophages, and disruption of the alveolar epithelium.

Although some patients will recover after this acute stage, other patients will enter a second phase known as the fibroproliferative stage. The time of onset of this stage is highly variable (3–10 days after initial onset of ARDS) but is typically characterized by the onset of lung architectural changes and persistent hypoxemia. Histologically, prominent interstitial infiltration by fibroblasts, myofibroblasts, and inflammatory cells (mostly of the mononuclear lineage) and increased collagen deposi-

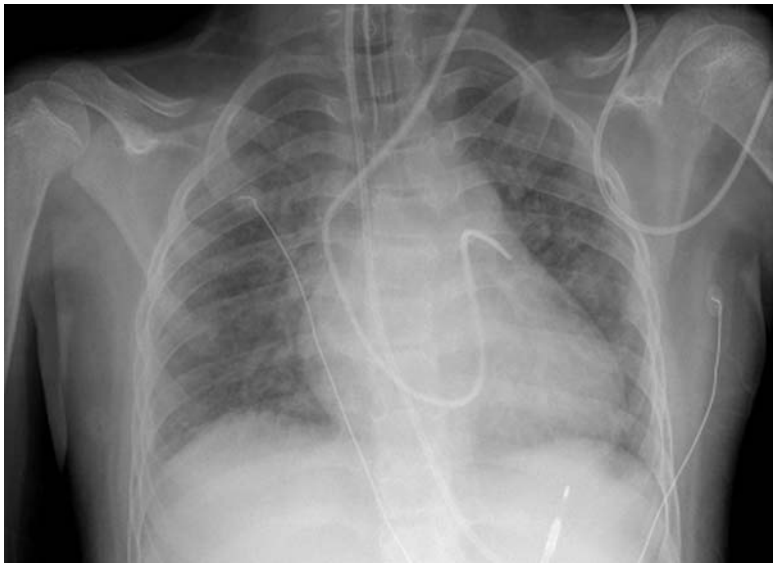

Fig. 1. Chest radiograph of a patient with ARDS illustrating bilateral diffuse alveolar infiltrates.

tion are seen. Other clinical features of the fibroproliferative stage include increased alveolar dead space and further decreases in lung compliance.

The final phase is the recovery phase, characterized by gradual resolution of the hypoxemia and improved compliance as the lung architecture is restored toward normal. The timing and duration of this stage are also highly variable. Some patients will have progressive lung fibrosis, irreversible loss of functional alveoli, and cyst formation, leading to death secondary to hypoxemia.

CAUSES AND OUTCOMES

The causative factors leading to ARDS can be broadly categorized as those that directly injure the lung and those systemic processes that cause indirect/secondary injury to the lung. Direct causes of ARDS include pneumonia, aspiration of gastric contents, lung contusion, fat emboli, near drowning, inhalation injury, and reperfusion injury. Systemic processes causing indirect/secondary injury to the lung include sepsis, multiple transfusions, pancreatitis, and polytrauma. Overall, it appears that the most common cause of ARDS is sepsis *(12,13)*.

Like the incidence, the exact mortality of ARDS has been relatively difficult to determine, with reported mortality in adults broadly ranging from 45 to 92% *(6,8,10)*. Factors that predict mortality in ARDS include chronic liver disease, multiple organ dysfunction, sepsis, and advanced age. Perhaps somewhat surprisingly, initial indices of oxygenation and ventilation (including the PaO_2/FiO_2 ratio and the LIS) do not seem to predict mortality. Failure to improve lung function during the first week of ARDS, however, is a highly negative prognostic factor *(14)*. Most deaths in patients with ARDS have been associated with multiple organ dysfunction or sepsis, rather than hypoxemic respiratory failure *per se*. As will be discussed below in the therapy sections, however, it appears that lung-specific protective strategies can reduce overall mortality secondary to ARDS.

PATHOPHYSIOLOGIC MECHANISMS IN ARDS

Cytokines

One of the difficulties in elucidating the pathophysiology at play in ARDS is the multiple etiologies that have been associated with its onset. In adults, common etiologies include aspiration of gastric contents, sepsis, and major trauma *(12,13,15)*; the leading etiologies of pediatric ARDS include viral pneumonia (especially respiratory syncytial virus), bacteremia, and near-drowning *(16–18)*. Interestingly, both direct and indirect causes of ARDS are characterized by a similar cascade of pathophysiologic events. Among the most consistent findings in both human and animal studies of ARDS is the presence of increased cytokines measured either locally [from bronchoalveolar lavage (BAL) samples] or systemically (in serum samples) *(19)*. Because of their presence and multiple effects, cytokine biology has been extensively investigated in ARDS.

Cytokines are a series of soluble proteins synthesized by numerous cells including virtually every cell type in the lung: alveolar epithelium, pulmonary vascular endothelium, alveolar macrophages, lymphocytes, and interstitial cells. This heterogeneous group of peptides and glycoproteins mediates a variety of biologic functions including intercellular communication, chemotaxis, leukocyte adhesion, production of oxygen- and nitrogen-based radicals, and cell signaling. Cytokines mediate their effects by binding to receptors on the surfaces of their target cells. The receptor-ligand interaction initiates a signaling cascade that can

result in either inhibitory or stimulatory responses by the target cell *(20)*. At low concentrations cytokines act locally by both autocrine and paracrine mechanisms; however, if cytokine concentrations increase substantially, as during ARDS, they can behave as classic hormones with an endocrine effect on distant organs and tissues. Finally, at the highest plasma cytokine concentrations, the inflammatory response to these chemical signals may directly injure host tissues *(21,22)*. The most extensively studied of these molecules in ARDS are tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8).

TNF-α and IL-1 are classified as early response cytokines and are produced in response to a variety of stimuli. The innate immune system, evolutionarily designed to protect the host from a number of pathogens, mediates this early response. Microorganisms express a series of highly conserved molecular patterns that distinguish them from the host. Examples include viral double-stranded RNA, unmethylated CpG dinucleotides common to bacterial but not vertebrate DNA, mannan binding proteins of yeast, glycolipids of mycobacteria, lipoproteins of bacteria and parasites, lipoteichoic acids of Gram-positive bacteria, and lipopolysaccharide (LPS) of Gram-negative bacteria *(23–28)*. The immune active cells of the host possess specific pattern recognition receptors to detect these pathogen-associated molecules and are critical in mediating expression of the cytokines that mediate acute lung inflammation.

LPS remains an important initiator of TNF-α production. The mechanism by which this occurs has been described in a recent series of seminal investigations. The mammalian Toll-like receptors (TLRs) are key signaling receptors of innate host defense that evolved from the *Drosophila Toll* gene *(23–27,29)*. Although initially identified as a mediator of dorsoventral polarization during embryogenesis, the cytoplasmic domain of the Toll receptor was found to be structurally homologous to that of the mammalian IL-1 receptor *(30)*. This supported the concept that both Toll and mammalian TLRs may share similar signal-transduction pathways that ultimately involve cytokine production. Subsequent work has confirmed the role of TLR4 as the receptor initiating LPS signal transduction on macrophages and monocytes. LPS recognition and triggering of early cytokine expression, however, is more complex than interaction with TLR4 alone. LPS binds to the serum protein lipopolysaccharide binding protein (LBP), which

transfers LPS to CD14 that is anchored to the cell membrane by glycosylphosphoinositol. As CD14 lacks a cytoplasmic domain for signal transduction, the LPS/CD14 complex uses TLR4 as a co-receptor *(29)*. In addition, a third molecule, MD-2, is constitutively associated with TLR4 and confers enhanced LPS responsiveness to TLR4 *(31)*. Thus, LPS recognition by the host is accomplished by a complex of at least three components, CD14, TLR4, and MD-2.

TNF-α is active as a trimer and mediates its effects by binding to one of two distinct receptors (55- and 75-kDa forms) that exist on most cell types studied thus far. Administration of recombinant TNF-α in vivo results in fever, hypotension, and impaired endothelial barrier function, resulting in pulmonary edema, whereas anti-TNF-α-neutralizing antibodies prevent shock when endotoxin or Gram-negative bacteria are administered to animals *(32–36)*. A role for TNF-α in ARDS was supported by findings of increased levels of TNF-α in BAL fluids of patients with ARDS *(37,38)*. The principal biologic effects of TNF-α are highlighted just below; it appears to play a proximal role in the cytokine cascade that is observed in ARDS, characterized by a predictable order of expression of subsequent cytokines *(39)*.

Peak TNF-α levels are followed by the appearance of IL-1, which exists as two species encoded by separate genes that share little homology. IL-1β is synthesized as a proform, which is proteolytically cleaved by the IL-1β converting enzyme (ICE; or caspase-1) to its active form that appears to be responsible for the biologic effects in the circulation and lung secretions *(40, 41)*. Both TNF-α and IL-1β independently, or synergistically, are capable of regulating expression of two subsequent cytokines, IL-6 *(42)* and IL-8 *(43)*. Although the role of IL-6 in ARDS remains incompletely understood, IL-8 has been shown to recruit and activate neutrophils during ALI *(44)*.

IL-8 is a member of a large family of chemoattractant cytokines, or chemokines (reviewed in ref. *45*). A new classification for chemokines has recently been reported that separates these molecules into four classes, the CXC, CC, C, and CX_3C chemokine families, which function as potent chemotactic factors for a variety of leukocytes including neutrophils, eosinophils, basophils, monocytes, mast cells, dendritic cells, natural killer (NK) cells, and T- and B-lymphocytes *(46)*. As just mentioned, CXC chemokines such as IL-8 (CXCL8), have been found to facilitate neutrophil infiltration into the lung in response to bacterial

challenge in experimental models. For example, substantial increases of CXC chemokines have been reported in animal models of *Escherichia coli* pneumonia in rabbits *(47)* and *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, and *Aspergillus fumigatus* pneumonia in mice *(48–54)*.

It remains unclear, however, as to whether neutralization of chemokines is of benefit, as they appear to be important for bacterial clearance. In trying to establish a role for CXC chemokines in the eradication of microorganisms in the lung, Greenberger and colleagues *(48)* observed that depletion of macrophage inflammatory protein-2 (MIP-2; or CXCL2/3) during murine *K. pneumoniae* pneumonia resulted not only in reduced neutrophil recruitment to the lung, but also in reduced bacterial clearance and increased bacteremia. Since CXC chemokines employ the CXC chemokine receptor CXCR2, similar studies have targeted the CXCR2 receptor to determine the importance of CXC chemokine ligand/CXCR2 biology during pneumonia. Standiford and colleagues blocked CXCR2 and found marked reductions in lung neutrophils in response to *P. aeruginosa (52), N. asteroides (54)*, and *A. fumigatus (51)* pneumonias that were accompanied with reduced clearance of the microorganisms and increased mortality.

There is substantial clinical evidence that IL-8 is present in the lungs of patients with ARDS, and increased BAL fluid levels of IL-8 were correlated with the number of neutrophils, the severity of injury, and mortality *(55)*. Thus, cytokines are clearly present in the setting of ARDS, and further refinement of our understanding of their biologic roles in this disease state is likely to lead to more rational therapeutic targeting.

Biologic Effects of Cytokines

The cytokines described above have a variety of biologic activities that in unison can serve as crucial modifiers of the immune response.

Autocrine Activity

Cytokines, especially the early response cytokines TNF-α and IL-1β, are key amplifiers of inflammation both as proximal mediators and through synergistic activities *(39,56,57)*. Additional cytokines remain to be fully identified that probably also provide autocrine stimulation. For example, in an immune complex-mediated model of lung inflammation, blocking of the CC chemokine MIP-1α decreased the

total BAL fluid TNF-α content, suggesting that MIP-1α might function as an autocrine activator of TNF-α expression *(58)*. This finding may be relevant, as early increases in MIP-1α were associated with poor outcome in neonatal respiratory distress syndrome *(59)*. Therefore, targeting proximal cytokine mediators may dampen this autoamplification observed in the acute inflammatory response.

Regulation of Adhesion Molecule Expression

One of the more important biologic roles for cytokines in ARDS is their mediation of the endothelial cell-leukocyte adhesion cascade. A hallmark of the autopsy findings of lungs from patients succumbing to ARDS is massive neutrophil infiltration. The mechanism by which leukocytes, in particular neutrophils, are recruited from the blood to the lung has been extensively studied over the past decade (reviewed in ref. *60*). In the initial phase of leukocyte adhesion called *rolling*, selectin family members of adhesion molecules (e.g., E-selectin) are expressed on the endothelial cell surface and interact with sialylated oligosaccharides constitutively expressed on neutrophils *(61–63)*. The second phase of adhesion results from the firmer interaction between cytokine-activated β_2-integrins (e.g., CD11a,–b, and –c/CD18) that are expressed on neutrophils and their counter-receptors (e.g. intercellular adhesion molecule-1 (ICAM-1)) expressed on the endothelium *(64)*.

Once they have adhered to the pulmonary vascular endothelium, neutrophils migrate to the alveolar space via chemotactic gradients generated by chemokine release (discussed in the next section) in a manner that is partially dependent on platelet-endothelial cell adhesion *(65)*. The subsequent release by leukocytes of oxygen- and nitrogen-based radical species, proteases, and arachidonic acid metabolites all contribute to cellular dysfunction, resulting in impaired endothelial barrier function and subsequent development of pulmonary edema. With this understanding of the role of adhesion molecules in ARDS, the possibility of using antiadhesion molecule therapy has been considered. Enthusiasm for an antiadhesion molecule strategy is rightfully tempered by the appreciation that the adhesion cascade is a critical innate immune response affording host protection against invading pathogens. In light of this caveat, inhibiting leukocyte adhesion in the setting of an infectious cause of ARDS could be detrimental to pathogen eradication and consequently to patient survival.

Leukocyte Chemotaxis and Transitional Inflammation

A key characteristic of the acute inflammation associated with the development of ARDS is the recruitment of predominantly neutrophils, followed by mononuclear cells, from the blood to the air spaces of the lung *(60)*. Although these leukocytes promote the eradication of an offending pathogen, the magnitude of infiltrating cells, combined with their release of mediators, leads to further amplification of acute inflammation and tissue injury. In addition, the maintenance of leukocyte recruitment necessitates intercellular communication between leukocytes and other structural cell types including the endothelial and parenchymal cells. This intercellular communication is mediated not only by cytokines and adhesion molecules, but also by the production of chemotactic molecules, including chemokines.

Early investigations identified a series of nonspecific chemotactic molecules such as *N*-formylmethionyl peptides from bacterial cell walls, the anaphylatoxin C5a, leukotriene B_4 (LTB_4), and platelet-activating factor (PAF) that were chemotactic for leukocytes *(66,67)*. Although these molecules are important in leukocyte extravasation, they lack specificity for particular subsets of leukocytes. It became increasingly apparent that the nature of the offending stimulus variably determined the subpopulation of leukocytes elicited during an inflammatory response. Thus, it was hypothesized that a more diverse set of chemotactic factors were likely to exist that possess specific activity for subsets of leukocytes.

Chemokines are a family of low-molecular-weight proteins that share a structural homology and possess a variety of biologic activities, most notably chemotactic activity for leukocytes (reviewed in ref. *68*). As mentioned in the previous section, the chemokines have been classified into four groups on the basis of their structural cysteine motifs: C (e.g., lymphotactin); CC (e.g., MIP-1α); the CXC chemokines (e.g., interleukin-8); and the CX_3C chemokines (e.g., fractalkine). The CXC chemokines (e.g., IL-8, MIP-2/GROα, KC), designated because the first two cysteine residues are interrupted by a nonconserved amino acid, are the principal neutrophil chemoattractants. In contrast, CC chemokines [e.g., MIP-1α, monocyte chemottractant protein-1 (MCP-1)], have their first two cysteine residues adjacent and are the principal mononuclear cell chemoattractants. In studies of immune complex-mediated lung inflammation, MIP-2 appeared to mediate activation and

recruitment of neutrophils into the alveolar space, whereas MIP-1α appeared to have an autocrine effect on TNF-α expression *(69,70)*.

In the context of ARDS, the acute infiltration of neutrophils is followed by migration of mononuclear cells into the lung. Both T-cells and monocytes appear to contribute to persistent lung inflammation and subsequent fibrosis in animal models of chronic lung inflammation induced by silica inhalation, as prior depletion of T-cells ameliorates the lung injury (T. Shanley, unpublished data). Recent clinical data suggest that this late mononuclear cell recruitment, or so-called transitional inflammation, is critical to the outcome of patients with ARDS *(71)*. In this study, mononuclear cell recruitment was correlated with MCP-1 levels, suggesting that this CC chemokine is a key mediator of this process. Additionally, the number of mononuclear cells in the BAL fluid between days 3 and 7 was correlated with impairment of oxygenation in patients with ARDS. Together, these studies demonstrate a role for chemokines in mediating ARDS. Further understanding of the regulation of expression of these chemokines may identify potential therapeutic targets for interrupting the inflammatory process in ARDS.

Regulation of Inflammation by "Antiinflammatory" Cytokines

Following their discovery, cytokines were considered strictly proinflammatory molecules on the basis of their contribution to the pathophysiology of disease states such as sepsis and ARDS. More recently, an accumulating body of data supports the role of a number of cytokines as antiinflammatory molecules. Included among this group of cytokines are IL-10, IL-4, IL-13, TGF-β and in some circumstances IL-6 and a related cytokine, IL-11. Perhaps the most well studied of the antiinflammatory cytokines is IL-10, which is a potent endogenous regulator of acute lung inflammation on the basis of its ability to downregulate cytokine production by macrophages *(72)*. For example, in the setting of ALI in rats, blocking of endogenous IL-10 caused increased inflammation and pulmonary edema in association with increased levels of TNF-α and IL-1β *(73)*. This finding was supported by the finding that in the IL-10 null mutant mouse, administration of a typically sublethal endotoxin dose resulted in 100% mortality *(74)*. A correlative finding in humans was demonstrated by Donnelly and colleagues *(75)*, who showed that patients with lower levels of IL-10 in their BAL fluid had a higher mortality rate from ARDS. Similar findings have been observed using TGF-β, IL-4, and IL-13 as "monocyte deactivating

agents" that are able to decrease proinflammatory cytokine expression from effector cells such as the alveolar macrophage. These data suggest that this family of molecules serves as important counter-regulatory molecules in the setting of acute inflammatory disease states such as ARDS.

It is important to recall that the biologic effects orchestrated by the proinflammatory cytokines are a critical component of the innate immune response directed against host invasion. Accordingly, it is imperative to not assume that all critically ill patients with ARDS will benefit from inhibition of the proinflammatory response, which may lead to untoward effects from immunosuppression. Already, clinical investigators have attempted to inhibit cytokine activity, and although this strategy has proved promising in preclinical studies, their ultimate clinical efficacy in human trials has been disappointing *(76,77)*. It may be that overexpression of antiinflammatory cytokines such as IL-10 may in fact contribute to host immunosuppression, thus impairing pathogen clearance. In this context, enhancing the proinflammatory cytokine response in patients suffering from infections may improve survival. In light of this complexity, it will be necessary for the host to maintain a homeostatic cytokine balance in its attempt to fight off infection, but not at the expense of lung tissue injury. The concept of balancing proinflammatory and antiinflammatory activity in the individual patient is depicted in **Fig. 2**.

Molecular Regulation of Cytokine Gene Expression

Because of the important role cytokines play in the development, promulgation, and eventual resolution of ARDS, their molecular regulation has been a target of active investigation over the past decade. It is hypothesized that a complete understanding of the mechanism(s) of gene expression and relevant signal transduction pathways necessary for their expression will provide investigators with additional therapeutic targets in combating ARDS. Although a full description of the signaling pathways that mediate cytokine gene activation in response to a variety of stimuli is beyond the scope of this chapter, it is important to review the most notable pathways.

NF-κB Signaling

Transcriptional activation factors are proteins that bind to DNA to facilitate the transcription of DNA to messenger RNA. A key transcrip-

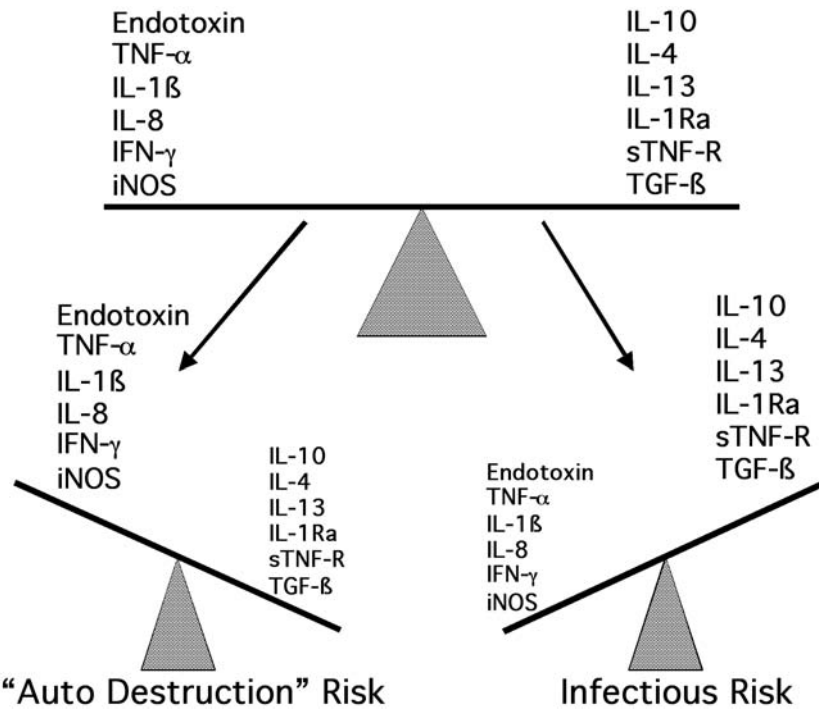

Fig. 2. Schematic depicting the balance of proinflammatory and antiinflammatory activity during ARDS. Dysregulated inflammation results in excessive production of proinflammatory mediators, causing tissue injury, whereas an overexuberant compensatory response results in immunosuppression with potential negative implications for pathogen eradication. IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthese; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; sTNF-R, tumor necrosis factor receptor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

tion factor activated by a number of stimuli associated with the onset of ARDS is nuclear factor κB (NF-κB). NF-κB is a member of the *Rel* family of transcription activation factors which is composed of two subunits (most commonly p50 and p65) that are anchored in the cytoplasm by an inhibitory subunit, IκB *(78)*. A number of stimuli can induce the nuclear translocation of NF-κB via a process that requires phosphorylation, ubiquitination, and subsequent degradation of IκB, initiated by the Iκ kinase complex *(79,80)*. Once in the nucleus, NF-κB binds to a consensus sequence of DNA in the promoter regions of a number of genes

to initiate the transcriptional process. NF-κB, either independently or in association with additional factors, regulates the transcription of multiple proinflammatory molecules including cytokines (e.g., TNFα), chemokines (e.g., IL-8), adhesion molecules (e.g., ICAM-1), and additional molecules such as inducible nitric oxide synthase (iNOS). Therefore, inhibiting this signaling pathway blocks the transcription of a number of products thought to contribute pathophysiologically to ARDS. NF-κB activation has been shown in alveolar macrophages from patients with ARDS, thereby strengthening the hypothesis that this pathway may be a valid therapeutic target *(81,82)*.

Redox State of the Cell in Relation to NF-κB

An obvious consequence of the standard therapy used to treat the hypoxemia associated with ARDS is an increase in the ambient oxygen concentration delivered to the lungs, which can contribute to oxidant stress on the lung tissue *(83)*. In addition to direct toxic effects, oxygen radicals contribute to the amplification of inflammation by inducing the transcription of proinflammatory genes such as TNFα *(84)*, IL-1, and IL-8 *(85,86)*. This oxidant-mediated pathway is at least in part dependent on activation of NF-κB *(87,88)*. As a further example of the redundancy of the regulation of the lung inflammatory response, cytokines can also mediate production of oxygen radical species. Thus, it is anticipated that targeting oxidants would ameliorate tissue injury in ARDS and blunt the inflammatory response.

MAP Kinase Pathways

Another key transcription factor that regulates the expression of a number of inflammatory molecules is activating protein-1 (AP-1). AP-1 is also a sequence-specific transcription factor composed of members of the *fos, jun,* and activating transcription factor (ATF) families (reviewed, in ref. *89*). In examining the gene products under transcriptional regulatory control by AP-1, it is clear that AP-1 and the associated upstream signaling pathways regulate a diverse set of cellular functions including inflammation, cell proliferation, apoptosis, and tissue morphogenesis. Therefore, it is necessary to understand fully the specific roles of this signaling pathway in ARDS in order to target it selectively for therapeutic intervention.

AP-1 transcriptional activity is the downstream result of a complex signal transduction cascade mediated by members of the mitogen-

activated protein kinases (MAPKs) and their upstream kinases (reviewed in refs. *90–92*). Among this set of protein kinases, three major pathways have been identified: the c-Jun NH_2-terminal kinases (JNK) pathway (also called the stress-activated MAPK or SAPK pathway); the extracellular-regulated protein kinase (ERK) pathway; and the p38 mitogen-activated kinase (p38 MAPK). All members of these MAPK families undergo activation via phosphorylation of threonine and tyrosine residues by upstream MAPK kinases (MKKs, or MEKs). These MKKs are in turn activated via phosphorylation by upstream MKK kinase (MKKKs or MEKKs). As is predicted by the complex nature of this cascade, a diverse set of stimuli can broadly influence a variety of cellular functions relevant to lung inflammation and ARDS.

Posttranscriptional Modification of mRNA

Altering the signal transduction pathways reviewed just above will have a predominant effect on the transcription of proinflammatory genes (i.e., the mRNA species). These mRNA species, however, must still undergo translation to the final protein product in order to exert their biologic activity. This process allows for posttranscriptional modification of the mRNA, thus influencing the amount of translation that can occur. An important mechanism that regulates the amount of translated product is mRNA destabilization. Many cytokines, including TNFα, IL-1β, and IL-8, possess copies of an AU-rich element (ARE) in the nucleotide base sequence of the 3'-untranslated region (3'-UTR) of their mRNA *(93,94)*. This ARE sequence provides a target for both stabilizing and destabilizing proteins (e.g., ribonucleases), thus serving to alter the length of time a transcriptional product can be translated to protein *(95)*. Because these sequences are most common in genes characterized as constitutively silent but acutely transcribed, such as cytokines, targeting this mechanism may prove selective for degradation of those molecules contributing to the pathophysiology in ARDS.

As a relevant example to the pathophysiology of ARDS, the posttranscriptional regulation of TNFα synthesis has been increasingly understood *(96)*. Recent investigations have identified a series of proteins that bind to an ARE sequence in the 3'-UTR of the TNFα transcript. These include tristetraproin (TTP) and AUF1, which destabilizes the mRNA, and HuR, which stabilizes TNFα mRNA. Members of the RNA recognition motif family of RNA binding proteins such as TIAR

and a related homolog, TIA-1, also appear capable of binding to the TNFα ARE and repressing the initiation of translation. Inhibition of the p38 and ERK MAP kinase pathways appears to regulate the interaction of these RNA binding proteins to the 3'-UTR, affecting the rate of transcription and possibly translation of the mRNA species. This tightly regulated system of posttranscriptional modification provides an additional therapeutic target in combating the cytokine production associated with pathologic disease states such as ARDS.

CONVENTIONAL THERAPEUTICS

Therapy for ARDS begins by addressing any treatable, underlying cause of ARDS, such as sepsis, pneumonia, or pancreatitis (reviewed in ref. *97*). Beyond this, with a few exceptions, most therapies specifically directed at the pathophysiologic mechanisms described just above remain experimental or have not shown any benefit in clinical trials (reviewed in ref. *8*). Thus, at present, most therapies for ARDS are primarily supportive. Furthermore, consideration of any therapy for ARDS must take into account the fact that most patients with ARDS do not die from respiratory failure, rather, as mentioned in the beginning, most patients with ARDS die as a result of sepsis or multiple organ failure. Nevertheless, it is expected that therapy specifically directed toward ARDS would have the potential to reduce the incidence of all causes of death associated with ARDS.

Conventional Mechanical Ventilation

As is the case for all patients with critical illnesses, maintaining adequate oxygen delivery is an important therapeutic goal in the management of ARDS. In the patient with ARDS this goal is achieved with the usual strategies of fluid management, achievement of adequate hematocrit, achievement of adequate oxygen saturation, and the use of appropriate inotropes and vasopressors to maintain adequate cardiac output. Unique to the patient with ARDS is the need for respiratory support, most typically in the form of mechanical ventilation.

A select group of patients with respiratory failure has the potential to be managed with noninvasive positive-pressure ventilation (NPPV). The most common indication for NPPV appears to be acute hypercapnic respiratory failure, particularly in the setting of chronic obstructive pulmonary disease *(98,99)*. Additionally, it has been suggested that

patients with hypoxemic respiratory failure do not receive the same benefit from NPPV as do patients with hypercapnic respiratory failure *(100)*. Nevertheless, there are reports of successful NPPV in the setting of ARDS *(101,102)*. Thus, NPPV may be considered in some selected patients with ARDS (i.e., patients with chronic obstructive pulmonary disease and patients with milder forms of ARDS), but it should be kept in mind that the vast majority of patients with ARDS require endotracheal intubation and mechanical ventilation.

Increasing mean alveolar pressure (mP_{alv}) is currently considered the key component of mechanical ventilation support for ARDS. Increased mP_{alv} allows for recruitment of alveoli and for reduction of FiO_2 to "nontoxic" levels (<60%). There are several ways to increase mP_{alv}, but PEEP appears to be the most effective with respect to lung mechanics and avoidance of ventilator-induced lung injury (VILI). Typically, PEEP levels are increased incrementally until the FiO_2 can be reduced below 60% while maintaining a systemic oxygen saturation > 90%. Recent literature has advocated the use of pressure-volume curves for the optimal setting of PEEP *(103)*. In this strategy, PEEP is set at or above the lower inflection point of the pressure-volume curve, with the goal of maintaining alveolar patency and eliminating repeated closure and opening of alveoli (the open lung approach). Thus, most patients with ARDS will require PEEP levels in the range of 10–15 cm H_2O. The efficacy of this strategy is being rigorously tested in a current trial conducted by the ARDS Clinical Network, which is comparing higher PEEP/lower FiO_2 with lower PEEP/higher FiO_2 in patients with ARDS (*see* www.ardsnet.org).

The main negative consequences of PEEP include barotrauma, alveolar distension with CO_2 retention, and decreased cardiac output secondary to increased intrathoracic pressure. Fear of barotrauma should not preclude the aggressive use of PEEP *a priori*, given the potential benefits of PEEP. Increases in $PaCO_2$ secondary to alveolar distension can be well tolerated physiologically (permissive hypercapnia); when they are excessive, they can be corrected by lowering PEEP as long as it does not compromise oxygenation. Finally, reductions in cardiac output can be overcome by appropriate augmentation of preload and appropriate use of intravenous inotropes. In this setting, data derived from a pulmonary artery catheter may provide valuable guidance.

Inverse Ratio Ventilation and High-Frequency Ventilation

Apart from PEEP, there are other available modalities for increasing mP_{alv} in the setting of ARDS. Inverse ratio ventilation (IRV) makes use of supraphysiologic inspiratory cycles such that the inspiratory-to-expiratory time ratio is greater than 1:1 *(104)*. This strategy substantially increases mP_{alv}, thereby increasing alveolar recruitment and improving oxygenation. Whether improvements in oxygenation are caused by increased inspiratory time *per se*, or increased intrinsic PEEP, is a matter of debate. Studies using historical controls suggest that IRV can improve the outcome of ARDS *(105–110)*. However, when considering the use of IRV, it should be kept in mind that there are no large, prospective randomized trials comparing IRV with conventional ventilation in ARDS.

High-frequency ventilation (HFV), in the form of either high-frequency jet ventilation (HFJV) or high-frequency oscillatory ventilation (HFOV), is another alternative means of increasing mPalv in the treatment of ARDS *(111)*. HFV has theoretical appeal in ARDS because it makes use of small tidal volumes, while maintaining alveolar recruitment, thus potentially reducing VILI. A large experience in adults with HFJV ventilation suggests that there is no benefit with respect to mortality *(111)*. Experience in pediatric patients, however, suggests that HFOV may provide some benefit *(112,113)*. Overall, there is continued enthusiasm for the use of HFV in the setting of ARDS, but its true benefit remains to be established.

Lung Protective Strategies

The use of mechanical ventilation presents a clinical paradox. On the one hand, it provides life-sustaining support to allow sufficient time for recovery. On the other hand, the use of high concentrations of oxygen and the stretching forces of positive pressure ventilation can be directly injurious to the lung.

Lung toxicity related to high concentrations of oxygen (hyperoxia) has been recognized for many years. Hyperoxia is directly toxic to lung parenchymal cells by the generation of oxygen-related radicals and by impacting the signal transduction pathways of lung parenchymal cells *(114)*. Indeed, in the words of Fridovich *(115)*, "The aerobic lifestyle offers many advantages but is fraught with danger." Although the "safe" level of oxygen during ARDS is not known, a reasonable goal

appears to be achievment of an $FiO_2 < 60\%$. Thus the general recommendation exists of titrating PEEP to a level that allows reduction of FiO_2 below 60%.

The concept of VILI secondary to mechanical forces has generated a great deal of clinical and investigative interest in the last decade. VILI is a manifestation of direct physical damage to lung parenchyma, as well as stretch-induced changes in lung parenchymal signal transduction pathways. This latter concept is embodied in the term *mechanotransduction*, which describes how physical forces change gene expression patterns in the lung, thus leading to potentially important negative consequences such as increased inflammation and alterations of ion channels. Multiple experimental models and clinical studies have documented the physiologic relevance of VILI *(116–125)*.

Recognition of the influence of VILI on the course of ARDS has led to the clinical use of lung protective strategies. These strategies seek to use mechanical ventilation forces in a manner that limits the degree of VILI. As described above, the appropriate use of PEEP to prevent cyclic opening and collapse of alveoli and the use of HFV are two examples of lung protective strategies. A more recent, and seemingly more successful, lung protective strategy has been to reduce tidal volume (6 mL/kg) below the more traditional volumes (12 mL/kg) used in clinical practice. Numerous studies have suggested clinical benefits of this low tidal volume strategy *(126–129)*. A recent trial conducted by investigators in the National Institutes of Health ARDS Clinical Network provides the most definitive evidence that a low tidal volume strategy is beneficial for patients with ARDS *(130)*. This trial enrolled over 800 patients who were randomized to a conventional ventilation group (using 12 mL/kg tidal volume) or an experimental group (using 6 mL/kg tidal volume). The trial was terminated early after a planned interim analysis because patients ventilated with the low tidal volume strategy had a mortality rate of 31.3% compared with 39.8% in the patients treated with conventional tidal volumes ($p = 0.01$). Details regarding the ARDS Network and this specific clinical trial can be found at the website: www.ardsnet.org. Based on these data, the use of low tidal volumes can now be considered as standard in the management of ARDS.

Permissive Hypercapnia

One physiologic consequence of a low-volume ventilation strategy is increased $PaCO_2$ *(128,129)*. Allowing $PaCO_2$ to rise in an attempt to limit VILI is known as *permissive hypercapnia*. An alternative term, coined by Robert S.B. Clark, is *submissive hypercapnia*, which describes extreme elevations of $PaCO_2$ in the most severe forms of ARDS. Although hypercapnia can cause pulmonary hypertension, increased intracranial pressure, and cardiovascular dysfunction, several clinical studies suggest that hypercapnia is well tolerated in patients with ARDS *(128,129)*. One study reported a mean $PaCO_2$ of 66.5 torr (range 38–158) and a mean pH of 7.23 (range 6.79–7.45), with a mortality rate of 26.4% *(129)*. Although most clinicians tolerate a pH of approximately 7.25, below this level there is considerably less consensus. When pH drops below this level, some clinicians will tolerate the lower pH, some will increase ventilation, and others will administer intravenous base agents. Which of the three approaches is most appropriate remains to be determined and for now should probably be dictated by the needs of the individual patient.

Prone Positioning

Chest computed tomography illustrates how heterogenously the lung parenchyma is affected in patients with ARDS. When in a supine position, the dependent areas (dorsal) tend to be fluid-filled and collapsed, whereas the nondependent areas (ventral) tend to be well ventilated, thus causing significant mismatch of ventilation and perfusion. Positioning patients in a prone position allows for improved ventilation/perfusion matching and has been shown in several clinical studies to improve oxygenation in patients with ARDS *(131–141)*. Complications of prone positioning include accidental extubation, pressure sores, catheter dislodgement, and, in some patients, worsening oxygenation. Overall, however, prone positioning is well tolerated and clinically feasible. The feasibility of positioning patients in the prone position is illustrated in **Fig. 3**. which depicts a 180-kg patient in the prone position. Our clinical experience indicates that the response to prone positioning is variable from patient to patient, with some patients achieving greater improvements in oxygenation than others, and other patients requiring relatively frequent changes from the supine position to the prone position. In the absence of a large prospective trial examining prone positioning in patients with ARDS, our most reasonable recom-

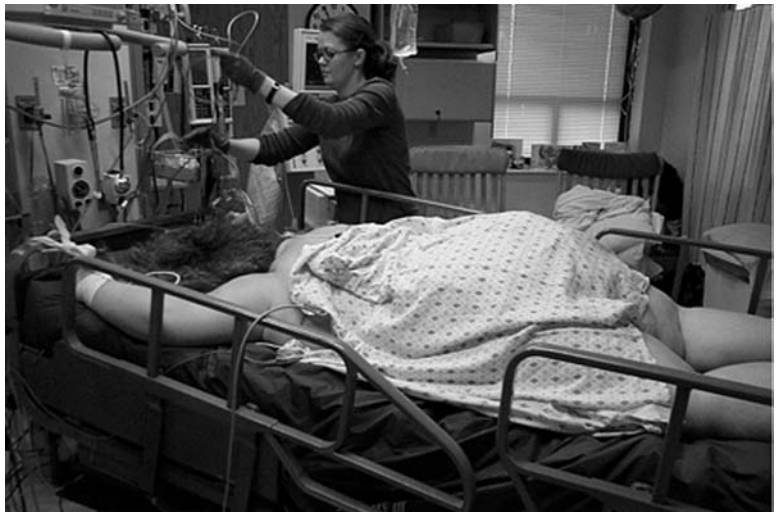

Fig. 3. A 180-kg patient in the prone position, illustrating that this position is feasible in virtually any patient with ARDS.

mendation is that the prone position should be considered in most patients with ARDS.

Fluid Management

Titrating preload and concomitant hemodynamic variables to supraphysiologic values in patients with ARDS cannot be recommended based on the current literature. Certainly, titrating these variables to normal seems to be a prudent recommendation. Furthermore, because pulmonary/alveolar edema is an important component of ARDS, it has been suggested that significantly reducing extravascular lung water, with fluid restriction and administration of diuretics, is beneficial for patients with ARDS. The literature supporting or refuting this approach is somewhat controversial and relies heavily on the use of pulmonary artery catheters, which has also come under strong criticism recently. Recent clinical data, however, suggest that pulmonary edema during ARDS results not only from an imbalance of alveolar-epithelial permeability but also from impaired alveolar fluid clearance by the alveolar epithelium *(142–144)*. In addition, patients with ARDS who have relatively normal alveolar fluid clearance have a better survival rate than patients who have a lower than normal alveolar fluid clearance rate

(143). Based on the available data, the "best" approach seems to be avoidance of hypervolemia and attempts to reduce of extravascular lung water to a level that does not compromise cardiac output (*euvolemic dehydration*, a term coined by Alan B. Fields). Admittedly, judging the latter can be problematic and relatively subjective. Future therapies aimed at restoring normal alveolar fluid clearance hold the promise of more specifically managing the pulmonary edema associated with ARDS.

Corticosteroids

There has been interest in the therapeutic use of high-dose corticosteroids in ARDS and septic shock since the early 1960s. This strategy is based on the sound pathophysiologic concept that a great deal of the organ injury seen in these clinical syndromes is a manifestation of dysregulated inflammation. Because corticosteroids are such potent antiinflammatory agents, it has been postulated that they can substantially attenuate organ injury associated with ARDS. Despite this background, however, a beneficial effect of corticosteroids has not been established in patients with ARDS *(144–146)*. Recently, there has been renewed interest in the use of corticosteroids in patients with "late" or "unresolving" ARDS *(147–149)*. The "new" strategy uses a longer course of therapy, at lower doses, compared with the original trials of corticosteroids in early ARDS. Meduri and colleagues *(149)* performed a randomized trial involving 24 patients with "unresolving" ARDS, defined as patients showing no improvement on day 7 of their disease process. Administration of corticosteroids to these patients had several benefits, including improvement in lung injury score, improvement in PaO_2/FiO_2 ratio, decreased multiple organ dysfunction, and decreased mortality. Although we await the results of the Late Steroid Rescue Trial being conducted by the ARDS Network (*see* www.ardsnet.org), the use of corticosteroids in unresolving ARDS may be considered in clinical practice.

EXPERIMENTAL THERAPIES

Targeting Cytokine Production

Although a number of cells produce cytokines, those of the mononuclear-leukocyte lineage, such as the peripheral blood monocyte (in indirect lung injury, e.g., sepsis) and the alveolar macrophage (in the

direct lung injury setting, e.g., pneumonia), appear to be the principle sources. As noted in the cytokine discussion above, a number of agents have shown promise in "deactivating" these cells as a means of inhibiting cytokine production. Antiinflammatory cytokines such as IL-10 *(72,150)* and transforming growth factor-β (TGF-β) *(151)* display potent monocyte deactivating properties and have been touted as therapeutic candidates in ARDS. IL-10 has demonstrated particular promise as it inhibits a variety of biologic functions that are fundamental to the development and promulgation of ARDS. First, it inhibits the synthesis of a number of cytokines, which would have an additional benefit of impairing the autocrine effect of these molecules *(72)*. Second, it inhibits the endothelial cell-leukocyte adhesion cascade by regulating adhesion molecule expression *(152)*. Third, it inhibits NF-κB nuclear activation via its ability to inhibit the Iκ kinase complex *(153,154)*. Fourth, IL-10 increases the expression of naturally occurring cytokine antagonists such as the IL-1 receptor antagonist (IL-1Ra) protein *(155)*. Finally, IL-10 may destabilize the mRNAs of cytokines, resulting in decreased translation *(156)*. In light of the multiple mechanisms by which IL-10 and other regulatory cytokines can regulate inflammation, exogenous administration of these molecules may be a potentially promising strategy.

Alternatively, increasing the endogenous production of these cytokines via pharmacologic means may accomplish similar functions. Several agents can increase the production of IL-10, including interferon-α, glucocorticoids, prostaglandin E_2, chlorpromazine, and cyclosporin (reviewed in ref *157*); however, the ability of these agents to control the inflammatory response in human ARDS remains to be determined.

Cytokine Neutralization

Because of the proximal role that cytokines play in the inflammatory cascade and their autocrine amplification effects, investigators have traditionally attempted to block their activity directly either by antibody neutralization (e.g., anti-TNF-α antibody) or receptor blockade (e.g., IL-1Ra). Although these strategies proved promising in preclinical trials, their ultimate clinical efficacy in human trials has been disappointing (reviewed in refs. *158* and *159*). The reasons for this are multiple, including inaccurate modeling of the human disease state, poor identification of underlying risk factors, and limitations on statistical power analysis. Other factors weighing against the success of

this strategy include the fact that cytokines are likely to be increased prior to the clinical presentation of a critically ill patient. Also, the cytokine cascade is redundant, making it unlikely that inhibition of a single cytokine will prove beneficial in the context of the limited clinical trials. Most importantly, as stated above, it is unlikely that all patients with ARDS are battling with uncontrolled proinflammation. It is probable that a subset of individuals are existing in a relatively immunocompromised state caused by overexpression of antiinflammatory molecules and cytokines, leaving the host at substantial risk for overwhelming infection as the cause of respiratory failure *(158,160)*.

Inhibition of Signal Transduction Pathways

Cytokine gene expression, and thus cytokine-induced biologic responses, are under the control of specific signaling pathways some of which were reviewed in the Pathophysiologic Mechanisms section above. As mentioned, the NF-κB pathway appears to be involved in the pathophysiology of ARDS. Consequently, investigators have employed a variety of agents to inhibit this pathway at different sites in order to modulate gene expression. Therapeutic targets have included the phosphorylation, ubiquitination, and proteasomal degradation of IκB. That the initial phosphorylation step can be targeted is supported by the observation that expression of a dominant-negative serine 32 (the amino acid targeted for phosphorylation) mutant IκBα blocked activation of NF-κB by TNFα *(161)*. Several novel compounds, including antioxidants, have been designed that also block the phosphorylation of IκBα independent of MAP kinase pathways *(162,163)*. The final step of proteasomal degradation has been successfully targeted for NF-κB inhibition, resulting in diminished cytokine production and cytokine-mediated adhesion molecule expression *(164,165)*.

An alternative strategy for NF-κB inhibition is overexpression of its inhibitory protein, IκBα. Adenovirus-directed expression of IκBα reduced expression of endothelial cell adhesion molecules, as well as the cytokines IL-1, IL-6, and IL-8 *(166)*. More recently, this pathway was shown to be dependent on tyrosine kinase- and phosphatidylcholine-specific phospholipase C in primary human alveolar macrophages, thus providing additional targets for inhibition with agents such as genistein and tyrophostin *(167)*. Also, as this pathway has been demonstrated to be exquisitely sensitive to oxidative stress (reviewed in ref.

168), several antioxidants including pyrrolidinedithiocarbamate (PDTC) *(163)*, *N*-acetylcysteine *(163)*, vitamin D *(169)*, and sesquiterpene lactones *(170)* have demonstrated the ability to block NF-κB inhibition and subsequent gene expression. Corticosteroids (described above in the therapy of ARDS) are also potent inhibitors of the NF-κB pathway.

Finally, an interesting characteristic of this signaling pathway has been its regulation by the heat shock or stress response. This is a conserved, cytoprotective response characterized by rapid expression of stress proteins, including heat shock proteins (reviewed in ref. *171*). Induction of the heat shock response has been shown to impact on the NF-κB signaling pathway. For example, in cultured human respiratory epithelial cells, heat shock decreased NF-κB translocation in a manner associated with increased IκB expression *(172)*. This strategy has been employed to inhibit induction of iNOS *(173)*. Although it is impractical to subject patients to thermal stress, nonpharmacologic inducers of this stress response such as prostaglandin A_1 *(174)* and zinc *(175)* may prove beneficial.

In light of the ubiquitous role of NF-κB in regulating a number of cellular functions, the practical nature of a strategy of NF-κB inhibition needs to be addressed in vivo; however, the current experimental data substantiate the potential role of the NF-κB pathway as a therapeutic target.

Posttranscriptional Strategies

In addition to conferring mRNA instability, the characteristic AU-rich sequence in the 3'-UTR of many cytokine mRNAs results in diminished translational efficiency, as has been shown for TNF-α *(176)*. These findings have prompted investigators to develop strategies for selectively degrading mRNA by decreasing mRNA half-life. As mentioned in the Targeting Cytokine Production section above, IL-10 has been shown to suppress cytokine production posttranscriptionally by promoting mRNA degradation *(151)*. Interestingly, thalidomide has been shown to decrease the half-life of TNF-α mRNA in a selective manner *(177)*. Investigators are actively developing systems that test the ability of pharmacologic agents to target this mechanism selectively *(178,179)*.

Some studies have addressed the role of genetic variation, or polymorphisms, in 3'-UTR of cytokine genes. It was felt that genetic alter-

ations in these sequences might contribute to the variable responses to inflammatory stimuli that are observed among individual patients. Although mutations in the 3'-UTR of the TNF-α gene in mice were shown to contribute to TNF-α-mediated diseases *(180)*, a subsequent study in a population of pediatric patients with autoimmune diseases was unable to confirm a significant frequency of mutations in this area *(181)*. Nevertheless, an increasing number of studies is aimed at determining the effect of polymorphisms in DNA sequences, which regulate cytokine gene expression *(182)*. For example, polymorphisms in the IL-10 promoter region as well as in the 5'-upstream sequence of TNF-α may regulate an individual's immune response to meningococcal disease *(183)* and heart transplantation *(184)*. It is anticipated that in the future genetic markers will assist the clinician in predicting the degree of inflammatory response each individual patient with ARDS is likely to display.

A novel approach for interfering with posttranscriptional processing of the mRNA for a gene product involves the introduction of an antisense oligonucleotide sequence. In using this strategy, DNA fragments that are complementary to the transcribed mRNA are introduced into the cell by a variety of transfection protocols. The complementary strand binds to the native mRNA, making it inaccessible to the translational machinery of the cell and thereby inhibiting protein expression. For example, in human umbilical vein endothelial cells and carcinoma cells, an antisense oligonucleotide for ICAM-1 mRNA inhibited its expression *(185)*. A similar antisense strategy has been employed by these same investigators to decrease functional protein expression of ICAM-1 in two in vivo models *(186,187)*. The antisense oligonucleotide delayed cardiac allograft rejection *(188)* and also inhibited LPS-induced neutrophil accumulation in the alveolar space *(189)*. Antisense strategies have been employed to block expression of other cytokine gene products as well *(190)*. The difficulties inherent in this strategy are analogous to those encountered with gene therapy: synthesizing an effective oligonucleotide, delivery of the sequence into the cell with specificity, and achieving sufficient inhibition of protein expression.

Blocking Adhesion Molecules

As our understanding of the role of adhesion molecule expression has unfolded, the goal of antiadhesion molecule therapy has become an

intriguing pursuit. Numerous preclinical animal trials have demonstrated that antiadhesion molecule antibodies such as anti-ICAM-1 *(187,191)*, anti-E-selectin *(192)*, anti-L-selectin *(193)*, and anti-P-selectin *(194)* are able to inhibit neutrophil accumulation in the lung and subsequent tissue injury. Despite these encouraging results, to date, no human trials have successfully used antiadhesion molecule strategies. The leukocyte-adhesion molecule cascade is a necessary host response, as evidenced by individuals who suffer recurrent bouts of infection as a result of leukocyte adhesion deficiency (LAD) syndromes 1 and 2. The molecular bases of these defects are absent expression of the β-integrins (counter-receptor for ICAM-1) in LAD-1 and absence of sialyl-Lewis X (carbohydrate ligand for selectins) in LAD-2 *(195)*. In light of this, disrupting this cascade in the setting of an invading organism may be detrimental to the host. Thus, antiadhesion molecule strategies are likely to face stringent safety trials.

Blocking of Chemokines or Chemokine Receptors

As mentioned in the Pathophysiologic Mechanisms section above, chemokines appear to play a central role in the activation and recruitment of neutrophils to the lung in ARDS. As such, chemokines have become important therapeutic targets in many inflammatory states including ARDS, rheumatoid arthritis, and AIDS. Currently two mechanisms are being employed. Monoclonal antibodies directed against IL-8 have been shown to decrease neutrophil influx and tissue injury in a number of animal models of lung injury *(196–198)*. Because of these encouraging preclinical results, anti-IL-8 antibody is likely to be tested in human ARDS in the very near future. In addition to antibody neutralization, targeting the chemokine receptors has become a hotly pursued field over the past 2 yrs (reviewed in ref. *199*). It is probable that chemokines will be successfully inhibited in both a selective and effective manner in the near future.

APPLICATION OF GENOMICS TO ARDS

As has been emphasized, ARDS is a highly heterogenous disease process with respect to both etiology and outcome. Variable outcomes are particularly frustrating when one considers that one patient with ARDS may survive, and another patient of similar age, having an identical cause of ARDS and similar comorbidities, may die. These highly

divergent outcomes can, at times, be explained by management strategies. Recent progress in genomics, however, suggests that the basis of these variable outcomes may lie in the genetic background of the individual patient. That is, some individuals with ARDS may have a genetic background predisposing him or her to a more severe manifestation of ARDS and are consequently more likely to die as a result of ARDS. The evolving field of genomics holds the promise of elucidating a genetic predisposition to ARDS *(200–202)*. Although no clear ARDS gene or marker has been established to date, there is good evidence that mutations, or polymorphisms, in surfactant protein genes can impart a phenotype characterized by the propensity to develop interstitial lung disease and/or ARDS *(203–205)*. In addition, polymorphisms of cytokine genes have been associated with increased mortality in sepsis *(206–208)*. Because the development of sepsis is so closely linked, clinically and pathophysiologically, to the development of ARDS, it is expected that similar associations will be found between cytokine gene polymorphisms and the course of ARDS.

Important tools for the application of genomics to the study of ARDS include the recent sequencing of the human genome, microarray technology, and bioinformatics *(200–202,209)*. The human genome sequence provides the blueprint for potentially understanding how an individual's genome impacts the development and outcome of virtually any disease process. Microarray technology allows for measuring the simultaneous expression of thousands of gene products and polymorphisms, which can subsequently be analyzed by the evolving field of bioinformatics. With these tools it may become possible to characterize the host response further during ARDS at the genomic level. These types of studies are eagerly awaited as they hold the promise of significantly increasing our understanding of ARDS. Specifically, it is hoped that individual patients can be more thoroughly characterized (i.e., immunophenotyped); as a consequence, immune- or inflammatory-modulating therapies can be more specifically tailored to the needs of the individual patient.

SUMMARY

ALI in its most severe form of ARDS continues to be a major cause of mortality in critical care medicine. It is clear that cytokines contribute to this pathophysiologic state via receptor-mediated signaling

pathways that effect target cell responses. The application of molecular biology techniques to the field of critical care medicine has both improved our understanding of this biologic response and identified a number of potential therapeutic targets. Although in vitro and animal model data have demonstrated the amelioration of the inflammatory response and lung injury by these strategies, the modalities that have been tested in humans thus far have proved ineffective. It is hoped that further understanding of the fundamental biology, improved identification of the individual patient's pro- versus antiinflammatory cytokine state, and application of therapies directed at multiple sites of action may ultimately prove beneficial for patients suffering from ARDS.

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11 Thermal Injury

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INTRODUCTION

The military relevance of thermal injury is indicated by the fact that burns perennially constitute about 5–20% of combat casualties (**Table 1**). The impact of this injury on combat readiness is magnified by the ability of small burns to incapacitate combatants and of extensive burns to consume large quantities of medical resources. The percentage of casualties sustaining thermal injury increases with the intensity of conflict and is particularly high during armored vehicular combat and war at sea. The widespread availability of fuel and the employment of aircraft, boats, and motor vehicles as delivery systems have increased the importance of thermal and inhalation injuries following terror attacks. Thus, the leading cause of injury among patients admitted to New York City hospitals on September 11–13 2001 as a consequence of the World Trade Center attacks was inhalation injury (52 patients, or 37%), and another 27 patients (19%) had a chief diagnosis of burns *(1)*. On the battlefield of the future, the recent 15th Conference on Military Medicine at the Uniformed Services University of the Health Sciences predicted an increased incidence of burns from novel weapons (thermobaric weapons and others), and from burning fuel, plastics, and light metals *(2)*. This chapter reviews the management principles and current research directions for the care of casualties with severe thermal injury. Chapter 12

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Table 1
Incidence of Burn Injury in Armed Conflict

Conflict	*Casualties*	
	%	*No.*
World War II (Hiroshima), 1945	65–85	45,500–59,500
Vietnam, 1965–1973	4.6	13,047
Israeli Six Day War, 1967	4.6	
Yom Kippur War, 1973	10.5	
Falkland Islands War, 1982		
British casualties	18.0	140
Argentine casualties	17.5	34 of 194
Lebanon War, 1982	8.6	
Operation Just Cause, Panama, 1989	2.3	6 of 259
Operations Desert Shield/Storm, 1990–1991	7.9	36 of 458

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reviews inhalation injury, which frequently accompanies cutaneous thermal injury and which increases the morbidity and mortality thereof.

In all respects, the burn wound is the central problem in the care of burn patients. Whereas nonthermal trauma models suffer from the inaccuracy of injury severity scores in predicting outcome, the magnitude of the physiologic changes induced by thermal injury reliably reflects, in a sigmoidal dose-response fashion, the percentage of the total body surface area burned. This fact makes thermal injury particularly suited for combat casualty care research, such that it has been termed the universal trauma model. Furthermore, the successful management of the burn wound and its effects on systemic physiology, from resuscitation through definitive surgery and rehabilitation, is the key to a satisfactory clinical outcome.

Depth of injury is a function of the temperature applied and the duration of exposure. Keratinocyte death following thermal injury may occur by heat fixation, apoptosis, or accidental cell death *(3)*. Jackson *(141)* described three zones in the burn wound: a necrotic zone of coagulation, an ischemic zone of stasis, and an inflamed zone of hyperemia. An important goal of patient care is to prevent the zone of stasis from progressing, by means of edema, hypoperfusion, desiccation, or infec-

tion, into a deeper wound. Furthermore, rescue of apoptotic cells, reversal of tissue ischemia, or prevention of secondary, mediator-induced injury may in the future represent valid strategies for salvaging the zone of stasis. Clinically, burn wounds are classified according to depth as first degree, second degree (partial thickness), or third degree (full thickness). Evanescent, erythematous first-degree burns are of little or no physiologic consequence and are not considered in burn size calculations. Accurate determination of the burn size is more important than prognostication of the burn depth during resuscitation. An initial estimate of burn size can be made with the rule of nines and then refined using the Lund-Browder chart *(4)*. This measurement should be made carefully. Referring hospitals often overestimate burn size, a practice that in a military setting can lead to erroneous triage and evacuation decisions and to excessive fluid resuscitation.

EMERGENCY CARE

Emergency field care and triage of burn patients during a mass casualty disaster or in combat can be performed as described as described in the *Emergency War Surgery NATO Handbook (5)*. A rapid primary survey, modified for burns, is performed *(6)*. With modern burn care, the lethal area 50% (the burn size that is lethal to 50% of patients) for young adults is over 80% *(7)*. Accordingly, even casualties with massive burns merit resuscitation. When the number of casualties overwhelms the available resources, patients with burns in excess of 80% total body surface area (TBSA) are triaged to the expectant/comfort care category. Inhalation injury and concomitant mechanical trauma each add 10% to the burn size for this calculation. (These considerations highlight the importance of careful burn size determination.) Aeromedical evacuation out of the theater of operations by burn-experienced personnel can be performed once casualties are stabilized *(8)*.

Because of the complex, multidisciplinary, and resource-intensive needs of burn patients, definitive surgery is best performed in a specialized center outside the theater of operations *(5)*. Burn center referral criteria have been developed, and recently revised, by the American Burn Association *(6)*: in brief, patients with burns of large *size* (10% TBSA or greater, or full-thickness burns of any size), of unusual *etiology*, on functionally at-risk *areas*, with associated *inhalation* injury, or in unusual patient *populations* merit transfer or at least consultation.

BURN SHOCK

Pathophysiology

Shock commonly occurs following cutaneous thermal injury to 10–20% TBSA or greater and mandates judicious fluid resuscitation. In patients successfully resuscitated with the modified Brooke formula, a characteristic time-course for hemodynamic changes is seen (**Table 2**). Successful fluid resuscitation does not require immediate correction of the evolving plasma-volume deficit; at the nadir, this deficit in appropriately resuscitated patients is about 20%. Restoration of the cardiac output occurs before restoration of the plasma volume, indicating the role of increased afterload in the early postburn decrease in cardiac output. Blood volume is not fully restored at the end of resuscitation, indicating that thermal destruction of red blood cells (RBCs) has occurred. From this, it can be seen that burn shock is the result of both elevated vascular resistance (increased afterload) and hypovolemia (decreased preload).

The postburn decrease in cardiac output and increase in vascular resistance are not uniformly distributed among the various organs, which results in differential changes in regional blood flow. The small intestine may be particularly vulnerable to such decreases; furthermore, aggressive fluid resuscitation and restoration of cardiac output may fail to restore blood flow fully in the mesenteric circulation *(9)*. A myocardial depressant factor of burn shock, affecting intrinsic myocardial contractility, is postulated as an additional postburn mechanism of impaired cardiac output. Horton and colleagues *(10)* have reported such contractility changes by means of a modification of the isolated, coronary-perfused heart (Langendorff preparation). Such decrements are unusual in vivo; for example, Goodwin et al. *(11)* documented hypercontractile left ventricular function during burn shock in patients by means of echocardiography.

The hypovolemia that occurs after thermal injury is caused primarily by the loss of plasma volume into the interstitium in both burned and unburned tissues; the result is edema formation. Transcapillary fluid flux is determined by the net effect of the three Starling forces: microvascular permeability, intravascular and interstitial hydrostatic pressure, and intravascular and interstitial colloid oncotic pressure. Each of these is affected by thermal injury and contributes to plasma loss and edema formation. Water, albumin, sodium, and RBCs accumulate within min-

Table 2
Hemodynamic Changes After Thermal Injury.

Variable	*Immediate effect*	*First postburn day*[a]	*Second–third postburn days*[a]
Cardiac output	Decreases to 50% of normal levels	Increases to 80% of normal levels by 6–8 h post burn	Increases to supranormal levels by 36 h post burn
SVR	Doubles	Decreases to less than normal by 6–8 h post burn	Remains about 50% of normal
PVR	More than doubles	Remains elevated	Decreases to normal by 48 h post burn
Blood volume	No significant immediate change	Decreases to a nadir at 18–24 h post burn	Increases, but remains below normal
Plasma volume	No significant immediate change	Decreases to a nadir at 18–24 h post burn	Increases to normal at 54 h post burn

[a]With adequate resuscitation.
SVR, systemic vascular resistance; PVR, pulmonary vascular resistance.

utes in burned skin, although the process of edema formation continues throughout the first 24–48 h post burn.

Arturson *(12)* showed that the rate of edema formation, the capillary filtration coefficient, and the diameter of the resistance vessels all increase most rapidly during the first 5–10 min post burn. He also found that the greatest increase in permeability occurred for large molecules, that this change was maximal at 1–3 h post burn, that it worsened with increased burn depth, and that it gradually resolved as the wound healed. Pitt et al. *(13)* determined the relative contribution to edema formation of pressure and permeability changes during the first 3 h post burn. Thermal injury caused a near-doubling of capillary pressure, from 24 to 47 mmHg, by 30 min post burn, secondary to a decrease in precapillary resistance with no change in postcapillary re-

sistance. Capillary permeability and the edema formation rate increased throughout the 3-h study. Until about 2 h post burn, most of the increase in fluid flux was explained by pressure increases, whereas thereafter permeability increases achieved greater importance. In addition, thermal injury alters the ultrastructure of the interstitium, creating what has been termed a dermal imbibition pressure, which contributes to the net balance of forces favoring edema formation *(14)*.

Aside from direct thermal injury to the endothelium and interstitium, several mediators have been implicated in the process of edema formation. Histamine is increased within 1 min of thermal injury (in proportion to the burn size) and causes vasodilation of the resistance vessels and increased microvascular permeability. Other mediators of local and systemic injury post burn include prostaglandins, complement, nitric oxide, the proinflammatory cytokines, and neutrophils.

Systemic release of these mediators and generalized hypoproteinemia induce edema formation in unburned tissue following burns in excess of about 25% TBSA. Demling and colleagues *(15)* defined the effect of a 30% TBSA burn on microvascular permeability in unburned tissues. There was an increase in lung lymph flow (Q_L) but no increase in pulmonary capillary permeability. By contrast, there was an increase in both Q_L and permeability in the flank areas, the latter resolving after 12 h post burn. Burn tissue Q_L was persistently elevated post burn for the duration of the 60-h study, and permeability remained elevated for 48 h post burn. Thus, it appears that unburned soft tissue microvascular permeability resolves before that of burned soft tissue *(15)*.

Resuscitation

Three factors laid the foundation for the development of fluid resuscitation formulae for burn shock: recognition that burn shock involves the gradual loss of large volumes of plasma into the interstitium, with resulting hemoconcentration and hypovolemic shock; recognition that these losses were proportional to the extent of burn; and the availability of plasma transfusions during World War II. Consequently, early formulae delivered various combinations of plasma and crystalloid; for example, the original Brooke formula called for 1.5 mL/kg/% burn of crystalloid and 0.5 mL/kg/% burn of colloid during the first 24 h post burn. In subsequent studies, the amount of colloid given during the first 24 h post burn, which was varied between zero and 50% of the total volume infused, had no greater effect than that of crystalloid fluid on the augmentation of

plasma volume *(16)*. Furthermore, the use of colloid immediately post burn was shown to promote the accumulation of extravascular lung water when edema fluid is reabsorbed, beginning on postburn day 3 *(11)*.

These findings gave rise to the modified Brooke formula, which is currently used by the authors: 2 mL/kg/% burn of lactated Ringer's solution is programmed for administration during the first 24 h post burn. During the second 24 h, restoration of microvascular permeability in unburned tissue allows for the use of colloid in order to reduce the total volume infused, and a 5% solution of albumin in normal saline is given at a dose of 0.3–0.5 mL/kg/% burn *(17)*. In children weighing less than 30 kg, the surface area-to-weight ratio is greater, and the volume requirements per percent burn are therefore greater. Thus, for children, the modified Brooke formula predicts a need for 3 mL/kg/% burn of lactated Ringer's solution for the first 24 h post burn, as well as 5% glucose and 5% dextrose and ½ normal saline in water at a maintenance rate *(18)*. The Parkland formula, which is also widely used, estimates fluid requirements as lactated Ringer's solution, 4 mL/kg/% burn for the first 24 h post burn.

The various formulae provide only an initial estimate of fluid requirements. The fluid infusion rate must be adjusted continually based on physiologic response, primarily the hourly urine output. A urine output of 30–50 mL/h in adults, or 1 mL/kg/h in children, should be achieved by 20% changes in the lactated Ringer's solution rate every hour or two. It is as important to decrease as it is to increase the fluid administration rate as needed, in order to avoid the complications associated with excessive edema formation. Typically, the actual volume given is greater than that predicted by the formulae. In one review, 2.88 mL/kg/% burn was the final dose administered under the modified Brooke formula *(17)*. In a recent multicenter review of experience with 50 patients, 5.2 mL/kg/% burn was the final dose given under the Parkland formula *(19)*. These data suggest that the Parkland formula overestimates the requirements of many patients and that its use may lead to excessive edema formation. A randomized, controlled trial comparing resuscitation formulae has not been performed.

The desire to limit fluid intake, and the recognition of the importance of sodium repletion, has led to studies of various hypertonic saline solutions for burn shock resuscitation. Monafo and colleagues *(20)*, and others, have argued that hypertonic saline solutions containing approximately 200 mEq/L of sodium reduce water requirements, without increasing sodium requirements, during burn resuscitation. By contrast,

Huang et al. *(21)* reported no sustained fluid-sparing effect, a twofold increase in mortality, and a fourfold increase in acute renal failure, for patients receiving a 290 mEq/L solution versus historical controls receiving lactated Ringer's solution. A recent Cochrane Collaboration review of such regimens found a total of four randomized controlled trials. The pooled relative risk of death in burn patients was 1.49 [95% confidence interval (CI) 0.56–3.95], such that these trials must be considered inconclusive *(22)*.

Various beneficial effects have been reported in animal models for hypertonic saline (7.5%) and dextran 70 (6%) solution (HSD), as a pharmacologic adjunct to standard resuscitation regimens, including improved cardiac function *(23)*, increased regional blood flow, and reduced lipid peroxidation *(24)*. However, HSD has provided early (8–12 h), but not sustained, fluid-sparing effects in large animal models, such that its role in burns remains to be defined *(25,26)*.

Based on observations such as a correlation between the base deficit or low oxygen delivery during resuscitation and increased subsequent mortality *(27)*, some authors have proposed a revision of resuscitation endpoints. It does not necessarily follow, however, that intentional augmentation of oxygen delivery by fluid loading will improve outcome, since the fundamental problem in burn shock is edema formation. As mentioned above, transcapillary fluid flux during the early postburn hours is primarily driven by pressure, such that fluid loading during this period is likely to result in massive edema. The devastating effects of large resuscitation volumes—to include the abdominal and extremity compartment syndromes and respiratory failure secondary to airway edema *(28,29)* are strong arguments against attempted hyperdynamic resuscitation of thermally injured patients.

By contrast, a pharmacologic approach to reducing the fluid resuscitation requirements of the thermally injured patient would be desirable. High-dose ascorbic acid (66 mg/kg/h) reduced lipid peroxidation, vascular permeability, burn and nonburn tissue edema, and fluid resuscitation requirements in animal models *(30)*. Intravenous ibuprofen improved blood flow while reducing edema formation in the burn wound *(31)*. Confirmatory studies are needed to place such treatment in clinical perspective.

Burn shock is associated with important end-organ effects. The approach to the care of burned extremities can be summarized briefly as exercise, elevate, evaluate, and (as needed) escharotomy. Edema beneath

constricting full-thickness eschar may impair arterial inflow and cause neuromuscular damage. The progressive diminution of arterial flow in the extremities, assessed hourly by Doppler flowmetry, is the primary indication for urgent escharotomy. This procedure is normally performed at the bedside. An incision through the eschar and into the subcutaneous tissue is made along the mid-medial and/or mid-lateral line of the affected limb. It is important to include all circumferential burn, particularly across affected joints, and to document pulse restoration after the procedure.

In the absence of antacid therapy, gastroduodenal stress ulceration (Curling's ulcer) has been diagnosed in 12% of burn patients; endoscopically, gastric mucosal ulceration can be visualized in 86% of patients with burns of over 35% TBSA beginning a few hours postburn *(32)*. Progression of these lesions to frank ulceration with bleeding and/or perforation is effectively prevented with antacids or H_2 antagonists. The authors' practice is to begin antacid therapy immediately, alternating a magnesium-containing antacid with an aluminum-containing antacid every 2 h, while also administering an intravenous H_2 antagonist such as cimetidine. The utility of proton-pump inhibitors in burn patients has not been determined.

Thermal injury may also alter intestinal permeability, which is increased on d 2 in those patients who later develop infection *(33)*. In animal models, this increase in permeability is associated with the translocation of micro-organisms and their products, which may lead to systemic inflammation, multiple system organ failure, and/or infection *(34, 35)*. Nonocclusive mesenteric ischemia and infarction is a rare but potentially devastating complication of thermal injury. Diagnostic peritoneal lavage and/or fiberoptic laparoscopy may be helpful *(36)*.

METABOLISM AND NUTRITION

Nutritional Requirements

One of the striking changes seen in patients during the weeks following extensive thermal injury is a hyperdynamic response to injury, which causes profound weight loss and lean body mass erosion. This phenomenon is attenuated—but not fully prevented—by the provision of adequate calories to meet greatly increased metabolic demands and of adequate protein to maintain a positive nitrogen balance. Thus, careful attention to the nutritional needs of burn patients is a cornerstone of burn care.

Resting energy expenditure (REE) during the first 1–3 wk post burn is a function of age, sex, TBSA, and body surface area in m^2 (BSA), as described, for example, by Carlson et al. *(37)*:

$$\text{Predicted REE (kcal/m}^2\text{/24 h)} = \text{BMR} \times [0.89142 + 0.01335 \times (\%\ \text{burn})] \times \text{BSA} \times 24$$

Age- and sex-specific preburn basal metabolic rate (BMR) can be estimated using the Fleisch equation:

$$\text{Male BMR} = 54.33782 - (1.19961 \times \text{age}) + (0.02548 \times \text{age}^2) - (0.00018 \times \text{age}^3)$$

$$\text{Female BMR} = 54.74942 - (1.54884 \times \text{age}) + (0.03580 \times \text{age}^2) - (0.00026 \times \text{age}^3)$$

This estimate of REE can then be multiplied by an activity factor (usually 1.25) to estimate estimated energy requirements, i.e., caloric needs, in burn patients. An earlier estimate of caloric requirements, also in common use, is the Curreri formula *(38)*:

$$\text{Calories required (kcal)} = 25 \times \text{weight (kg)} + 40 \times (\%\ \text{burn})$$

As the burn wound heals, the REE decreases. Milner et al.*(34)* measured REE in 20 patients weekly until the wounds were closed or the patient discharged and compared these results with the REE predicted by the above (Carlson) equation. During the first 30 d post burn, predicted REE and measured REE were similar. After 30 d post burn, because of variations in REE related to time post burn and other factors, REE is best estimated by indirect calorimetry. At the time of discharge, measured REE was still 25% above normal BMR, even though wounds were closed *(39)*. These findings were recently extended by Hart et al. *(40)*, who measured REE, lean body mass by dual-energy X-ray absorptiometry, and isolated leg protein kinetics at 6, 9, and 12 mo after burn in children. REE remained elevated above the Harris-Benedict prediction and gradually fell throughout the 12-mo period. Net protein balance in the leg remained abnormally low, and lean body mass continued to fall, until after 9 mo post burn. Protein balance returned to normal, and lean body mass began to rise at 12 mo. Thus, hypermetabolism and catabolism persisted long after discharge in these patients *(40)*.

The clinician must also meet the burn patient's nitrogen requirements. In burn patients, rates of whole-body protein synthesis and breakdown were both increased above those of uninjured subjects and

correlated with TBSA burned *(41)*. Although protein requirements are commonly estimated such that a nonprotein kcal/nitrogen ratio of 150:1 is provided *(42)*, actual nitrogen requirements are highly variable. The nitrogen intake required to maintain a positive balance declines over time, as the burn wound heals. Matsuda et al. *(43)*, having estimated caloric requirements based on the Curreri formula, found that a kcal/nitrogen ratio of 100:1, compared with a ratio of 150:1, was necessary to maintain positive nitrogen balance in patients with larger burns. Because of this time- and burn size-related variability and because urinary nitrogen losses comprise the major route of nitrogen excretion, it is important to measure 24-h urinary nitrogen losses once a week in the care of patients with large burns. The nitrogen balance is then calculated; small deficits can be made up by an increase in the enteral feeding rate, but large deficits are made up by boluses of a protein formula. Milner et al. *(44)* found that the urinary urea nitrogen (UUN), which is easily measured, closely predicted ($r^2 = 0.88$) the total urinary nitrogen (TUN), which is not easily measured:

$$\text{TUN}_{\text{predicted}} = \text{UUN} \times 1.25$$

Waxman et al. *(45)* measured protein losses across open burn wounds and found them to be significant. During the first week post burn, the 24-h protein loss in grams is:

$$\text{Wound losses}_{\text{predicted}} = 0.3 \times \text{BSA} \times (\%\ \text{burn}) \times 0.8$$

where BSA is body surface area in meters squared. After the first postburn week, protein is lost at approximately one-third this rate:

$$\text{Wound losses}_{\text{predicted}} = 0.1 \times \text{BSA} \times (\%\ \text{burn}) \times 0.8$$

The 0.8 factor in both formulae is a correction factor for the use of topical antimicrobials; Waxman et al. *(45)* found that silver sulfadiazine cream decreased protein losses. These formulae are commonly used to estimate protein losses across the burn wound. Finally, an estimate of 2 g/d is commonly used for fecal losses. These three major routes of nitrogen excretion (urine, wound, estimated fecal) allow us to write the following equations:

$$\text{Nitrogen balance} = \text{nitrogen intake} - \text{nitrogen output}$$

$$\text{Nitrogen output} = \text{TUN}_{\text{predicted}} + \text{wound losses}_{\text{predicted}} + \text{fecal losses}_{\text{predicted}}$$

Various visceral protein levels (albumin, prealbumin, retinal binding protein, and transferrin) correlate poorly with nitrogen balance. Nitrogen balance was better predicted (r^2 = 0.59) by a multiple logistic regression equation incorporating nitrogen intake, postburn day, TBSA burned, and age, but the UUN measurement remains necessary *(42)*.

The loss of the cutaneous barrier to evaporation causes considerable insensible water loss post burn, the volume of which is a function of burn size. After resuscitation is completed, these losses must be replaced and can be estimated by the following formula *(46)*:

$$\text{Insensible water loss (cc/h)} = (25 + \%\ \text{burn})(\text{BSA})$$

where BSA is body surface area in m^2. In the absence thereof, hypernatremia owing to inadequate replacement of insensible water loss is the most common electrolyte disturbance of burn patients. In addition, an apparent resetting of the hormonal control mechanisms over plasma tonicity occasionally results in hyponatremia secondary to a syndrome of inappropriate secretion of antidiuretic hormone (SIADH). Thus, the serum sodium must be carefully monitored, and water intake must be adjusted accordingly, in order to avoid dangerous hyper- or hyponatremia.

Augmentation of oral intake with enteral or parenteral nutrition is commonly required in adult patients with burns in excess of 30% TBSA, as they are unable to consume enough orally to meet their needs. The well-known immunosuppressive effects of parenteral nutrition and the benefits of enteral feeding lead most authors to prefer the enteral route whenever possible. Discontinuation of gastric feedings because of gastric ileus, or to avoid perioperative aspiration, causes a significant caloric deficit in many patients fed via intragastric tubes *(47)*. For these reasons, and to minimize the risk of gastric aspiration, the authors prefer to place a nasoenteral tube past the pylorus and into the jejunum whenever possible. Bedside fluoroscopy (C-arm) or endoscopy is employed to facilitate nasoenteral tube placement, as needed. Also, initiation of enteral feeding immediately post burn is recommended by some; however, net absorption of tube feeds may be sufficiently low that predicted caloric needs are not met for several days post burn *(48)*. Thus, the authors initiate enteral feeding at the close of the first 48 h post burn, once return of bowel function and resolution of ileus have occurred. Continuation of jejunal tube feedings during sur-

gery has been successfully performed and helps to prevent inadequate caloric intake in patients undergoing multiple operations.

Mechanism of Hypermetabolism

Thyroid function is normal in burn patients, whereas elevated catecholamine levels are a major mechanism for postburn hypermetabolism. Urinary catecholamine excretion correlated well with metabolic rate in burn patients. In some patients, an increase in metabolic rate owing to environmental cooling led to an increase in catecholamine excretion. Other patients (all of whom later died) became hypothermic and had decreased catecholamine production, following environmental cooling. β-Blockade with propranolol decreased metabolic rate in burn patients, but α-blockade did not; epinephrine infusion increased it in normal subjects. Thus, catecholamines are important mediators of postinjury hypermetabolism *(49)*. Infusion of the three counterregulatory (stress) hormones cortisol, glucagon, and epinephrine in normal subjects during a 4-d period reproduced many of the metabolic responses seen following injury and demonstrated that these hormones acted synergistically.

These changes included increases in minute ventilation, $\dot{V}O_2$, $\dot{V}CO_2$, metabolic rate, urinary nitrogen excretion, glucose and insulin levels, and endogenous glucose production *(50)*. Skeletal muscle intracellular glutamine concentrations were lower during stress hormone infusion, but free amino acid levels in arterial blood and forearm amino acid efflux were unchanged; thus, hormonal changes alone do not reproduce all features of postinjury skeletal muscle proteolysis *(50)*.

Later work has evaluated the role of the proinflammatory cytokines in postinjury metabolism. For example, infusion of tumor necrosis factor (TNF) into animals produced changes such as hypotension, decreased skeletal muscle transmembrane potential, increased lactate efflux from the extremities, and increased stress hormone levels in a dose-responsive manner *(51)*. The proinflammatory cytokines appear to influence the hypothalamic-pituitary-adrenocortical (HPA) axis. Intravenous TNF-α stimulated adrenocorticotropic hormone (ACTH) and corticosterone secretion in a dose-dependent fashion. This effect was inhibited by a corticotropin-releasing hormone (CRH) antiserum *(52)*. Antibodies to interleukin-6 (IL-6), TNF, and IL-1 receptor each blocked the production of ACTH following lipopolysaccharide infu-

sion, although at different time points *(53)*. The inflammatory cytokines may cross (or produce changes on the other side of) the blood-brain barrier *(54)*; in addition, peripheral inflammation may activate the HPA axis, via nociceptive, visceral, or somatosensory afferent neurons *(54,55)*.

At the molecular level, the breakdown of myofibrillar proteins (actin and myosin) proceeds as follows: (1) they are released from the myofibrils; (2) they are tagged with a marker protein, ubiquitin; and (3) they are degraded by a barrel-shaped proteolytic particle, the 26 S proteasome. Several mediators known to be elevated in burn patients, especially cortisol, but also TNF-α and IL-1, have been implicated in this process *(56)*. In the case of the cytokines, this effect probably involves the upregulation of ubiquitin genes *(57)*. Also, thermal injury results in the upregulation of ubiquitin-conjugating enzyme ($E2_{14k}$), which may be a rate-limiting enzyme in the process *(58)*, in an increased rate of ubiquination of proteins *(59)*, and in increased expression of genes for components of the 26 S proteasome *(60)*. Thermal injury also activates skeletal muscle apoptosis *(61)*.

Novel Approaches

This process of continued lean body mass erosion may cause undesirable consequences such as impaired wound healing, respiratory muscle weakness, difficulty performing physical and occupational therapy, prolonged hospitalization, and delay in return to work. Several approaches have been taken in an effort to prevent such catabolism. β-Blockade with propranolol attenuated hypermetabolism and muscle-protein catabolism *(62)*. Provision of human growth hormone *(63)*, insulin-like growth factor-I (IGF-I) *(64)*, or insulin *(65)* has also been effective. By combining IGF-I with its principle binding protein, IGF-I-binding protein 3 (IGFBP-3), adverse effects of IGF-I therapy such as hypoglycemia can be avoided *(66)*. Another promising option is the oral anabolic steroid oxandrolone *(67)*. On the one hand, testosterone is immunosuppressive and estrogen-immunoprotective, in several models of injury and infection *(68)*. On the other hand, an agent that speeds wound healing and maintains lean body mass may increase survival in burn patients, in whom successful wound closure is the prerequisite for survival. Thus, the net effect of anabolic steroids on infection rates and on survival, particularly in patients with massive burns, is unknown, and more research is needed.

Following the demonstration by Alexander et al. *(69)*, of enhanced immune function in burned children receiving high levels of protein intake, there has been much interest in developing immune-enhancing diets (IEDs). The primary components of commercially available IED formulas are glutamine, arginine, and Ω-3 fatty acids. Glutamine is a major precursor for hepatic gluconeogenesis and for the production of reduced glutathione (GSH) and nucleic acids. It fuels rapidly dividing cells in the intestinal mucosa and the immune system *(70,71)*. It also fuels fibroblasts *(72)* and stimulates production of collagen by fibroblasts *(73)*. Following thermal injury in adults, glutamine becomes a conditionally essential amino acid *(70)*. This relative glutamine scarcity, as well as increased requirements, may impair lymphocyte, macrophage, and neutrophil function *(71)* as well as gut barrier function and energy charge *(74)*.

Arginine is another frequent IED component. There is evidence that arginine supplementation improves wound healing, via a nitric oxide effect *(75)*. In addition, arginine supplementation may downrregulate postburn production of the proinflammatory cytokines *(76)* and may enhance T-cell proliferation *(77)*.

Ω-3 Fatty acids, in particular α-linolenic acid, are frequent IED components. These, unlike Ω-6 fatty acids, are not converted by cyclo-oxygenase into prostaglandins. Because of their location on the cell membrane, fatty acids are in a position to influence a variety of signaling processes. Their net effect on immune function includes downregulation of proinflammatory cytokine production, decreased polymorphonuclear leukocyte chemotaxis, decreased L-selectin and adhesion molecule expression, and decreased production of nitric oxide and superoxide *(78)*. Clinical trials of IEDs in critically ill patients and burn patients have yielded mixed results *(79–82)*. Thus, despite enticing preclinical studies, the precise role of IEDs in burn patient care remains to be defined.

IMMUNE FUNCTION

Thermal injury causes several changes in immune function, which may predispose to infection. Since infection is the leading cause of death in patients with thermal injury who are admitted to burn centers, elucidation of a complete picture of these immunologic changes is critical.

Neutrophils

Neutrophils play a central role in innate immune response, for example, to bacteria and fungi. Moore et al. *(83)* described complement-mediated, systemic activation (i.e., priming) of neutrophils following burn *(83)*. Systemic neutrophil activation is potentially harmful, because it can both impair chemotaxis and cause endothelial and end-organ damage *(84)*. Indeed, this concept of maladaptive neutrophil priming is consistent with the second-hit hypothesis *(85)*. Agents that may prime neutrophils in burn patients include complement, endotoxin, TNF-α, granulocyte/ macrophage colony-stimulating factor, platelet-activating factor, and IL-8 *(86)*. One feature of postburn neutrophil function is a supranormal oxidative burst *(87)*. Another is abnormal locomotion, secondary to abnormal microtubule assembly *(88)* or to failure of actin to undergo the cyclic polymerization and depolymerization required for normal motility *(89)*.

T-Lymphocytes

T-lymphocytes play a central role in adaptive immunity, by means of their ability to recognize and respond to antigens presented by monocytes, macrophages, dendritic cells, and others. Examples of burn-induced failure of adaptive immunity include prolonged skin allograft survival, inhibited delayed-type hypersensitivity reaction, and reduced peripheral blood lymphocyte proliferation in the mixed lymphocyte reaction. Such immunosuppression may be explained by decreases in T-cell number or by alterations in function.

With respect to numerical changes, it is well known that thermal injury is associated with leukocytosis, lymphopenia, monocytosis, and decreases in the total number of T-cells *(90)*. Several authors have found a decrease in the helper-to-suppressor ($CD4^+$ to $CD8^+$) T-cell ratio after burn; the decrease in $CD4^+$ is variably seen either with or without a lesser decrease in the $CD4^+$ count. This change, when seen later in a patient's course, has been associated with an increased frequency of sepsis and mortality *(91)*. The finding of a decreased helper-to-suppressor ratio was later questioned by several groups on the grounds that burn injury produces morphologically abnormal granulocytes and monocytes, which may be indistinguishable from lymphocytes by means of earlier flow cytometric techniques *(92)*. When such abnormal nonlymphocytes were excluded, Burleson et al. *(90)* found in the rat model that the helper-to-suppressor ratio was preserved follow-

ing burn, and decreased only after induction of burn wound infection. The process that leads to decreased T-cell numbers following injury may involve T-cell apoptosis *(93)*. It is proposed that macrophage-derived Fas ligand may induce T-cell apoptosis *(94)*. Also, glucocorticoids may cause T-cell apoptosis in burn models *(95)*. Indeed, infusion of hydrocortisone in normal humans reproduced the T-cell helper-to-suppressor ratio changes seen in burn patients *(96)*.

Functional derangements may also explain T-cell failure after burn. Xu et al. *(97)* evaluated the ability of purified burn patient T-cells to take up tritiated thymidine (a functional assay) in response to three conditions: spontaneously, following mitogen stimulation (with phytohemagglutin) and following antigen stimulation. In each case, and analogous to the of Burleson et al. *(90)*, the removal of nonlymphocyte cells corrected these measures of T-cell function to normal levels *(97)*. Zapata-Sirvent and Hansbrough *(98)* examined the time sequence of antigen expression on the surface of T-cells from burn patients. Certain antigens—HLA-DR, IL-2 receptor (CD25), and transferrin receptor (CD71)—are associated with T-cell activation. These authors found a decrease in these activation antigens as early as d 1 post burn, which may be indicative of impaired cell-mediated immunity *(98)*. Others have described, however, increases in the activation markers CD25, CD69, and CD71 *(99–101)*. In an effort to arrive at a unifying hypothesis, Deitch et al. *(102)* proposed that burn injury may cause early, nonspecific activation of the cellular immune system, which in turn may impair later specific T-cell responses to a challenge.

Another cause of T-cell functional failure following burn is decreased production of IL-2. IL-2 is principally a product of T-lymphocytes and specifically of $CD4^+$ T-helper cells; activation of T-lymphocytes via the T-cell receptor (TCR) causes IL-2 production. IL-2, in turn, stimulates T-cell clonal expansion (blastogenesis) in response to a mitogen and enhances nonspecific (natural killer) and specific T-cell cytotoxicity. Wood et al. *(103)*, and others, found that IL-2 production by burn patient lymphocytes in response to a mitogen was decreased, with further decreases during sepsis. As expected, blastogenesis was concomitantly decreased and correlated with IL-2 levels *(103)*. Replacement with recombinant IL-2 restored T-cell response to mitogen and improved survival in burned mice *(104)*. IL-2 receptor (IL-2R, CD25), is a product of a gene activated by IL-2. Decreased IL-2R production and production of nonfunctional receptors also occur

after burn *(105)*. An important mediator of postburn T-cell immunosuppression is prostaglandin E_2 (PGE_2). PGE_2 inhibits T-cell function in vitro, to include IL-2 production and consequently T-cell activation *(106)*. Postburn inhibition of PGE_2 production by indomethacin improves IL-2 production by splenocytes *(107)*, and also improves the mitogen responsiveness of peripheral blood mononuclear cells *(108)*.

Two functionally distinct types of $CD4^+$ T-helper cells, Th1 and Th2, have been described, in terms not of their cell surface markers, but of the cytokines they produce. Th1 cells produce cytokines such as IL-2, TNF-β, and IFN-interferon-γ Th2 cells produce cytokines such as IL-4, IL-5, IL-6, and IL-10. Broadly speaking, selective activation of Th1 cells enhances cell-mediated immunity, and selective activation of Th2 cells enhances humoral immunity. Following injury, a shift in the helper cell phenotype from Th1 to Th2 has been observed *(109,110)*. It has been proposed that this may reflect a "compensatory antiinflammatory response syndrome" (CARS), which occurs following the initial systemic inflammatory process unleashed by the burn *(92,111)*. One effect of this shift should be a decrease in total T-cell numbers, because of decreased IL-2 production *(112)*. Shifting the balance back toward the Th1 phenotype may be therapeutic following major thermal injury. In patients, increased IL-10 production was associated with infection; in burned mice, anti-IL-10 antibody improved survival after cecal ligation and puncture *(113,114)*. On the other hand, replacement of IL-12, which induces the Th1 phenotype, increased survival following cecal ligation and puncture in burned mice *(115)*.

Monocytes and Macrophages

Monocytes and dendritic cells, considered part of the innate immune system, nevertheless play a critical role in adaptive immunity by presenting foreign antigens to T-lymphocytes in the context of expressed MHC class II (HLA-DR) antigens. Burns have been associated with various abnormalities in monocyte-macrophage function, including reduced phagocytic capacity, decreased presentation of antigens to T-lymphocytes, and increased cytokine production *(116)*. Thus, impaired monocyte/macrophage function may result in impaired T-cell function. Several groups have now described decreased expression of HLA-DR antigens on blood monoctyes of burn patients, particularly in association with infection *(117)* or mortality *(116)*.

B-Lymphocytes

Plasma levels of immunoglobulin (Ig), in particular IgG, have been noted to decrease following thermal injury in humans. Decreased in vivo levels may arise from resuscitation-related dilution, decreased production, increased losses across the burn wound or into the interstitium, increased consumption *(118)*, and/or burn excision with associated blood loss and transfusion *(119)*. The results of in vitro studies of IgG and IgM production by B-lymphocytes, spontaneously or in response to stimulation, have been quite variable *(120)*. The question is further complicated by the fact that B-cells are dependent on T-cell help; thus, changes in cellular immunity will influence humoral immunity as well. Several studies have documented abnormalities of B-cell activation, proliferation, differentiation, and synthetic activity in burn patients *(118,121,122)*. PGE_2 and transforming growth factor-β (TGF-β) have been identified as mediators of these changes *(123)*.

INFECTION

In the face of these changes in immunologic function following thermal injury—superimposed on massive soft tissue injury—it is not surprising that infection is the leading cause of death in burn patients. At the same time, advances in burn wound care have largely overcome the problem of invasive Gram-negative burn wound infection, such that the location and microbiology of lethal postburn complications have shifted *(124)*.

Topical Therapy

Prior to the introduction of topical mafenide acetate (Sulfamylon®) cream in 1964, invasive Gram-negative burn wound infection with systemic involvement (burn wound sepsis) was the leading cause of death in burn patients. The burn wound, a reservoir of necrotic tissue in extensive contact with both the environment and the circulation, provides an ideal medium for the growth of pathogens. *Pseudomonas* is the most common invasive pathogen in patients who do not receive adequate topical antimicrobial therapy *(125)*. Mafenide was shown to convert a uniformly lethal burn wound infection into a uniformly survivable one, if applied to an experimental wound within 24 h of seeding. Following its introduction into clinical burn care, mafenide had a dramatic effect on mortality. Comparing patients treated with

conventional exposure therapy (1962–1963) with those treated with mafenide topical therapy (1964–1966), Pruitt et al. *(126)* reported a decrease in all-cause mortality from 38 to 20%, a decrease in the incidence of burn wound sepsis from 22 to 2% of patients and a decrease in burn wound sepsis as a percentage of total deaths from 59 to 10%.

Mafenide is structurally related to the sulfonamide class of antibiotics, but its mechanism of action is distinct. It has an unequalled record of efficacy against *Pseudomonas*: none of the 8500 strains analyzed over a 25-yr period (1967–1992) at the U.S. Army Burn Center were resistant to mafenide at concentrations seen topically (Dr. A.T. McManus, unpublished data). Mafenide concentrations in the burn wound decline to subinhibitory concentrations in about 10 h, mandating reapplication at least twice daily. Mafenide and its metabolite are carbonic anhydrase inhibitors, such that twice-daily use of mafenide may be associated with metabolic acidosis, particularly in those patients with extensive partial-thickness burns, decreased glomerular filtration rate (the metabolite is cleared renally), and impaired ventilation. Mafenide's ability to penetrate avascular tissue makes it useful not only in the treatment of full-thickness burn wounds and infected burn wounds, but also in the prevention of suppurative chondritis of burned ears.

The other major approach to the prevention of invasive burn wound infection has employed various formulations of silver, an element with a long history as an antimicrobial. The "oligodynamic" action of silver includes bacterial cell wall disruption, disruption of key bacterial enzymes such as cytochromes, and interaction with nucleic acids *(127)*. Silver can be delivered as an aqueous solution of silver nitrate ($AgNO_3$), as silver sulfadiazine cream, or as a silver-impregnated dressing. A 0.5% solution of $AgNO_3$ (29.4 mEq/L) in water is an effective topical antimicrobial, without epidermal toxicity. Thick gauze dressings are used; the dressings are kept continuously wet with $AgNO_3$ solution by reapplication every 3–4 h, and are changed at least once (preferably twice) daily. Evaporative water loss causes an increase in the local $AgNO_3$ concentration, which may thus reach tissue toxic levels. Another complication of $AgNO_3$ therapy is loss of sodium, chloride, potassium, and other ions from the body into the hypotonic solution, following the concentration gradient. Frequent electrolyte determinations are therefore necessary. Finally, and in contrast to mafenide, the precipitation of silver salts that occurs upon contact with burn wound

chloride ions and proteins limits the efficacy of $AgNO_3$ to surface contamination only; it has no efficacy against established infection.

Silver sulfadiazine in burn care is a water-insoluble compound that forms when $AgNO_3$ reacts with sulfadiazine, a topical antibiotic that *per se* suffers from a significant resistance pattern. Silver sulfadiazine is devoid of the side effects seen with $AgNO_3$. Ag^+, but not sulfadiazine, enters the bacterial cell wall and combines with bacterial DNA. Silver sulfadiazine dissociates at a moderate rate, in essence serving as a slow-release formulation of Ag^+. Unlike mafenide, penetration by silver sulfadiazine cream into eschar is limited, which may explain observed discrepancies between in vitro and in vivo efficacy. Also, silver sulfadiazine use is occasionally associated with a decrease in the white blood cell and granulocyte counts, which has been attributed to toxicity toward bone marrow granulocyte-macrophage progenitor cells *(128)*. This effect rarely requires cessation of silver sulfadiazine therapy and usually resolves despite continued use.

Recent additions to the burn wound armamentarium are dressings coated with elemental silver. Silver-nylon cloth (Argentum Medical, Lakemont, GA) was effective in preventing and treating invasive burn wound infection in the rat model. Therapeutic efficacy was enhanced by application of weak direct current to the silver cloth, with the cloth acting as an anode; this serves to liberate Ag^+ from the cloth *(129)*. Acticoat® (Smith & Nephew, Largo, FL) is another silver-impregnated dressing. A binary alloy of silver (97%) and oxygen is sputtered onto a polyethylene mesh. Water is applied approximately every 6 h, and the dressing is changed once every 48–72 h. This dressing provides a continuous release of Ag+ and, in addition, may release complexes of nonionic Ag as well (Dr. R. Burrell, personal communication). In a clinical study, Acticoat compared with $AgNO_3$ resulted in a decreased incidence of infection (defined as $> 10^5$ organisms per gram of tissue from burn wound biopsies) *(127)*.

The authors' current practice is to alternate mafenide acetate and silver sulfadiazine creams, applying the former in the morning and the latter 12 h later. This maximizes the advantages and minimizes the disadvantages of each preparation.

Fungal Burn Wound Infection

With the control of invasive Gram-negative bacterial burn wound infection, invasive fungal infection has attained greater prominence.

Candida sp. are the most common colonizers of the burn wound but rarely cause invasive infection; in fact, such invasion may indicate collapse of host defenses and is often a preterminal event. By contrast, the filamentous or "true" fungi are more aggressive invaders of the subcutaneous tissues. These organisms can be broadly classified by their morphologic appearance on wound biopsy. At present, *Aspergillus* and *Fusarium* are the most common fungal organisms causing invasive wound infection at the U.S. Army Burn Center; they feature long filamentous hyphae that branch at 45-degree angles. On the other hand, the Phycomycetes (primarily *Mucor*, *Absidia*, and *Rhizopus*) feature broad, nonseptate hyphae that branch widely. The Phycomycetes invade rapidly and frequently spread along and cross fascial planes. Thus, it is not surprising that amputation is frequently required in patients requiring surgery for burn wound infections caused by these organisms.

Management of Burn Wound Infection

Regardless of the causative organism, burn wound infection is best diagnosed by histopathologic examination of biopsy specimens. Quantitative culture of wound biopsies is sensitive but not specific for infection *(130)*. In other words, culture cannot distinguish between true infection and heavy colonization, whereas both the natural history and treatment of these entities are divergent. In addition, culture results are obtained slowly, but a rapid section technique for biopsy histopathology can be performed in as little as 4 h *(131)*.

Patients with known or suspected burn wound infection require aggressive care in an intensive care unit (ICU). Gram-negative infections are treated with topical application of mafenide acetate cream, institution of two broad-spectrum anti-pseudomonal intravenous antibiotics, and prompt subeschar injection (clysis) of one-half the daily dose of an anti-pseudomonal semisynthetic penicillin (such as piperacillin), diluted in a volume appropriate for the size of the involved wound. Clysis is repeated 8–12 h later, and the wound is then excised to fascia to remove all infected tissue. Fungal burn wound infections are treated with intravenous amphotericin B and wide excision. Newer triazole antifungals such as voriconazole may also be useful.

Other Infections

The declining incidence of burn wound infection has heightened the importance of other infections, especially pneumonia and bacteremia,

in burn patients. Postinhalation injury pneumonia typically develops beginning 5–6 d post burn, whereas pneumonia in patients without inhalation injury presents somewhat later in the hospital course *(132)*. Bacteremia in burn patients may be particularly common during burn wound manipulation. Mozingo et al. *(133)* documented a decrease in bacteremia between studies published in 1979 and 1997, reflecting advances in wound care, earlier excision, and improved patient isolation. Bacteremia was seen only in patients with burns over 40% TBSA and was less common during the first 10 postburn d than thereafter *(133)*.

Infection Control

In addition to effective topical antimicrobial therapy, other factors have contributed to the reduction in infection-related mortality in burn patients. Redesign of the U.S. Army Burn Center in 1983 to permit isolation of patients with extensive wounds and increased emphasis on hygiene were associated with reductions in mortality and bacteremia and with the elimination of endemic strains of multidrug-resistant *Providencia* and *Pseudomonasn (134)*. Furthermore, the increased mortality previously associated with bacteremia in the open ward was not observed following conversion to an isolation ward *(135)*. This environmental change was also associated with a striking further reduction in the incidence of bacterial burn wound infection, whereas the incidence of fungal burn wound infection remained constant *(136)*.

The authors' current approach to infection control and surveillance is as follows. Thrice-weekly cultures of wounds, sputum, urine, and stool are obtained. These surveillance cultures are used in part as a quality-control tool in the ICU, to ensure that patient isolation is maintained and to enable rapid identification of cross-contamination with resistant organisms if it is not. Furthermore, when infection is diagnosed, antibiotics are selected based on existing surveillance data. Antibiotic choice is refined based on cultures obtained at the time of diagnosis of infection. Prophylactic antibiotics are not used, except perioperatively.

Surgery

The surgical treatment of the wound is, likewise, a critical element in reducing the incidence of infection and improving the survival of patients with extensive thermal injury. Traditionally, wounds were grafted once the eschar had been removed by daily debridement and

spontaneous separation and granulation tissue had formed. This method gave way in the early 1980s to tangential excision of deep partial-thickness burns and excision to fascia of extensive full-thickness burns. The authors' current practice is to make every effort to excise all full-thickness eschar within 7 d of injury in extensively burned patients. Beginning about 48 h after injury, staged operations are performed every day or two, during each of which 20% of the total body surface area is excised and grafted. In the absence of sufficient donor sites from which to obtain autologous skin grafts, biologic dressings such as cadaver allograft are used to close the excised wound temporarily *(137)*. Such dressings re-establish skin barrier function; in particular, they prevent desiccation-induced necrosis of the wound surface and prevent contamination of underlying tissue with exogenous bacteria. In areas of allograft adherence, wound bacteria counts decrease. However, if allograft is placed over nonviable tissue, subgraft suppuration will occur, and the grafts will not adhere. Every biologic dressing should be inspected on a daily basis. If suppuration occurs beneath the grafts, they should be removed and replaced as frequently as necessary until suppuration ceases and the biologic dressing becomes adherent. Synthetic skin substitutes, which are more likely to develop submembrane suppuration than are naturally occurring biologic dressings, can be safely applied at the time of burn wound excision but should not be applied to wounds with retained nonviable tissue.

A recently developed temporary skin substitute, TransCyte™ (Smith & Nephew, London, UK), is based on a bilaminar product, Biobrane™ (Dow B. Hickam, Sugarland, TX), which consists of an inner nylon mesh and an outer silicone membrane bonded to the mesh. Human newborn fibroblasts are then cultured with this mesh, yielding various growth factors that reportedly hasten the healing of partial-thickness burns *(138)*. Other new skin substitutes are designed to replace the dermis permanently. One such product, Integra® (Integra LifeSciences, Plainsboro, NJ) consists of a dermal analog (collagen-glycosaminoglycan matrix) and a temporary epidermal analog (silicone). After vascularization of the dermal analog, the silicone layer is replaced by a thin split-thickness skin graft in a second operation. Another product, Alloderm® (LifeCell, Branchburg, NJ), employs decellularized and freeze-dried cadaver dermis, over which a thin split-thickness skin graft is placed at the same operation. These products develop a more functionally and cosmetically appealing dermal layer and accelerate

donor site regeneration; but there is an increased risk of subgraft suppuration. Cultured keratinocytes are another approach to wound closure for the extensively burned patient. Because of poor engraftment rates and long-term functional results in some studies *(139)*, recent research has focused on providing a combined dermal matrix with a cultured keratinocyte layer *(140)*.

CONCLUSIONS

Improvements in our understanding of the multisystem response to thermal injury and advances in the critical care of burn patients have only heightened the importance of a multidisciplinary team approach to burn care, and of continued research. Substantial work remains in areas such as reduction of resuscitation fluid requirements, prevention of lean body mass erosion, control of the systemic inflammatory response to injury, enhancement of immunocompetence, and rehabilitation of patients to productive lives. Meanwhile, the battlefield of the future will, as in the past, almost certainly challenge military providers with thermally injured casualties.

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12 Inhalation Injury

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INTRODUCTION

Approximately 15–30% of patients admitted to burn centers have sustained inhalation injury. The importance of inhalation injury is indicated by the fact that it independently increases the risk of death in burn patients over that predicted by age and burn size alone by up to 20%. Inhalation injury also increases the risk of pneumonia; in turn, pneumonia acts independently to increase the risk of death by up to 40%. These contributions to mortality risk are greatest at the midrange of age and burn size *(1)*. Inhalation injury is more common during fires that take place in enclosed spaces, such as residential and vehicular fires. Consequently, in combat casualties, inhalation injury is particularly common during armor engagements, shipboard fires, and military operations in urban terrain. As mentioned in Chapter 11 (on thermal injury), smoke inhalation injury was the leading diagnosis in patients hospitalized (37%) and in those treated and released (50%) following the attack on the World Trade Center on September 11, 2001 *(2)*. There is now growing concern that certain widely available toxic industrial chemicals may be used as weapons of opportunity by terrorists *(3,4)*. The inhalation of these compounds produces injuries similar in several respects to the inhalation of smoke and is discussed here as well.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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CLASSIFICATION

Inhalation injury can be classified anatomically as producing (1) injury to the upper airways, including the glottis; (2) injury to the lower airways and pulmonary parenchyma; and/or (3) systemic toxicity, by means of the inhalation of carbon monoxide, of cyanide, or of gases that cause methemoglobinemia. These three classes of inhalation injury frequently coincide. Upper airway injury may cause life-threatening airway obstruction soon after injury. If this process is properly managed, upper airway edema usually resolves without sequelae in a few days. Subglottic injury is typically a chemical injury rather than a thermal injury, caused by the inhalation of the toxic products of combustion. Direct thermal injury to the subglottic airway and parenchyma is rare because the airways exchange heat very efficiently; accordingly, such injury usually implies the inhalation of a gas with a high heat content such as superheated steam.

SMOKE

The composition of smoke is highly variable and depends on the nature of the burning material and on the temperature of the fire; it also changes over time during the course of the fire. Heat, toxic gases (carbon dioxide, carbon monoxide, and cyanide), and low ambient oxygen levels interact to cause death at the scene in structural fires. Several autopsy studies indicate that the toxic gas of greatest importance in fire deaths is carbon monoxide; these studies have found lethal levels of carboxyhemoglobin (COHb) in most nonsurvivors. By contrast, the true incidence of severe cyanide poisoning in fire victims is a matter of debate; this is because cyanide has a short in vivo half-life and can be either generated or consumed post mortem. In the review by Barillo et al. *(5)* of fire fatality data from the state of New Jersey, 195 of 433 casualties (45%) had lethal COHb levels (≥50%). Only 31 of 364 (8.5%) had lethal (>3 mg/L) cyanide levels, and only 8 of 364 (2.2%) had lethal cyanide levels but sublethal COHb levels *(5)*. Contrary findings were reported by Baud et al. *(6)* for Paris, France. There are data to support the concept that sublethal doses of carbon monoxide, combined with sublethal doses of cyanide, can act synergistically to produce increased toxicity *(7)*.

Other common toxic smoke constituents are aldehydes (including formaldehyde and acrolein), ammonia, hydrogen sulfide, sulfur diox-

ide, hydrogen chloride and fluoride, phosgene, nitrogen dioxide, and organic nitriles *(8)*. Furthermore, particulate material is an important component of smoke. Removal of solid-phase material from smoke, by means of filters with 0.3-μm-diameter pores, essentially eliminates the pathogenicity of smoke in some models *(9)*. Persistent free radicals are also present in smoke *(10)*. The origin and composition of smoke greatly influence the pathophysiology of the injury and the outcome of studies in this field *(11,12)*.

PATHOPHYSIOLOGY

Hubbard et al. *(13)* described the morphologic changes seen at several time points following subglottic exposure of sheep to various doses of smoke. Inhalation injury caused necrosis and sloughing of respiratory tract epithelium beginning 15 min after exposure. Less severe injury featured loss of cilia. With severe injury (high smoke doses), full-thickness ulceration of the epithelial surface was occasionally seen. Mucus production was increased by 12 h. Beginning 2 h after injury and peaking at 24 h, an acute inflammatory reaction, featuring neutrophilic infiltration into the airways, followed. Extensive pseudomembranes formed within the airways. Ultimately, these pseudomembranes nearly occluded the major airways of many sheep. Atelectasis developed distal to terminal airways obstructed by debris and edema. This was associated with subsequent bacterial colonization at 72 h, followed by pneumonia. Parenchymal changes were less prominent than airway changes. Alveolar edema was a delayed phenomenon, seen after 24 h. Vascular endothelial changes were not seen in this model. Electron microscopy revealed changes in type I and type II (surfactant-producing) pneumocytes.

Ventilation-perfusion (V_A/Q) mismatch is a principal intrapulmonary cause of hypoxia in a variety of conditions, including inhalation injury. Nieman et al. *(14)* identified abnormalities in V_A/Q matching during the immediate postinjury period in a unilateral inhalation injury model. Pulmonary vascular resistance increased in the uninjured lung but not in the injured lung. There was a gradual increase in blood flow to the injured lung, which reached significance at 2 h post injury. These findings suggest that mediators released by smoke inhalation cause vasoconstriction in uninjured segments, whereas other processes cause impairment of local vasoconstriction in injured lung segments *(14)*. In

ovine studies, Shimazu et al. *(15)* used the multiple inert gas elimination technique to rigorously define the effect of inhalation injury on V_A/Q distribution. Smoke caused time- and severity-related decreases in PaO_2. This hypoxia was associated with an increase in blood flow distribution to low V_A/Q compartments ($0 < V_A/Q < 0.1$), at the expense of blood flow to normal V_A/Q compartments ($0.1 < V_A/Q < 10$). The likely explanation for these changes is the airway obstruction observed histologically *(15)*.

Clinically significant pulmonary edema is infrequently seen in patients with inhalation injury during the first 48 h post burn. Elevations in the pulmonary vascular resistance, seen after inhalation injury as well as after cutaneous thermal injury, may protect against increases in pulmonary hydrostatic pressure. When present before 48 h post burn, such edema may indicate a more severe injury. Potential causes of pulmonary edema after inhalation injury include increased endothelial and/or alveolar permeability and increased hydrostatic pressure. In sheep, Herndon et al. *(16)* described increases in lung lymph flow (Q_L), lymph-to-plasma protein concentration ratio (C_L/C_P), and lung transvascular protein flux at 12 h post injury, consistent with an increase in lung endothelial permeability to protein. These findings were refined by studies in which the endothelial surface area was held constant by maximizing the pulmonary venous pressure *(17,18)*. In one such study, the contribution of hydrostatic pressure was also determined. Endothelial permeability increases were more prominent than pressure increases at 24 h, whereas the reverse was true at 48 h. Increased alveolar permeability to aerosolized radioactive tracers has also been documented *(19,20)*.

Atelectasis is a prominent feature of inhalation injury and is caused by small airway obstruction as well as surfactant depletion. By in vivo microscopy, Nieman et al. *(21)* observed focal atelectasis within minutes of wood smoke inhalation, even before significant cellular infiltration or bronchiolar obstruction. Most likely, this represented a smoke-induced increase in alveolar surface tension, representing rapid decreases in quantity and quality of surfactant. These authors also found that wood but not cotton smoke caused these changes in surface tension and that in vitro surfactant replacement restored surface tension following injury *(12)*. Indeed, immediate postinjury intratracheal instillation of calf surfactant but not synthetic surfactant improved static

compliance and PaO_2 in smoke-injured dogs. This difference probably reflects the role of the surfactant proteins, absent in the synthetic compound used, in decreasing surface tension *(22)*.

SECONDARY LUNG INJURY

A hematogenous route for secondary lung injury following smoke inhalation is probable. Unilateral smoke exposure caused increases in contralateral lung microvascular permeability *(23)*. Ablation of the bronchial circulation attenuated smoke-induced changes in pulmonary vascular resistance, lung lymph flow, endothelial permeability, wet-to-dry ratio, and oxygenation *(24)*.

Neutrophils contribute to secondary lung injury following inhalation injury *(16)*. Preinjury leukocyte depletion with nitrogen mustard attenuated smoke-induced changes in pulmonary artery pressure, pulmonary vascular resistance, pulmonary lymph flow, PaO_2/FiO_2 ratio, plasma conjugated dienes, and the consumption of antiprotease in lung lymph *(25)*. A synthetic antiprotease (gabexate mesilate), given post injury, had similar protective effects *(26)*. Tasaki et al. *(27)* evaluated the effect of Sulfo Lewis C, a putative ligand of E-selectins. Free radical production by granulocytes was increased after smoke exposure in both treated and untreated groups, but treatment improved oxygenation and reduced V_A/Q mismatch *(27)*. A monoclonal antibody against L-selectin attenuated late but not early increases in lung lymph flow *(28)*. In another study, this drug also preserved the PaO_2/FiO_2 ratio and the lung wet-to-dry ratio *(29)*. Antibodies to interleukin-8, a neutrophil chemotactic factor, decreased the permeability of the alveolar-capillary membrane to protein *(20)*. Pentoxifylline, an anticytokine agent that inhibits multiple steps in neutrophil-mediated inflammation, was evaluated by Ogura et al. *(30)* in the ovine model. Beneficial effects included improved oxygenation and V_A/Q matching; lower lung wet-to-dry ratios; and reduced levels of polymorphonudeai leukocytes (PMNs), protein, and conjugated dienes in bronchoalveolar lavage (BAL) fluid and levels of conjugated dienes in plasma.

Oxygen-derived free radicals are both found in smoke *(10)* and, more importantly, produced by neutrophils, macrophages, and other cells following injury. They have been extensively implicated in the pathophysiology of inhalation injury. For example, Demling et al. *(31)*

in a rat model found that the degree of lung lipid peroxidation (malondialdehyde levels) correlated with mortality, as did the degree of decrease in lung catalase levels. In sheep, these authors also noted increased lung lipid peroxidation following smoke inhalation, along with decreases in plasma catalase and glutathione levels. Thus, pulmonary injury decreases systemic levels of circulating antioxidants *(32)*. After smoke inhalation, free iron release, leading to increased hydroxyl radical production, may be a mechanism of oxidative injury *(33)*. Platelet-activating factor (PAF) acts via multiple pathways to activate neutrophils and platelets and to cause increased production of eicosanoids and free radicals. An antagonist of PAF prevented smoke-induced increases in blood, lung, and bronchoalveolar lavage fluid levels of malondialdehyde and reduced V_A/Q mismatching *(34)*.

Nitric oxide (NO) may play a role in secondary injury following smoke inhalation. In addition to its role as a vasodilator, NO serves as a chemotactic factor for neutrophils and combines with oxygen-derived species to form peroxynitrite. Treatment with N^G– nitro-L-arginine methyl ester (L-NAME) or induction of neutropenia reduced indices of oxidative injury and of lung permeability to I^{125}-albumin in a rat model of inhalation injury and systemic inflammation (peritonitis) *(35)*. Similarly, mercaptoethylguanidine (MEG; an inhibitor of inducible nitric oxide synthetase and a free-radical scavenger) reduced indices of oxidative injury and intrapulmonary shunt fraction in smoke-injured sheep *(36)*. In combined smoke and cutaneous thermal injury, MEG reduced microvascular permeability of lung but not of burned tissue *(37)*.

Eicosanoids contribute to the pathogenesis of V_A/Q mismatch and edema following inhalation injury. Levels of thromboxane B_2 (a metabolite of thromboxane A_2, a potent vaso- and bronchoconstrictor) were increased in tracheobronchial exudates and, to a lesser extent, in lung lymph. Levels of 6-keto prostaglandin $F_{1\text{-}\alpha}$ (a metabolite of prostacyclin, a vasodilator) were elevated in plasma and lymph *(38,39)*. In smoke models featuring high levels of acrolein, lipoxygenase inhibition decreased smoke-induced increases in lung lymph flow, lymph protein flux, and wet-to-dry ratios. This implicates leukotrienes in the pathogenesis of pulmonary edema following inhalation of acrolein *(11)*. Smoke also increased activity in the lung of phospholipase A_2 (PLA_2), the enzyme responsible for the production of the eicosanoid precursor arachidonic acid *(40)*.

Inhalation injury predisposes to pneumonia not only by causing small-airway injury, atelectasis, and ciliary impairment, but also by altering pulmonary immune function. Alveolar macrophages, obtained by BAL from smoke-injured sheep, demonstrated reduced phagocytosis and killing of *Pseudomonas aeruginosa* organisms following ingestion. Such macrophages also demonstrated reduced phagocytosis of apoptotic PMNs. PMNs incubated in media conditioned by smoke-exposed alveolar macrophages showed increased apoptosis rates *(41)*. Similarly, alveolar macrophages obtained by BAL from smoke-injured rabbits showed decreased adherence and decreased phagocytosis of opsonized bacteria. On the other hand, they showed increased basal production of superoxide and lipopolysaccharide (LPS)-stimulated release of tumor necrosis factor (TNF). Thus, smoke injury decreased essential functions while increasing potentially deleterious functions *(42)*.

SYSTEMIC EFFECTS OF INHALATION INJURY

Inhalation injury also affects other organs; for example, regional blood flow to the splanchnic organs is selectively decreased, independent of changes in cardiac output or systemic oxygen delivery *(43)*. Because severe inhalation injury and extensive cutaneous burns frequently coincide, the inter-relationship of the two injuries is clinically relevant. Patients with inhalation injury often require a larger fluid resuscitation than those without; for example, one group of patients resuscitated with the modified Brooke formula (which estimates 2 mL/kg/% burn for the first 24 h post burn) actually required over 5 mL/kg/% burn *(44)*. The addition of smoke injury to a 15–18% TBSA burn caused oxygen consumption to become delivery-dependent, without causing changes in oxygenation *(45)*. This combined injury model also featured an increase in fluid balance and in lymph flow in burn tissue, nonburn tissue, and lung *(46)*. The effect of the combined injury on oxygen consumption, fluid requirements, and positive fluid balance increased with increasing smoke dose *(47)*. A quantitative histologic scoring system of the extent of airway injury correlated well with these changes, indicating again the primary role of the airway in the pathophysiology of inhalation injury *(9)*.

Cutaneous thermal injury may, conversely, increase the extent of pulmonary dysfunction following smoke inhalation. Clark et al. *(48)* noted that 12% of patients with inhalation injury alone required endotracheal

intubation, versus 62% of those with both inhalation injury and burns. Tasaki et al. *(49)* studied the effect of a severe inhalation injury (carboxyhemoglobin level 90%) with or without a 40% full-thickness scald injury on pulmonary function in sheep. The smoke-plus-burn group demonstrated greater hemodynamic changes and lung malondialdehyde levels than the smoke-only group, but there was no difference during the 48-h study in PaO_2, lung wet-to-dry ratio, or V_A/Q mismatch *(49)*.

DIAGNOSIS

Early diagnosis of inhalation injury is important, to identify those patients who merit close intensive care unit (ICU) observation, prophylactic intubation, and transfer to facilities with special resources such as high-frequency percussive ventilation. Clinical findings in one series included facial burns (96% of patients), wheezing (47%), carbonaceous sputum (39%), rales (35%), dyspnea (27%), hoarseness (26%), tachypnea (26%), and cough and hypersecretion (26%) *(50)*. However, no one physical or historical finding is sufficiently sensitive or specific. Chest radiographs are obtained on admission in order to provide a baseline and to rule out other injuries, since they are initially normal in 92% of patients with inhalation injury *(51)*. Shirani et al. *(1)* reported an accurate logistic predictor of inhalation injury based on the presence or absence of closed-space injury, the presence or absence of facial burns, the burn size, and the age. In practice, though, flexible fiberoptic bronchoscopy is used to diagnose subglottic inhalation injury. Typical findings include erythema, soot, edema, and/or ulceration. This procedure also enables the direct evaluation of airway patency and permits awake endotracheal intubation over the bronchoscope. When the clinical scenario strongly supports the diagnosis of inhalation injury, it may be useful to repeat the study 24 h after injury, at which time restoration of airway perfusion and progression of inflammation may facilitate the diagnosis. $Xenon^{133}$ lung scanning can also be used in questionable cases. Pulmonary function testing, which demonstrates decreased peak flow and decreased flow at 25, 50, and 75% of vital capacity in patients with inhalation injury, can be used as a screening tool *(52)*.

TREATMENT

The most important early priority in the management of patients with inhalation injury is control of the airway. Although many mini-

mally symptomatic patients with inhalation injury can be safely watched in an ICU, prophylactic intubation of such patients prior to interhospital transport is usually recommended. Other indications for elective intubation include the presence of extensive cutaneous injury, with or without facial burns, since progressive edema formation during burn resuscitation places the airway at risk. This risk is greater in children under 2 yr of age, even in the absence of inhalation injury *(53)*. Because airway loss can be catastrophic in the edematous burn patient, it is exceedingly important to use a fail-safe method of securing the endotracheal tube, such as cotton ties (umbilical tape) rather than adhesive tape. Also, obstruction of the endotracheal tube by mucous plugs, debris, or clot may mandate emergency replacement (**Fig. 1**).

Approximately 19% of patients admitted to burn centers require intubation for longer than 24 h *(44)*. In the authors' practice, burn patients requiring prolonged translaryngeal intubation are converted to a tracheostomy after approximately 10–14 d. An earlier tracheostomy may be required to facilitate pulmonary toilet in patients who develop obstructing tracheobronchial casts and clots. Predischarge pulmonary function testing may be used to screen patients for upper airway sequelae of inhalation injury and prolonged intubation, with definitive diagnosis by fiberoptic bronchoscopy *(44)*.

The most important recent development in the care of patients with inhalation injury has been the introduction of high-frequency percussive ventilation by means of the volumetric diffusive respiration ventilator (VDR-4, Percussionaire, Sandpoint, ID). The VDR, in essence, superimposes a high-frequency waveform on a low-frequency waveform (**Fig. 2**). In 1989 Cioffi et al. *(54)* described the use of this ventilator as salvage therapy for patients with severe inhalation injury. Use of the VDR was associated with improved PCO_2 and/or PO_2 levels over those achieved with conventional ventilators. These authors then evaluated the VDR as prophylactic therapy. In comparison with recent historical controls, 54 smoke-injured patients treated with the VDR within 24 h of intubation experienced a halving of mortality and a near-halving of the pneumonia rate *(55)*. In a baboon model of inhalation injury, Cioffi et al. *(56)* compared VDR, high-frequency oscillatory ventilation, and conventional volume-controlled ventilation. The VDR group demonstrated a lower barotrauma index (versus the conventional group) and decreased parenchymal damage (versus both groups) *(56)*.

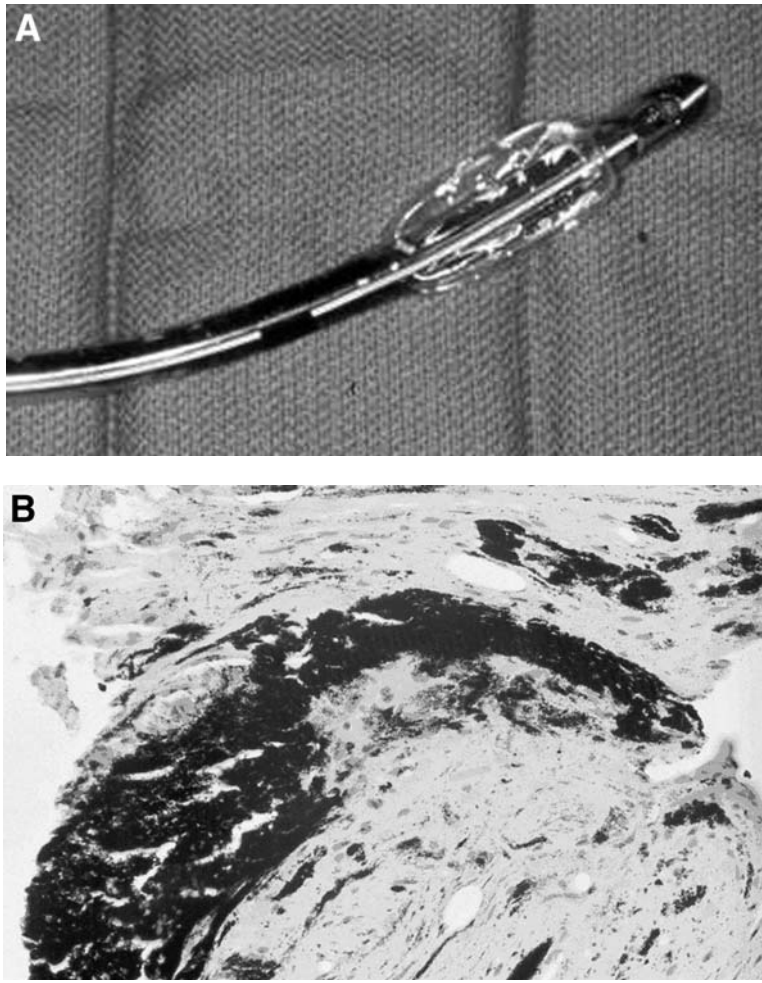

Fig. 1. (**A**) Endotracheal tube completely obstructed by tenacious mucous material and carbonaceous debris. (**B**) Photomicrograph of obstructing material.

Subsequent clinical reports have confirmed the VDR's ability to improve oxygenation and ventilation with less barotrauma *(57,58)*.

Bearing in mind that a primary pathophysiologic feature of inhalation injury is small airway obstruction, with resultant V_A/Q mismatch, atelectasis, and pneumonia, VDR ventilation probably recruits and keeps open these small airways *(56)*. In addition, high-frequency

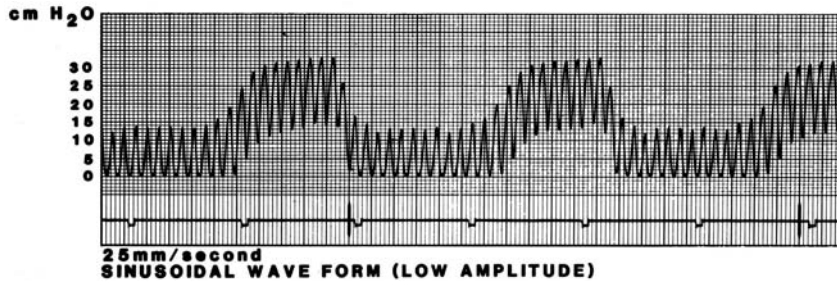

Fig. 2. VDR-4 high-frequency percussive ventilator: typical pressure-time waveform measured by a manometer at the endotracheal tube. The high-frequency breaths, at a rate of about 600/min, facilitate the clearance of secretions and debris, recruit and maintain collapsed alveoli, and improve gas exchange across the alveolar-capillary membrane. The low-frequency breaths, at a rate of about 10–20/min, accomplish bulk gas movement into and out of the lungs. (Reproduced with permission from Percussionaire, Inc., Sandpoint, ID.)

molecular motion probably enhances gas exchange, and the percussive effect enhances clearance of secretions and debris. Thus, the VDR is employed prophylactically by the authors for all patients with inhalation injury.

Another method commonly employed to limit barotrauma in burn patients with lung failure is permissive hypercapnia *(59)*. Partial correction of the blood pH by infusion of a sodium bicarbonate solution, unless contraindicated on the basis of hypervolemia, is performed as needed and may attenuate certain effects of hypercarbia such as increased intracranial pressure *(60)*.

Two porcine studies employing perfluorocarbon for partial liquid ventilation (PLV) following inhalation injury have yielded differing results. In a 24-h study in which the perfluorocarbon (LiquiVent™, Alliance Pharmaceuticals, San Diego, CA) was instilled immediately after injury, PLV was associated with improved compliance, oxygenation, and survival compared with conventional ventilation *(61)*. On the other hand, in a 72-h study in which the drug was instilled 2 or 6 h after injury, opposite results were obtained *(62)*. Thus, a clinical role for PLV following inhalation injury remains to be defined.

Inhaled β_2-agonists are used as needed in patients with inhalation injury who develop bronchospasm. Inhaled dexamethasone and inhaled gentamicin demonstrated no benefit following inhalation injury in clin-

ical trials and are to be avoided *(63)*. By contrast, inhaled heparin, by reducing the formation of obstructing small and large airway casts, may improve survival following smoke inhalation. Nebulized heparin, nebulized dimethylsulfoxide (DMSO; a free-radical scavenger), or both were evaluated in the ovine model. In this small study, the two-drug combination reduced mortality over that of controls *(64)*. Also in the ovine model, intravenous heparin (titrated to an activated clotting time of 250–300 s) reduced airway cast formation and barotrauma and improved oxygenation and lung wet-to-dry ratios. There was no reduction in pulmonary leukosequestration or in plasma or lung conjugated diene levels, suggesting that heparin acted primarily as an anticoagulant rather than an antiinflammatory agent in that setting *(65)*. In a subsequent clinical study in children with inhalation injury, the combination of nebulized heparin alternating with nebulized *N*-acetylcystine reduced mortality and reintubation rates in comparison with historical controls *(66)*. The authors currently use inhaled heparin in smoke-injured patients with potentially obstructing clots and casts; systemic anticoagulation is rarely observed during this therapy *(67)*.

Inhaled NO improves blood flow to well-ventilated lung segments; in the ovine model, this drug produced a statistically significant but clinically modest improvement in oxygenation. Also, the pulmonary vascular resistance decreased without a significant improvement in the cardiac output *(68)*. Several small trials of inhaled NO in burn patients have likewise suggested a slightly beneficial effect of inhaled NO *(69,70)*.

In small case series, extracorporeal membrane oxygenation (ECMO) has been employed for pediatric burn patients with severe lung failure *(71,72)*. In one series, hemorrhage from unexcised burn wounds characterized nonsurvivors, whereas survivors tended to have developed lung failure after successful excision and grafting. Because of the technical difficulty and potential complications associated with ECMO, Zwischenberger and colleagues *(73)* have developed a method of total arteriovenous CO_2 removal ($AVCO_2R$). By means of a low-resistance oxygenator, the use of a pump is avoided *(73)*. Typically it is possible to remove 95% of total CO_2 production and thus reduce greatly minute ventilation and peak airway pressures. Even so, oxygenation (PaO_2/FiO_2 ratio) is typically improved *(74)*. Flow through the $AVCO_2R$ circuit is about 12% of cardiac output and is well tolerated in these animals. An

improvement in survival has been achieved in sheep *(75)*, and initial clinical experience has been reported *(76)*.

CARBON MONOXIDE

Carbon monoxide (CO) is produced by the incomplete combustion of many fuels, especially cellullosics such as wood, paper, and cotton *(77)*. The predominant toxic effect of CO is its binding to hemoglobin to form COHb *(78)*. This makes hemoglobin unavailable for the transport of oxygen. In addition, COHb shifts the oxygen-hemoglobin dissociation curve to the left and makes its shape less sigmoidal and more hyperbolic (the Haldane effect). Thus, oxygen bound to hemoglobin does not dissociate as readily at the capillary level. Finally, CO binds to hemoglobin 200–250 times more avidly than does oxygen *(79)*. These changes cause CO hypoxia, that is, reduced delivery of oxygen to the tissues. The most vulnerable tissues include the central nervous system and the heart. Evidence in support of this concept was provided by a study in which total body asanguinous hypothermic perfusion, by removing all of the COHb, resuscitated comatose dogs in 20 min *(80)*.

In addition, CO binding to intracellular cytochromes and to other metalloproteins (such as myoglobin) probably contributes to CO toxicity. Such extravascular binding accounts for 10–15% of total body CO stores and may explain the two-compartment pharmacokinetics that have been observed for COHb elimination *(81)*. That COHb alone is not responsible for the toxic effects of CO was suggested by the persistence of CO-induced visual-threshold changes, despite normalization of COHb levels. Also, animals were bled and then transfused with COHb-poisoned red blood cells to a mean COHb level of 64%, with no ill effect *(82,83)*. Chance et al. *(84)* used spectrophotometry to document the binding of CO to mitochondrial cytochrome c oxidase (cytochrome a,a_3, the terminal cytochrome on the electron transport chain). Brown and Piantadosi *(85)*, using transcranial spectrophotometry of the intact brain, demonstrated in vivo binding of CO to cytochrome c oxidase, and the reversal thereof by oxygen at 3 atmospheres.

Despite the elimination of COHb from the blood by oxygen therapy, recovery of phosphocreatine levels, intracellular pH, and cytochrome c oxidase oxidation levels was delayed *(86)*. Cytochrome c oxidase (complex IV) activity in lymphocytes of patients was inhibited by CO

poisoning both acutely and several days after elimination of COHb from the blood *(87)*. Electron transport chain dysfunction, induced by CO binding, may also cause leakage of electrons, the generation of superoxide, and mitochondrial oxidative stress; restoration of tissue oxygenation may in addition cause a reoxygenation injury *(88)*. Coburn et al. *(89,90)* have demonstrated that CO also binds to myocardial and skeletal muscle myoglobin, which may impede transport of oxygen within muscle cells.

Patients with CO exposure present with findings referable to the central nervous and cardiovascular systems. These range in severity from mild constitutional symptoms, through compensatory tachycardia and tachypnea, to coma, seizures, dysrrhythmias, myocardial ischemia, and hypotension. Classic findings of cherry-red lips, cyanosis, and retinal hemorrhages are, in fact, rarely seen *(91)*. Patients with pre-existing cardiac or pulmonary disease may be particularly vulnerable to CO poisoning *(92)*. The fetus is also vulnerable, since affinity of fetal hemoglobin for CO is greater than that of adult hemoglobin *(93)*. A delayed neuropsychiatric syndrome, with onset 3–240 d after exposure, has been observed. It consists of cognitive and personality changes, parkinsonism, incontinence, dementia, and/or psychosis; spontaneous resolution occurs in many *(91,94,95)*.

Pulse oximeters fail to distinguish between oxyhemoglobin and COHb and will give falsely high SpO_2 readings in patients with CO poisoning. Arterial blood gas analyzers, which estimate the SO_2 based on measurement of the dissolved PO_2, also fail to detect elevated levels of COHb. Thus, diagnosis of CO poisoning requires the direct determination of COHb levels in arterial (or venous) blood by cooximetry. Magnetic resonance imaging of the brain, and other modalities, have been used to evaluate symptomatic patients; common findings include lesions of the globus pallidus and other basal ganglia, deep white matter, and cerebral cortex *(96–98)*.

For adult males, the half-life of COHb at room air is 240 min, with 100% oxygen at 1 atmosphere (atm) it is 47 min, and with 100% oxygen at 2.5 atm it is 22 min. The half-life for females is about 30% shorter at each pressure *(99)*. Accordingly, the mainstay of treatment of CO poisoning is 100% oxygen until levels are below 10–15% and, if clinically indicated, mechanical ventilation. The additional decrement in half-life gained by the use of hyperbaric oxygen has been the primary rationale for its use. The ability of hyperbaric oxygen to acceler-

ate the dissociation of CO from cytochrome a,a_3 and to increase tissue PO_2 despite impaired hemoglobin function is also cited. However, there are essentially no well-designed trials of this therapy. Also, hemodynamically unstable burn patients are at increased risk of significant complications during hyperbaric oxygenation, such as progressive hypovolemia, seizures, aspiration, and so on, and it may be impossible to provide adequate critical care inside the chamber *(95)*. Given the efficacy of 100% oxygen at 1 atm, the risks of hyperbaric oxygenation often outweigh the benefits for patients with CO poisoning and extensive thermal injury—particularly in the absence of an in-house chamber. Normobaric, normocapnic hyperventilation with 4.5–4.8% CO_2 in O_2 (carbogen) also accelerates clearance of CO and has been recently reintroduced by one group *(100)*.

CYANIDE

Cyanide (CN) is produced during the combustion of synthetics such as plastics, foam, varnishes, and paints and of some natural fibers such as wool and silk *(101)*. Cyanide binds to cytochrome c oxidase, at a site distinct from that binding CO. This produces dose-dependent inhibition of mitochondrial electron transport. Clinical findings involve the central nervous, cardiovascular, and respiratory systems and include dyspnea, tachypnea, vomiting, bradycardia, hypotension, coma, and seizures. The diagnosis of CN poisoning is difficult because no rapid assay is widely available, although several have been described recently. According to Baud et al. *(6)*, in patients with smoke inhalation but without burns, an elevated lactate level (10 μmol/L) demonstrated a sensitivity of 87% and a specificity of 94% for toxic cyanide levels (40 μmol/L). Also, an elevated mixed venous saturation, indicative of a failure of mitochondrial oxygen utilization, is suggestive of cyanide toxicity but is not universally seen.

Cyanide is metabolized by hepatic rhodanese, which catalyzes the donation of sulfur from the sulfane pool to cyanide to form nontoxic thiocyanate. The half-life of cyanide in humans is about 1–3 h *(6,102)*. There are three approaches to the treatment of cyanide poisoning. Amyl and sodium nitrite oxidize hemoglobin to methemoglobin, which chelates cyanide to form cyanmethemoglobin. By causing methemoglobinemia, these drugs reduce oxygen-carrying capacity; they are also vasodilators and can cause hypotension *(103)*. Thus, they should be

used with caution in patients with concomitant CO poisoning or burn shock. Sodium thiosulfate provides sulfur for the detoxification of cyanide by rhodanese and is free of significant side effects. Finally, hydroxocobalamin is a cyanide chelator without significant side effects; unfortunately, it is not currently available in the United States in the doses required for cyanide therapy *(101)*. The authors' current practice is to consider the use of sodium thiosulfate for patients with smoke inhalation who have persistent, unexplained metabolic acidosis despite adequate fluid resuscitation and cardiopulmonary support *(5)*.

ACQUIRED METHEMOGLOBINEMIA

Inhalation of gases containing strong oxidants such as nitrogen dioxide (NO_2), NO, or benzene can cause oxidation of one of the four iron moieties in each hemoglobin molecule from its usual state, Fe^{2+}, to Fe^{3+}. This forms methemoglobin (MetHb). Fe^{3+} is incapable of carrying oxygen; also, the affinity of the remaining Fe^{2+} moieties for oxygen is greatly increased, shifting the oxygen-hemoglobin dissociation curve leftward and impairing oxygen delivery. Methemoglobinemia can also be caused by the use of nitrites as a treatment for cyanide poisoning (see Cyanide section above), by inhaled NO therapy, or by a variety of drugs and other chemicals.

The diagnosis is suggested by central cyanosis and by blood that, when placed on filter paper, appears chocolate brown in color. At MetHb levels of 30% or greater, symptoms of cardiac and neurologic dysfunction are seen. The PaO_2 may be normal, and pulse oximeters provide readings no lower than about 85% in patients with high levels of MetHb. Thus, co-oximetry is required for diagnosis, with confirmation by the specific Evelyn-Malloy test. Treatment is methylene blue (1–2 mg/kg by i.v. injection every 30–60 min up to a maximal dose of 7 mg/kg), which serves as an electron donor for NADPH-dependent MetHb reductase. Subsequent co-oximetric determination of MetHb levels may be inaccurate because of similarities in the MetHb and methylene blue spectra. This therapy should not be used in patients with glucose-6-phosphate dehydrogenase deficiency *(104, 105)*. Consultation with a poison control center is recommended to assist in management.

TOXIC INDUSTRIAL CHEMICALS

Because of their widespread availability and high toxicity, there is growing concern that toxic industrial chemicals may be used as weapons by terrorists *(3,4)*. For several of these agents, inhalation injury is an important mechanism of action; there are both similarities and differences in the injuries induced by each of these agents and that induced by smoke; more research is needed on both pathophysiology and treatment.

Chlorine

Chlorine gas (Cl_2) is used abundantly in industrial processes, was used extensively as a weapon during World War I, and causes death within minutes at concentrations of 1000 ppm (0.1%) or greater. Cl_2 dissolves in water to produce hydrochloric (HCl) and hypochlorous (HOCl) acids; all three species participate in pathogenicity. Intracellularly, chloride ions react with a variety of functional groups; chlorine may generate free oxygen radicals as well *(106,107)*. Gunnarson et al. *(108)* developed a porcine model of graded Cl_2 inhalation injury that featured, in the severe group, progressive hypoxemia, pulmonary artery hypertension, and decreased compliance. Histologic features 6 h after injury included sloughing of the bronchial epithelium and early white blood cell infiltration but largely intact alveoli. Also in swine, these authors demonstrated an improvement in cardiopulmonary function 6 h after injury in animals treated immediately with nebulized corticosteroids *(109)*. However, these findings have not been replicated in longer term studies.

Phosgene

Phosgene ($COCl_2$) is another gas used during World War I as a chemical weapon *(110)*. Inhalation produces severe pulmonary edema, characterized by oxidative stress and an influx of neutrophils into the lung. A delay in onset of 6–24 h between inhalation and onset of pulmonary failure is frequently described. Antiinflammatory treatment with agents such as ibuprofen *(111)* and colchicine *(112)* were effective in reducing pulmonary edema in animal models.

Hydrogen Sulfide

Hydrogen sulfide (H_2S) enters the bloodstream rapidly, binding to hemoglobin, myoglobin, and, most importantly and analogously to cyanide, cytochrome c oxidase *(113)*. Sudden inhibition of brainstem

mitochondrial function can cause sudden loss of consciousness (a phenomenon known as knockdown); other findings may include seizures and myocardial ischemia. The gas also causes alveolar damage and pulmonary edema, as well as keratoconjunctivitis (gas eye). Treatment is primarily supportive, although some authors have claimed a role for nitrite or hyperbaric therapy *(114)*.

Ammonia

Anhydrous ammonia (NH_3) is a gas that, when transported under pressure, assumes a liquid state. It causes cutaneous, ocular, and pulmonary injuries. Upon dissolution in water, it forms ammonium hydroxide, a strong base; in addition, it is stored at cold temperatures (–28°F) such that cold injury is often superimposed on a strong alkali burn. When inhaled, ammonia can rapidly produce laryngeal injury and obstruction; upper tracheobronchial mucosal necrosis with sloughing; and severe pulmonary edema. Damage of type I alveolar epithelial cells, manifested by marked edema, has been described *(115)*. Treatment of the pulmonary injury is primarily supportive, whereas the cutaneous and ocular burns must be treated with early and copious irrigation with water *(116,117)*.

CONCLUSIONS

Smoke inhalation injury is a threat to military personnel, particularly those injured in enclosed spaces such as ships, armored fighting vehicles, and buildings. Terrorist attacks employing conventional fuels and explosives or toxic industrial chemicals place the civilian population at risk as well. Despite dramatic recent improvements in survival, smoke inhalation injury continues to exert an additive effect on the morbidity and mortality of patients with cutaneous burns.

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13 Traumatic Brain Injury

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INTRODUCTION

Traumatic injury to the central nervous system (CNS) accounts for a significant proportion of combat casualties *(1,2)* as well as civilian trauma *(3)*. Among those casualties reaching medical care, neurotrauma victims comprise a substantial fraction of the combat fatalities *(2)*. In the United States, trauma is the most common cause of death and permanent disability, with neurologic injuries accounting for most of this morbidity and mortality *(3,4)*. These injuries mostly consist of blunt and penetrating trauma to the head and spinal cord. Because of the complexity and delicacy of the nervous system, injuries of this type remain the most perplexing to treat, and very often, despite impeccable management, the outcome is disappointing. The human nervous system is an unforgiving entity, and insults to its integrity, whether through direct trauma or the sequelae of metabolic aberrancy, often result in irreversible dysfunction. For these reasons many treatment providers regard the nervous system as overwhelmingly daunting and are timorously apprehensive when medical intervention is required. This chapter focuses on brain injury caused by trauma and includes discussions on its epidemiology, pathophysiology, diagnosis, treatment, and prognosis.

EPIDEMIOLOGY

Neurologic trauma is the leading cause of mortality and disability sustained by young civilians (aged 15–44 yr) as well as by combatants

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(3). In the United States in 1990 nearly 150,000 people died of acute traumatic injury, a figure that accounts for about 8% of all deaths that year *(5)*. Although it is difficult to determine what proportion of the acute traumatic mortality is caused by neurologic injury, some studies suggest that the figure is approximately 50% *(6)*. One study cites a brain trauma mortality of 50,000 in 1992, with 44% of these deaths attributable to firearms and 34% to motor vehicle crashes *(7)*. The significantly higher number of deaths involving firearms attests to the fact that, among civilians, firearm-related brain injuries cause a fatality of more than 80% *(8)*; the Wound Data and Munitions Effectiveness Team (WDMET; Vietnam) suggests that the fatality from bullet wounds to the head made by assault rifles is 95% *(2)*. Most firearm victims expire either at the scene or en route to the hospital *(9)*.

The precise mortality figure owing to neurotrauma is obviously very difficult to attain, as the immediate cause of death in a multitrauma victim is often multifaceted. Many victims die at the scene without any medical evaluation. Examination of the brain injury epidemiologic literature reveals an apparent marked variability and inconsistency in patient outcome among studies. Some studies consider all head injuries (including scalp lacerations and skull fractures), whereas others do not attempt to account for the multitrauma victims who were pronounced dead at the scene. Thus it is only possible to formulate a best estimate of the morbidity and mortality in multiple trauma attributable to neurologic injury. The incidence of traumatic brain injury (TBI) ranges from 132[10] to 367[11]/100,000/yr, with a mortality range of 14–30/100,000/yr *(5,6,10–14)*.

On the battlefield, neurologic injuries account for a significant proportion of the morbidity and mortality as well. During Operation Desert Storm, injuries to the head accounted for 20% of U.S. Marine casualties requiring evacuation *(1)*. A study on the war in Croatia from 1991 to 1992 reported that more than 10% of the wounded persons treated at a regional hospital facility had injuries to the head *(15)* and that their mortality rate was nearly 40% *(16)*. It is important to note that demographic data from recent wars on the specific causes of bodily injury in war (i.e., gunshot wounds versus bomb fragments) and the victimized populations (i.e., combatants versus civilians) vary greatly *(15,17)*. Thus it is difficult to draw conclusions and assemble general statements regarding injury in wartime. Furthermore, there is a general

consensus that the evolution of war fighting is undergoing a major paradigm shift. Future wars will probably involve weapons capable of greater lethality and mass destruction as well as highly technical military hardware designed to cripple populations as opposed to individuals. However, the literature continues to support the contention that head injury will account for a significant proportion of combat morbidity and mortality.

For patients who survive to the hospital or higher echelon treatment facility, there has been a significant decline in mortality over the last few decades. About 30 years ago, nearly half of all patients who suffered a TBI died, whereas more recent reports show TBI mortality to be closer to 25% *(18,19)*. Even more encouraging is the fact that this decline in mortality from TBI has come without a corresponding increase in the proportion of brain-injured persons remaining in a severely disabled or vegetative state *(20)*.

Most experts in head injury feel that these improved patient outcomes are largely attributable to better understanding of neurotrauma and especially to treatments focused on optimizing physiology *(20)*. In other words, many aspects of modern medical and surgical care are contributing to better outcomes in TBI, with the optimization of physiology in the head-injured patient credited for most of the improvement. Additional specific, yet extremely significant, advances in head trauma management include the advent of computed tomography in the 1970s, dramatic improvements in the emergency medical system (EMS) in the 1980s, and the establishment of uniform and consistent management guidelines in the 1990s. The institution of management guidelines was a significant contribution to TBI outcome improvement because it provided a consistent set of recommendations for all practitioners of trauma management, thus bringing even the smaller medical centers up to standard.

PATHOPHYSIOLOGY AND CLASSIFICATION OF BRAIN INJURIES

The CNS is injured either directly or indirectly. Direct injuries consist of blunt or penetrating insults, and indirect injuries are the result of acceleration, deceleration, and rotational forces. In the combat zone, the overwhelming majority of TBI battle casualties involve penetrating

missile wounds that result either from explosive munitions or from bullets fired by small arms *(2)*. Gunshot wounds account for nearly all penetrating injuries to the brain in civilian populations *(21)*, whereas shrapnel accounts for a significant proportion of penetrating head injuries in combatants *(1)*. The consequent injury from blunt trauma to the head is usually from indirect acceleration, deceleration, or rotational forces applied to the brain within the rigid cranial vault as the brain impacts on the inner surface of the skull. Blunt injuries that occur in the combat zone are usually caused by accidents and not hostile action *(2)*.

The current understanding of TBI broadly categorizes the ensuing nervous system lesions that result from both direct and indirect forces as either primary or secondary neuronal injuries *(19)*. Primary neuronal injury is the immediate damage resulting from insults to nervous tissue. At worst, the destruction is instantaneous, irreversible, and usually considered untreatable. Obviously, preclusion or minimization of the injury in the first place through the employment of better helmets, armor, and safety standards is the only way to mitigate primary neuronal injury.

Secondary neuronal injury begins minutes to days after the primary injury; it may be potentially prevented or at least attenuated *(19)*. In fact, there is good evidence demonstrating that *most* of the subsequent nervous tissue dysfunction after TBI is the manifestation of secondary neuronal injury and not the result of the immediate impact. In other words, initial damaging forces do not usually bring about sweeping structural degeneration of neurons, glial cells, or axons *(20,22–24)*, rather, this damage follows after the primary injury and is thought to be mediated by processes such as excitotoxicity, ischemia, membrane dysfunction, cellular transport aberrancies, and ionic gradient disruption *(20)*. **Table 1** summarizes the processes that lead to secondary neuronal injury. It is these components of secondary injury in the early period following TBI that novel therapies will target, with the goal of ameliorating brain dysfunction.

A better understanding of the pathophysiology of traumatic brain injury has contributed significantly to the increased survival of head injury patients. Although a detailed discussion of the physiologic processes involved in traumatic brain injury is beyond the scope of this chapter, some physiologic concepts warrant attention. For example, it is

Table 1
Processes and Factors Leading to Secondary Posttraumatic Brain Damage

Intrinsic
Mass lesion, brain shift, herniation
Intracranial hematoma (EDH/SDH/ICH/SAH)
Focal brain swelling
Cerebral ischemia
Reduced cerebral perfusion pressure
Decreased mean arterial pressure
Intracranial hypertension
Pyrexia
Hypoxemia/anemia
Cerebral vasospasm
Posttraumatic seizures
Infection
Hyponatremia
Extrinsic or iatrogenic
Inadequate resuscitation of circulatory shock
Inadequate oxygen delivery
Over-hyperventilation
Anesthetic agents, alcohol, other drugs
Nosocomial infections

EDH, epidural hematoma; SDH, subdural hematoma; ICH, intracranial hemorrhage; SAH, subarachnoid hemorrhage.

Modified from refs. *20* and *30*.

generally accepted that the deleterious effects of head injury are exacerbated when other physiologic parameters in the body cannot be maintained *(25,26)*. It is critically important to maintain hemodynamic stability following a head injury *(26)*. As cerebral perfusion pressure (CPP) is directly related to mean arterial pressure (MAP) as well as cerebral blood flow (CBF), adequate brain perfusion is maintained by ensuring that mean arterial pressure is sufficient. The current general guidelines mandate that systolic blood pressure be maintained at or above 120 mm Hg (MAP > 90 mm Hg) *(19)* and never fall below 70 mm Hg *(25)*. However, the other critical CPP parameter in is intracranial pressure (ICP), as shown in the following well-known equation:

$$CPP = MAP - ICP$$

The concept of CPP is only significant in that it provides an estimation of the most critical parameter in nervous system physiology, CBF. Because CPP is directly proportional to CBF and because it can be readily measured at the bedside, it provides for a useful mathematical estimation of CBF. The relation of CPP to CBF is shown by the following formula:

$$\text{CBF} = k\,(\text{CPP} \times d^4)/8lv$$

where k is a constant, d is the diameter of the blood vessel, l is the length of the blood vessels (which is practically constant), and v is the blood viscosity *(27)*.

The vasculature of the central nervous system is exquisitely sensitive to alterations in cerebral blood flow and through *autoregulation* is able to adapt and modify over a wide range of changes in ICP and MAP *(28–30)*. This ability to autoregulate maintains the CBF within an acceptable range under most conditions and CPP at 50–150 mmHg. However, under extreme conditions (i.e., after a TBI in which ICP may be markedly elevated), autoregulation often becomes impaired. Localized ischemia secondary to inadequate CBF or even regional hyperemia may ensue. Insufficient CBF after severe head injury is associated with a poor prognosis *(31,32)*. Additionally, any episode of hypoxia has been shown to worsen the outcome following a head injury *(25,33)*. One study demonstrated that mortality rates in patients with hypoxia or hypotension doubled compared with patients with similar demographics, incidence of intracranial hematoma, and frequency of intracranial hypertension *(34)*. Finally, it is well recognized that pyrexia is detrimental following TBI. However, many studies have failed to show that therapeutic hypothermia is beneficial as a treatment in TBI and may even be associated with increased complications *(35–37)*.

Types of Head Injury

Several distinct injuries result from trauma to the brain. These mainly consist of skull fractures and intracranial hemorrhage. The brain is a viscoelastic substance, and injuries result when it accelerates or decelerates within the rigid cranial vault and impacts on the inner surface. The various types of intracranial bleeding include subdural hematoma, epidural hematoma, intraparenchymal hemorrhage or contusion, and traumatic subarachnoid hemorrhage. Any type of head trauma can bring about any of these intracranial injuries. In general,

subdural hematoma is the most common traumatic intracranial bleed, accounting for approximately 50% of admissions for head injury; epidural hematoma accounts for about 1–3% of such admissions *(38,39)*. When there is an associated skull fracture, especially at the temporoparietal junction, then the incidence of epidural hematoma tends to increase, usually because of disruption of the middle meningeal artery as it exits its bony groove at the pterion *(38,39)*.

Gunshot wounds in civilian and military populations tend to differ based on dissimilarities in the inflicting weapon. Civilian injuries typically involve low-velocity bullets fired at close range, whereas military injuries tend to be either low-velocity, high-mass shrapnel injuries or high-velocity, variable-caliber, long-range bullet wounds *(21,40)*. Because the amount of energy imparted into the brain from a gunshot wound is directly proportional to the velocity of the bullet, higher velocity weapons cause greater brain damage and thus higher mortality *(41,42)*.

DIAGNOSIS AND GRADING OF HEAD INJURY

History and physical examination remain the cornerstone of initial evaluation of head injury. However, more so than in most other injuries, radiologic evaluation plays a crucial role in quickly determining the course of action in head trauma management. Evaluation of the trauma victim begins with standard initial management protocols as prescribed by the advanced trauma life support (ATLS) guidelines *(43–45)* (*see* Treatment section below for a more detailed discussion).

After the patient is stabilized, a thorough neurologic exam begins with a determination of the level of consciousness and the mental status. A change in consciousness is indicative of dysfunction of both cerebral hemispheres and/or the reticular activating system in the brainstem. Neurologic examination continues with an assessment of the cranial nerves, with particular attention to the optic and oculomotor nerves, especially if the patient is unconscious. A pupillary defect is indicative of an optic nerve injury, and dilated pupils either unilaterally or bilaterally signify ominous neurologic injury most likely caused by brain herniation *(46)*. Examination of the cranial nerves is followed by motor function, sensory, and gait assessment if the patient can cooperate with the exam or if other injuries do not preclude these assessments.

The diagnosis of TBI portends a highly variable outcome. On the one hand, a mild brain injury or concussion may have little or no long-term consequences, whereas the most severe of head injuries will lead to death or persistent vegetative state.

The most widely used classification scheme for grading head injury is the Glasgow Coma Scale (GCS); *see* **Table 2** and refs. *39*, *42*, and *47*). Originally described in 1974, the GCS assigns a neurologic function score based on evaluation of the patient's ability to open the eyes, follow commands, or speak. Although a great deal of controversy exists regarding its reproducibility and interobserver variability, the GCS has withstood the test of time and remains one of the most reliable measures for assessment of head trauma *(39)*. The categorization of head injury severity breaks down to minimal [GCS = 15 with no loss of consciousness (LOC) or amnesia], mild [GCS = 14 or 15 with either brief LOC (<5 min) or impaired alertness or memory], moderate (GCS = 9–13 or LOC > 5 min or focal neurologic deficit), severe (GCS = 5–8), or critical (GCS = 3–4) *(39)* The GCS has been most useful in predicting outcome based on the initial postresuscitative GCS score *(38,42,48–51)*, but it also provides the added benefit of serving as a quantitative metric of how a patient is faring (i.e., getting better or worse) and can therefore be used to follow the neurologic course.

Our group has created a novel neurotrauma scoring system specifically designed for the combat field medic, who often has only a rudimentary medical background and who is expected to function under the austere conditions of the battlefield. This system, the Neurotriage Scale, provides a score based on the presence or absence of scalp laceration, clinical skull fracture, disorientation, limb paralysis, and abnormal pupillary exam. In a retrospective review of charts at a level I trauma center, we demonstrated that the Neurotriage Scale correlates significantly with length of stay, abnormal radiologic findings, and unfavorable prognosis *(52)*.

For minor head injuries, the GCS is usually too insensitive. Minor head injuries, often referred to as concussion [defined as a temporary alteration in mental status *(53)*], are better classified by the American Academy of Neurology's system for concussion grading *(54)*. This system classifies concussion into three grades: grades 1 and 2 demonstrate confusion without LOC that resolves within or lasts longer than 15 min, respectively; grade 3 concussion is marked by any loss of consciousness *(54)*. Undue emphasis should not be placed on grading of concus-

Table 2
Glasgow Coma Scale

Parameter	*Score*
Eye-opening (E)	
Spontaneous	4
To voice	3
To pain	2
No response	1
Best motor response (M)	
Obeys commands	6
Localizes	5
Withdraws to pain	4
Flexion posturing (decorticate)	3
Extensor posturing (decerebrate)	2
No response	1
Verbal response (V)	
Oriented conversation	5
Confused, disoriented	4
Inappropriate words	3
Incomprehensible sounds	2
No response	1
Total score = E + M + V	

Modified from ref. *47*.

sions *(39)*, as these categories were developed mostly for the purpose of determining when it is safe for head-injured athletes to return to play *(39,53–55)*. On the battlefield, a head-injured soldier with a grade 1 or 2 concussion will probably return to combat, whereas a soldier with a grade 3 concussion will probably have to be evaluated at a higher echelon treatment station.

TREATMENT

As noted above in the section on diagnosis, treatment of the head-injured patient begins with a rapid initial assessment and then subsequent management as outlined in the ATLS guidelines. First and foremost, management begins with maintenance of the airway and cervical spine precautions. A cervical spine injury should be assumed in any trauma victim, especially with blunt injury above the clavicle *(19)*.

Once it is established that the patient has a patent airway, then it must be determined whether the patient is spontaneously breathing or requires mechanical ventilation. Severely head-injured patients, defined as having a GCS of 8 or less, will require the placement of a definitive airway, as they are unable to protect their own. The finding of nonpurposeful motor responses also strongly suggests the need for definitive airway management *(19)*.

Next, the circulatory system can be quickly assessed by checking for a cardiac pulse and determining the blood pressure. Since hemorrhage is the most common cause of preventable death after trauma *(19)*, any hypotension following a traumatic injury should be assumed to be hypovolemic in origin until proved otherwise *(19)*. This should be corrected rapidly with available fluids, and necessary measures should be taken to control hemorrhage. Maintenance of the blood pressure is of paramount importance in the head-injured patient, because early postinjury episodes of hypotension or hypoxia leading to brain ischemia greatly increase morbidity and mortality from severe head injury *(26,33,56,57)*.

One of the most important goals in treatment of the head-injured patient is to prevent brain ischemia. This is best accomplished by ensuring that CPP is constantly maintained. A general guideline is to keep CPP > 70 mmHg. As described above, MAP should be maintained at >90 mmHg. Furthermore, optimal brain perfusion is achieved if the ICP is also kept within normal limits. Elevated ICP compromises CPP (and hence CBF) through the relationship CPP = MAP − ICP. Controversy remains as to when it is appropriate to use an ICP monitor; however, many experts posit that ICP monitoring is indicated with severe head injury (GCS 3–8) and an abnormal computed tomography (CT) scan (showing hematomas, contusions, edema, or compressed basal cisterns) *(57)*. The advantages of ICP monitoring include (1) earlier detection of intracranial mass lesions; (2) minimization of the indiscriminate use of therapies to control ICP, which can themselves be potentially harmful; (3) reduction of ICP by cerebrospinal fluid drainage; and (4) assistance in determining prognosis; and (5) potential improvement in outcome *(57)*.

The recommended guideline to initiate treatment of elevated ICP is at an upper threshold of 20–25 mmHg. Even though this is considered a general guideline, in actuality there is insufficient evidence to support

this recommendation as a standard. Whether or not ICP monitoring has been initiated, if the patient demonstrates signs of transtentorial herniation or progressive neurologic deterioration not attributable to systemic pathology, then it is appropriate to treat for elevated ICP. Such treatment begins with drainage of cerebrospinal fluid through a ventriculostomy when this is available. If elevated ICP persists, then the pharmacologic agent of choice is generally mannitol (effective dose: 0.25–1 g/kg body weight) or other such osmotic agents *(57)*. Prior to the use of mannitol, other treatments, beginning with elevation of the head of the bed (20–30°) and use of sedation, may be appropriate. Treatment of elevated ICP with hyperventilation should be avoided during the first 24 h after severe TBI because it can further compromise cerebral perfusion during a time when CBF is probably reduced *(57)*. Furthermore, multiple second-tier options for management of intractable elevation of ICP exist, including the use of barbiturates. In cases of severe refractory elevated ICP and CPP < 70 mmHg, some experts recommend treatment with a decompressive craniectomy *(58)*.

The goals of emergency surgery in head injury are generally limited to decompression of the brain parenchyma by removing a mass lesion, which is usually a hematoma *(2)* and wound debridement in penetrating injuries *(59)*. Occasionally lobectomy can be a life-saving procedure to prevent herniation *(60)*. The utility of decompressive craniectomy on improvement of outcome in TBI remains controversial, but currently a randomized prospective multicenter trial is under way to answer this question definitively *(61–63)*.

Other important factors to consider are nutrition, deep venous thrombosis prophylaxis, and ulcer prophylaxis. The prevention of early seizures in severe TBI (GCS 3–8 or the presence of an intracranial lesion, e.g., subdural hematoma) with a 1-wk course of either phenytoin or valproate is recommended by most head trauma experts *(64,65)*. Finally, studies have also proved the benefit of early physical and occupational therapy in long-term improvement *(57,66)*. However, there is uncertainty as to whether a difference exists between intense inpatient rehabilitation and outpatient therapy *(67)*.

In summary, the fundamental goals of resuscitation of the head-injured patient center on the prevention of secondary neuronal injury, by restoration of circulating volume, blood pressure, oxygenation, and ventilation *(57)*. These basic but critical parameters of patient manage-

ment have all been shown to play a major role in determining the final outcome from a neurologic standpoint.

OUTCOME AND PROGNOSIS

TBIs have highly variable outcomes. Even within the various degrees of severity based on the GCS, there is inconsistency. Most patients with a very low GCS will have an unfavorable outcome; however, there are those few who will have a satisfactory outcome. One of the most widely relied on measures of outcome following a TBI is the Glasgow Outcome Scale (**Table 3**). Despite criticism over its low sensitivity for detecting subtle change in recovery, this scale remains a fairly useful quantification of outcome *(68)*. Only 30–40% of patients who sustain a gunshot wound to the head achieve 'satisfactory outcomes' (defined as good, or moderately disabled) *(69)*.

Factors associated with worsened outcomes have been studied (**Table 4**). These include demographic factors (advanced age), complications (disseminated intravascular coagulation, hypotension, hypoxemia, and increased intracranial pressure), and wound characteristics (multilobar or bilateral wounds, transventricular penetrating wounds, and wounds crossing the midsagittal or midcoronal plane) *(42,70)*. Although the mortality rate is high for patients sustaining penetrating head injuries, for those who reach medical care and inpatient rehabilitation, the chance of achieving functional improvement is also high *(69)*.

TBI remains one of the most devastating misfortunes a person can suffer. The best case scenario is a mild head injury or concussion with complete recovery. At the other end of the spectrum are those who suffer TBI and either die or remain in a persistent vegetative state. Thanks to enormous advances in the treatment of patients with severe brain injury, many persons who would have otherwise died or been left severely disabled have now achieved satisfactory outcomes. Progress in our understanding of neurobiology, physiology, and pathology has led to advances in prehospital care, radiologic imaging, critical care, and rehabilitation, which have all contributed to a reduction in mortality and an improvement in quality of life.

Table 3
Glasgow Outcome Scale Score

Category	*Description*
1	Dead
2	Vegetative state: no meaningful responsiveness; cannot obey simple commands or communicate
3	Severe disability: dependent on others for daily activities; cannot function independently because of significant physical or cognitive impairment
4	Moderate disability: able to function independently but cannot return to preinjury level of functioning because of physical or cognitive deficits
5	Good recovery: able to resume all preinjury functions, although slight physical or cognitive impairments may lead to a lower level of functioning

Modified from ref. *20*.

Table 4
Summary of Factors that Portend Worsened Prognosis

Factor	*Level*
Extremes of age	<2 or >60 yr
Glasgow Coma Score	
Total score after resuscitation	<9
Motor score	<3
Eye opening	<2
Verbal response	<2
Pupils	Dilated or abnormal response to light
Oculocephalic or oculovestibular reflexes	Absent
Injury severity scale	>40

Modified from ref. *20*.

SCIENTIFIC ENDEAVOR AND TRAUMATIC BRAIN INJURY

A great deal of basic science and clinical research is dedicated to the advancement of our understanding of head trauma. Animal models have been the mainstay, with the aim of understanding TBI from a behavioral and gross anatomic perspective. These models have been crucial in increasing our understanding of the physiologic pathways and pathophysiologic processes involved in traumatic neuronal injury, damage to connections, and regeneration or repair (*71*). More recently there has been an overwhelming increase in interest in the mechanisms governing TBI at the cellular and molecular levels. Several models are currently in use that mimic TBI and examine its effects at various levels.

One of the most useful and widely studied head injury systems is the fluid percussion injury model employed in rats (*72–74*). This system causes TBI by rapidly transmitting a fluid energy pulse delivered through a fluid cylinder and a flexible catheter to the epidural space from a weighted pendulum (*75*). The ensuing pressure pulse can been calibrated to approximately 1.5–3.0 atm depending on the desired severity of injury. Using this model, myriad subsequent studies have been devised whereby the behavioral, histologic, and pathophysiologic processes have been examined. This model has also been used by several groups to evaluate the effects of pharmacologic agents on recovery after TBI.

Our group has achieved promising preliminary data with the rat fluid percussion injury model using a novel compound that selectively inhibits an excitatory glutamate receptor [glu(R)5] (*76*). This compound, which was originally designed as an antiepileptic agent and which has demonstrated safety in human trials, is hypothesized to minimize subsequent excitotoxicity through its glutamatergic antagonism. Excitotoxicity is thought to be a key component of secondary neuronal injury (see discussion on secondary neuronal injury in the Pathophysiology section above) (*77*). When an intravenous glu(R)5 antagonist is administered 15 and 30 min after fluid percussion injury, rats perform better in neurologic testing, show decreased numbers of apoptotic cells, and demonstrate a smaller histologic injury penumbra than matched injured controls administered normal saline at corresponding times (*76*). Naturally this is a very exciting finding, as no pharmacologic agent to date has demonstrated a significantly improved outcome when

administered after injury. Experiments are under way to determine whether these positive effects can be reproduced in injured animals when drug administration is extended to even greater time points, which would more closely model the typical time-course for head-injured patients or soldiers presenting to medical caretakers.

Many groups are also studying the mechanisms of TBI at the cellular and molecular levels. Some of these studies are examining the genetic components of head injury, including up- and downregulation of gene transcription following a TBI. Other groups are focusing on specific proteins that are thought to play crucial roles in mediating neuronal damage after head injury. For instance, members of our group are studying the effects of dopamine transporter-1 (DAT-1) transcription on enkephalin production. Enkephalin is hypothesized to be integrally involved in the pathophysiology of head injury. DAT-1 and its protein product are demonstrating significant involvement in neuronal injury through their functional effects on enkephalin. These molecules may potentially provide useful targets for therapeutic intervention.

We have chosen to list and briefly discuss only a few relevant examples from the scientific discovery and current basic science of TBI. Along with other areas of neuroscience research that evolved in the latter half of the last century with phenomenal rapidity, including vast increases in neurobiologic, neurophysiologic, and neuroanatomic knowledge, the subgroup of brain injury research made extraordinary strides. TBI research will undoubtedly continue to be an extremely fast-moving area of scientific endeavor, and our best guess is that the future of TBI research will provide many exciting and useful approaches for bettering the outcome in this horrendous public health problem.

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14 Red Blood Cell Storage

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INTRODUCTION

Blood transfusion has played an important role in military casualty care since World War I. In November 1917, Capt. Oswald H. Robertson of the Harvard Medical Unit in Camier, France, took 22 quart bottles of universal donor red blood cells (RBCs) suspended in citrate and glucose to a British Casualty Clearing Station behind the Western Front during the Battle of Cambrai. There he demonstrated for the first time the ability of stored blood to resuscitate soldiers in profound shock and to save lives *(1,2)*. Today, packed RBCs are the most important blood product for combat casualty care *(3)*. They are part of every major military deployment.

Keeping adequate supplies of RBCs in remote theaters is a formidable military logistic and medical housekeeping task. RBC units must be collected where volunteer donors can be found, tested for infectious diseases, labeled for interstate shipment, moved to blood trans-shipment centers at air hubs, shipped on ice while staying between 1 and 10°C, dispatched to medical units, and maintained until they are needed or until they outdate. Many aspects of this process are subject to federal regulations, industry standards, and military directives. The responsibility for blood availability and safety frequently falls on young medical officers in small surgical units. Knowledge of blood use and storage is important in these resource-limited environments.

Research on blood storage can improve the options of medical offi-

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cers in the field in several ways. It can lead to more robust products, such as RBCs with a longer shelf-life, which therefore require less handling. It can produce alternative products such as frozen RBCs, which are compatible with prepositioning. Finally, it can lead to knowledge of how to deal with unusual or difficult situations, such as what to do if RBCs are accidently allowed to warm. This chapter discusses work in all these areas and also provides background information about military and emergency blood usage to give the non-blood-bank specialist a sense of why the data and inventions are important.

BACKGROUND

The Nature of Military and Emergency Blood Use

Approximately one admission in six to a combat theater hospital receives blood *(4)*. Most of these casualties receive 2 or 3 U of RBCs. However, a few of the injured receive a great deal of blood, and therefore the number of units transfused to a casualty who receives blood averages between 6 and 8 U. In the largest published series, about 5% of transfused casualties received more than 25 U of RBCs and about 1% more than 50 U.

Forward surgical teams and combat support hospitals deploy with a small number of RBC units stored in styrofoam boxes on wet ice or in small thermocouple refrigerators. A forward surgical team may take 30 U of RBCs, and a combat support hospital usually takes 40–120 U depending on the expected level of activity. In the continuous care of casualties, surgical teams use less than 1 U of RBCs each hour. In Mogadishu, three teams operated for 36 h each and used about 80 U of blood total *(5)*. Two British teams operated for 34 h each after the battle of Port Stanley in the Falklands War and used about 40 U of RBCs *(6)*. Even at the height of the Vietnam War with a hundred surgical teams in the country, blood usage never exceeded an average of 1200 U of RBCs a day *(8)*.

The experience with blood use in good military medical units over the last 35 yr is no different from that seen in modern level 1 trauma centers. In a review of blood usage in the Trauma Center at the Ben Taub General Hospital in Houston in 1998, it was noted that 18% of 2997 admissions received blood products. *(9)* Fifty percent received 3 U of RBCs or less. The average number of RBC units received by an individual receiving blood was 8 U. Surgeoner and his colleagues *(10)*

at the Center for Blood Research at Harvard have shown that this pattern of blood usage, with a low modal number of RBC units used and a markedly right-skewed distribution, can be shown for many surgical procedures and the treatment of many diseases.

Historic Methods of Red Blood Cell Storage

When Robertson *(1)* first demonstrated RBC storage during World War I, he collected 300 mL of whole blood into 700 mL of anticoagulant (citrate) and nutrient (glucose) solution. RBCs were only 12% of the volume of the suspension. By the beginning of World War II, blood bankers were collecting more blood and using less anticoagulant and nutrient solution, so that the ratio of blood to nutrient approached 1:1, and the RBC volume fraction, the storage hematocrit, approached 20%. Healthy young casualties tolerated the extra volume. As blood was increasingly shipped by air in World War II, a need for lighter blood products became obvious. In response to that need to facilitate air transport, the first modern blood storage solution, acid citrate dextrose (ACD), was developed *(11)*. The acidity of ACD (pH 5.5) allowed the solution to be heat-sterilized without caramelizing the glucose. Its low volume ratio (1:4 with whole blood for ACD-A; later 1:7 for ACD-B) defined the volume of blood collected and the proportion of storage solution, which are used to this day (63 mL of anticoagulant to 450 mL of blood).

ACD-B and its successor, citrate phosphate dextrose (CPD), were the standard solutions used for 3-wk blood storage until 1979. The Korean and Vietnam Wars brought the problem of 3-wk blood storage to military attention, but it was also a problem in civilian hospitals. Almost a third of the blood collected in the United States outdated in blood bank refrigerators and was thrown away. The addition of adenine to blood storage solutions by Shields *(12)* at the Army Medical Research Laboratory at Ft. Knox was the basis for citrate phosphate dextrose adenine (CPDA-1), which permitted RBCs to be stored for up to 5 wk without unacceptable changes. This extended storage time produced a revolution in civilian blood banking, allowing more efficient inventory management and greater than 90% utilization rates.

Meanwhile, patterns of blood usage were changing. Before 1970, almost all the blood used was whole blood. After that time, most blood was separated into components: packed RBCs, platelets, and plasma. This change was driven by the need for platelets to treat cancer patients,

the commercial demand for plasma for albumin and clotting factor production, and the desire to reduce the infused volume to patients who just needed RBCs for oxygen-carrying capacity. However, it soon became clear that removing more plasma led to denser RBC concentrates. These RBC concentrates flowed more slowly when infused, and the RBCs had reduced survival in patients *(13)*.

To address this problem, nutrient additive solutions for RBCs were developed. In these systems, 450 mL of blood is drawn into 63 mL of CPD anticoagulant, the bag is centrifuged to concentrate the approximately 200 mL of RBCs in the bottom, and about 75% of the plasma (about 250 mL), is removed. Then, 100 mL of the nutrient additive solution, a mixture of saline, adenine, glucose, and mannitol (SAG-M) or saline, adenine, glucose, and monosodium phosphate (SAG-P) is added to the RBCs *(14)*. The resulting "packed" RBCs have a storage hematocrit of about 55%, so they flow well through intravenous needles in rapid resuscitation situations, and they allow storage of the RBCs for 6 wk. This whole blood collection and component manufacturing process can be accomplished in a closed, sterile, quadruple-bag blood collection system, which can be manufactured for only a few dollars each and can be sterilized by autoclaving after packaging. With these additive solution systems, civilian blood banking has become almost 95% efficient nationally, and in large hospitals, more than 99% efficient for RBC units.

However, for the military, even these systems proved inefficient in the Gulf War. In that conflict, over 82,000 U of RBCs were sent, and over 67,000 units (81%) outdated. Maintaining the Stabilization Force in Bosnia has required almost 6000 U of RBCs, and only 80 U have been used. Longer RBC storage would reduce wastage in these and similar situations.

It is possible to store RBCs for long periods by freezing them *(15)*. In the licensed versions of this process, glycerol is added to the packed RBCs to prevent ice crystals from destroying the cells, and they are frozen in the bag in a −80°C freezer. RBCs can be stored in this manner for 10 yr. The problems with frozen RBCs, apart from the expense of storage, occur mostly when the units are thawed. The glycerol must be removed to protect the cells and the recipient. The cell washing process necessary to reduce the glycerol concentration from 40% to less than 1% requires a special machine, uses several liters of salt solutions, takes at least 30 min for each unit, looses about 15% of the RBCs, and

exposes the unit to potential bacterial contamination in handling. The Food and Drug Administration (FDA) has ruled that such thawed RBC must be used within 24 h or destroyed. Thawed RBCs are too labor-intensive and short-lived to be of much use in most military settings. Nevertheless, they are now deployed on some U.S. Navy ships and in two U.S. Army blood depots in Korea.

Two other methods of RBC storage have been proposed, but they have not been made into practicable systems. The first would involve freezing RBCs in a directly infusible frozen storage solution, such as hydroxyethyl starch (HES). Unfortunately, HES is a poor cryoprotectant, and at least 3% of the cells break in the freeze/thaw process*(16)*. This percentage is well above the FDA standard of less than 1% hemolysis required for licensing other RBC storage systems. The second proposed method of RBC storage, freeze-drying, is even less well developed. Sterile systems to process such cells do not exist, and laboratory prototypes of the product still have 15% hemolysis or greater *(17)*. It seems unlikely that useful alternatives to liquid stored or glycerol frozen RBCs will be developed in the near future.

LIQUID RED CELL STORAGE

John Collins *(18)*, a surgeon and national expert on blood product usage, succinctly described the requirements for RBC storage systems when he said that red cells must be safe, available, effective, and cheap. *Safety*, in the context of storage (that is, once the blood is collected from a healthy donor and is in the bag), is matter of ensuring continued sterility and preventing the blood from breaking down into toxic products. Since hemoglobin itself is toxic in moderate quantities, the FDA requires that RBC storage systems average less than 1% hemolysis. *Available*, in the context of emergency blood use, means being able to reach into a refrigerator or ice chest, take out a unit of universal donor blood, and hang it. This present standard does not leave much room for more complicated systems. *Effective* means that the cells need to work in the recipient. They need to survive and deliver oxygen. The FDA has defined 24-h survival as the critical measure and insists that at least 75% of transfused cells survive that long. *Cheap*, in the context of modern blood banking, means that blood storage systems (bag, needle, and chemicals) should cost only a few dollars to make. When packaged, sterilized, and delivered, a blood collection bag costs about $20.

A new blood storage system has to compete economically with that present cost as a clinical added value against a system that is already 99% efficient. There is little leeway for expensive developmental or licensure costs.

Several research groups have been working on improving RBC storage, and each has made significant contributions. In 1986, Dr. Harry Meryman and his colleagues *(19)* at the American Red Cross showed that RBCs could be stored for as long as 14 wk in a hypotonic solution containing ammonia that he called "solution 6." Unfortunately, they only reported the data from a few volunteers, and when other workers repeated the experiments, they did not get equivalent results. Also, the ammonia would have to be washed out of the suspending solution before the RBCs could be infused, so the solution did not meet the Collins criteria of available and cheap. However, Meryman and his colleagues *(20)* persisted and showed that RBCs could be stored for as long as 34 wk when the storage hematocrit was lowered to 4%. While Meryman's work did not lead to a practical storage solution, it did clearly show that the present limits on RBC storage at 6 wk are limitations of the storage systems and not of the red cells themselves.

Following some of Meryman's ideas, Dr. Claes Hogman *(21)* made a neutral (pH 7) additive solution called RAS2 and showed in well-designed clinical trials that it allowed 7-wk storage. The higher pH came from buffering the phosphate. He separated the glucose and phosphate in different parts of the bag system so that the bags could be autoclaved without caramelizing the glucose. The system is sold in Europe as ErythroSol®. At the same time, Walker and his colleagues *(22)* produced an alternative 7-wk additive solution called PAGGS-mannitol. The solution uses mannitol and phosphate in the same additive and has the added micronutrient guanine. PAGGS-mannitol is available in Europe. Lastly, Dr. Tibor Greenwalt of the Hoxworth Blood Center in Cincinnati and his colleagues *(23)* have carried out a systematic review of the components of storage solutions. He has produced 200-mL additive solutions that appear to work for 8 wk *(23)*. However, the solutions have contained investigational compounds, which would make licensure difficult.

At the Blood Research Detachment of Walter Reed Army Institute of Research, we started by investigating a commercial variant of Meryman's solution 6. We compared storage in a standard additive solution for 6 wk with storage in the experimental additive solution for 8 wk in

a randomized crossover trial *(24)*. The RBCs stored for 8 wk were white-cell-reduced in hopes of further extending storage. As endpoints, we measured the fraction of stored, radioactively labeled, and reinfused cells that survived in the original donors for 24 h, called the recovery, and the hemolysis at the end of storage. The hypotonic solutions did not provide acceptable recoveries after 8 wk of storage, but the leukoreduced cells had less hemolysis after 8 wk of storage than conventionally stored cells had after 6 wk of storage. The studies taught us that there is a large variability in the recovery and hemolysis observed between individuals. This difference between the RBCs of different volunteers was the largest source of variability in the studies.

In our next studies, we examined how warming blood, as might happen if a refrigerator failed or the ice ran out in a box of blood, affected the quality of the RBCs. In civilian life, such blood is thrown away and fresh supplies are obtained, but in certain military situations, it may be the only blood available. We first examined the effect of 24 h at room temperature on RBCs stored in the 5-wk solution CPDA-1 *(25)*. We collected 24 U of blood, pooled the units in groups of three, aliquoted each pool into three study units, and used a unit from each pool in each arm of a three-armed study that compared early or late warming with conventional cold storage. Warming increased the rate of glucose consumption and led to a more rapid decrease in pH. This in turn led to lower RBC adenosine 5-triphosphate (ATP) concentrations, the best correlate of recovery. Recovery could not be measured on the pooled blood because of the infectious risk of transfusion. To measure recovery, we conducted a second study using a 6-wk additive solution and a randomized crossover design, so that the volunteers only received their own blood *(26)*. The studies taught us that in vitro pooling studies can provide very precise measures of normal RBC metabolic events and that in vivo crossover studies can provide comparable measures of recovery, the accepted clinical endpoint. Since human use committees want to see an in vitro study before each in vivo study, the whole process of conducting a study cycle can take a year.

To speed and broaden the scope of the testing process, we formed a collaboration with Dr. Tibor Greenwalt and his group in Cincinnati. In their lab we focused on the question of whether the storage volume was important for the function of their 200-mL experimental additive solution (EAS) that had allowed 8-wk storage *(27)*. To simplify the experiment, we removed the experimental compounds but left the high-pH

disodium phosphate. The final solution thus contained only saline, adenine, glucose, mannitol, and disodium phosphate. We then stored aliquots of pools of RBCs in 100, 200, 300, or 400 mL of this new EAS, EAS-61. The results of this simple experiment were dramatic.

In a strictly dose-dependent manner, storage of RBCs in increasing volumes of EAS-61 led to higher RBC ATP concentrations, better RBC morphology, and less hemolysis at all time points out to 9 wk. Most of the benefit occurred with the increase in additive solution volume from 100 to 200 mL. To confirm that this benefit observed in vitro translated into useful additional storage, we conducted a human trial of RBC storage for 7 and 8 wk, measuring autologous RBC recovery and hemolysis during storage. The recovery fractions were 85% (n = 10) at 7 wk and 81% (n = 10) at 8 wk. The hemolysis was 0.4% in both groups. Because the RBC ATP concentrations and low hemolysis suggested that even longer storage was possible, we repeated the study at 8 and 9 wk *(28)*. The second study had essentially equivalent results: 85% (n = 10) recovery at 8 wk and 81% (n = 10) at 9 wk, with hemolysis of 0.26 and 0.35%, respectively.

We then looked at the potential of 300-mL additive solutions *(29)*. The greater additive solution volume would mean that the storage hematocrit would be only 36–40%. This lower hematocrit would of course require greater administered volume to achieve equal therapeutic benefit, but we rationalized that the higher volume would be well tolerated by healthy young soldiers. We did reduce the concentrations of glucose, phosphate, and mannitol to reduce the amount delivered to patients. The new solution was called EAS-64. It worked in vitro as its predecessor had, providing better RBC ATP concentrations and morphology and lower hemolysis with each successive increase in volume from 100 to 400 mL. In a clinical crossover trial, comparing 10-wk storage in 300 mL of EAS-64 with 6-wk storage in 100 mL of AS-1 (the current standard), both groups averaged 84% RBC recovery when measured with a double isotope $^{51}Cr/^{99m}Tc$ technique. Hemolysis was 0.43% in the 10-wk arm and 0.63% in the 6-wk arm.

Because the high pH conveyed by the disodium phosphate appears to be critical to the function of these solutions, we explored the effect of further manipulations to maintain high pH. These manipulations include replacing some of the sodium chloride in the solutions with sodium bicarbonate *(30)*. Beutler and West *(13)* had demonstrated the utility of this approach with the original 100 mL additive solutions two

decades ago. Adding sodium bicarbonate to blood storage solutions raises pH in two ways: first, it is alkaline, and second, as it breaks down, a proton is removed from solution by the carbonic anhydrase reaction while excess CO_2 generated in the process diffuses out through the plastic bag. Again, adding sodium bicarbonate to the storage solutions raised the pH in a dose-dependent manner, and in a small clinical trial, 79% (n = 9) recovery was recorded with 0.70% hemolysis.

Our laboratories continue to work on ways to improve blood storage, and the recent effort has demonstrated practical methods to increase liquid RBC storage from 6 to 11 wk. Such an extension could contribute to reducing seasonal shortages for RBCs, improve autologous blood systems in which patients donate blood for their own surgeries, and improve blood availability in remote nonmilitary locations such as American Samoa. The improved storage was accomplished using only water and ingredients that are already approved for use in RBC storage solutions. Thus, the cost of obtaining regulatory approval for the formulae should be modest. The total cost of the 6-yr research effort in two labs was less than the value of the 67,000 U of blood lost in the Gulf War.

FROZEN BLOOD STORAGE

RBCs frozen in 40% glycerol may be stored for 10 yrs for routine use. However, the techniques of preparing fresh RBCs for freezing and thawed cells for infusion have required opening the blood bag. Once thawed, the cells had to be used within 24 h or discarded.

The development of sterile connection technology for blood bag tubes, in which a hot knife is passed through two tubes and the ends welded together, made possible the design of systems for sterile glycerol addition and glycerol removal. To glycerolize cells, a large 0.22-μm filter is attached in a sterile fashion, and glycerol is added. To remove glycerol, the storage bag is attached in a sterile fashion to the washing harness, which has integral 0.22-μm filters for the wash and storage fluids. Blood washing systems can use either centrifugation or tangent-flow diafiltration to remove the glycerol.

Shortly after the technique for connecting tubing in a sterile fashion was developed, we demonstrated that 3-wk post-thaw storage was possible *(31)*. We collected units from 40 volunteers, froze them, washed them conventionally, stored them for 2 or 3 wk, cultured samples of the

units to make sure there were no bacterial contaminants, and measured the recovery and hemolysis. Both a commercial AS and a high pH EAS allowed 3-wk storage with greater than 75% recovery.

Then, using the federal Small-Business Initiative Research (SBIR) program, we solicited companies to make sterile systems for glycerolizing and deglycerolizing RBCs. The military supported the development of a system based on diafiltration, but a company that made mobile blood component collection devices based on centrifugation modified their machine for processing frozen blood. We participated in a multi-institution evaluation of this machine, the Haemonetics 215, and the device was recently approved for sterile processing of frozen blood and licensed for 2-wk post-thaw storage *(32)*.

Finally, we have used this new machine to evaluate the physiology of the cell washing process. In these tests, washing the RBCs in conventional acidic wash solutions reduced the pH of the RBCs to a degree that glycolysis was slowed and concentrations of ATP decreased *(33)*. The study suggested better ways to formulate wash solutions with the potential of prolonging liquid storage of previously frozen RBCs to 3 wk or more.

CONCLUSIONS

The U.S. Army has played a major role in the development of methods for red blood cell storage. This effort has been driven by requirements to provide RBCs in distant locations and to support the frequently episodic nature of combat blood usage without undue blood wastage. The program has succeeded because the limited military research resources were used to bridge gaps in the national blood program, at a time when RBC storage was viewed as a mature technology. U.S. military forces will continue to use RBC transfusion to support surgical care of combat casualties. New scientific understanding and technical procedures will continue to require technical development to function optimally in combat casualty care.

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15 Bioterrorism

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INTRODUCTION

The September 11, 2001 terrorist attacks on the World Trade Center and the Pentagon, followed shortly thereafter by the dissemination of anthrax through the U.S. mail, has drawn considerable attention in both the medical and lay communities to the risks of bioterrorism. In this chapter we review the biologic agents that are most likely to be employed in terrorist attacks, emphasizing the most likely clinical presentation of victims, diagnostic approaches, and management.

A biologic agent may be defined as a living organism with the ability to replicate (virus or bacteria), or the nonreplicating product of a living organism (a toxin or physiologically active protein or peptide). Bioterrorism may be defined as the deliberate use of a biologic agent to cause death, disease, or disability in humans, animals, and/or plants. Biologic agents are often lumped with chemical agents, and sometimes even with nuclear agents as well, as weapons of mass destruction. Each of these types of agents differs greatly from conventional weaponry, and there are also significant differences between the three categories of weapons of mass destruction. An act of nuclear terrorism, whether the detonation of a nuclear weapon or an attack on a nuclear power plant, is heralded by a massive explosion, initial traumatic injuries, and consequent radiation-induced illness. Similarly, a chemical agent, usually manufactured by a chemical process, will usually result in prompt, profound morbidity and/or mortality.

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Biologic agents, on the other hand, often have an onset of action that is delayed by days to weeks, potentially facilitating surreptitious dispersal by terrorists who could then escape before detection of the act. In addition, biologic agents are generally found in nature, and the clinical syndromes they produce often mimic other common illnesses such as influenza, which can sometimes make it difficult to discern an act of bioterrorism from a naturally occurring epidemic with any degree of certainty. Another potential appeal of biologic agents to terrorists is that they can be acquired or produced relatively easily and inexpensively, without the technologic capabilities often needed for the use of conventional or nuclear weapons.

On the other hand, the factors that make biologic agents attractive to terrorists may make them less attractive to nations waging war. Nations, especially those having the audacity to wage war, tend to have some financial resources, so the lesser expense of biologic agents is not as significant. In addition, the delayed onset of many biologic agents probably makes them less appealing, especially on the rapidly changing modern battlefield, than the acute impact generated by nuclear or chemical agents. Nations at war have less need to be surreptitious. Therefore, although a deployed army is in some ways comparable to a midsized city, and the individual effects of biologic agents can be expected to be similar in soldiers and civilians, this chapter is oriented more toward acts of bioterrorism than the traditional battlefield. However, it is also important to recognize that future conflicts are less likely to occur on a traditional battlefield and more likely to be the type of urban warfare experienced in Mogadishu. Two factors favor this development. First, urbanization of the world's population is accelerating. Second, the U.S. military is so far superior to the rest of the world on the traditional battlefield, as evidenced by the Gulf War, that adversaries are unlikely to engage in such futility, and instead will fight using guerrilla tactics or the kind of assault made on September 11, 2001. In this respect, the bioterrorism risks described here are indeed applicable to the future battlefield.

HISTORICAL PRECEDENTS

Although biologic agents have probably received more media attention after September 2001, than ever before, it is useful to examine the many historical precedents, to prepare better for future incidents; in

fact, this examination was already well under way in medical and government circles before 2001. Crude initial bioterrorist incidents probably included the use of arrows treated with manure or blood, contamination of food or water supplies with animal or human carcasses, and throwing venomous snakes onto enemy ships, all before the time of Christ *(1)*. In the mid-14th century, Tatars laying siege to Kaffa catapulted plague-infected cadavers into the city, although it is debated whether the subsequent outbreak of plague could be directly attributed to this effort, or was the result of coincidental rat-and flea-transmitted disease under poor conditions of sanitation *(2)*. Likewise, it remains unclear whether a 1763 smallpox epidemic in Native Americans can be attributed to deliberate spread of disease by gifts of infected blankets, or coincidental spread of disease by person-to-person contact *(2)*.

Scientific advances, particularly the development of modern microbiology, is probably in large part responsible for the far greater number of attempts to use biologic agents during the past century. Both sides employed chemical weapons to deadly effect in World War I; the Germans also attempted to spread plague in Russia *(1)* and had variable success in transmitting anthrax and glanders to livestock in the United States, Argentina, and Russia *(3)*. The 1925 Geneva Protocol prohibited use of, but not research on, biologic agents, and many nations experimented with biologic agents between the two world wars *(2)*. The Japanese attempted to spread plague, cholera, typhoid, and anthrax in Manchuria in the 1930s and early 1940s, also with variable success; in doing so they infected thousands of their own troops *(1)*. After World War II, the Cold War spurred combatants on both sides to continue research until President Nixon renounced the use of biologic weapons in 1972. Although the Soviets officially denied having a biologic warfare program, this was belied by the 1979 accidental release of anthrax in the city of Sverdlovsk, which resulted in at least 77 cases (66 deaths) of inhalational anthrax *(2)*.

Over the past century, biologic agents have been used or their use has been threatened far more often for terrorist or criminal intent than in warfare. This is especially true since 1990 and is probably a harbinger of what to expect in the 21st century; advances in genetic engineering may lead to a new and more frightening implementation of biologic agents, modified to be resistant to many antibiotics and/or able to survive under a greater range of environmental conditions.

Among the many terrorists worldwide, several stand out in their influence on the attention the U.S. government has paid to the threat of chemical and biologic agents since 1990. First, Iraq developed a variety of biologic and chemical warfare agents, and in fact used chemical weapons against both Iranian soldiers and Kurdish citizens of their own country in the 1980s; they also possessed but elected not to use such agents during the Gulf War *(4)*. Second, the Japanese sect Aum Shinrikyo unsuccessfully attempted to spread anthrax and botulism before releasing the fatal nerve agent sarin in Matsumoto and in the Tokyo subway system *(5)*. Among many other threats and hoaxes, a package sent to the international headquarters of B'nai B'rith in Washington, DC, in April 1997, is notable *(5)*. In this case, it was claimed that a package contained anthrax, but in fact it contained a related, but far less pathogenic species, *Bacillus cereus*. What was remarkable about this case was that cordoning off two blocks in the center of downtown Washington for 9 hours while authorities were trying to determine the contents of the package resulted in major traffic disruptions throughout the city when a real threat was not even present.

The unique features of each biologic agent may influence how likely they are to be considered for use by terrorists, and in turn how likely they are to result in illness. Among the key characteristics are the infectivity, lethality, methods available for dissemination, incubation period, risk for person-to-person transmission, and hardiness of the organism under varying environmental conditions. Finally, consideration must be given to the goals terrorists may have in using biologic agents to kill or incapacitate individuals or groups: to perpetrate economic damage, to induce psychological trauma, or to make a statement or garner attention for their cause. Although many organisms could be used for terrorism, a handful warrant greater attention because they have characteristics that meet the goals of terrorists and/or it is relatively easy for terrorists to acquire and disseminate them.

ANTHRAX

Bacillus anthracis is a spore-forming Gram-positive bacteria found in soil throughout the world that infects both wild and domesticated animals, as well as humans. The spores can last for decades in the soil, demonstrating remarkable resistance to environmental conditions such as heat and light. Anthrax has considerable historical significance. It is

suspected to represent the Fifth Plague of Egypt described in Exodus, the first disease for which Koch established a microbial origin in 1876, and for which Pasteur developed an effective live vaccine in 1881 *(6)*. Cutaneous anthrax has been known for centuries, but the first cases of inhalational anthrax were not identified until the late 1800s in association with sorting wool from infected animals, perhaps the first instance of occupational respiratory infectious disease *(6)*.

At least several nations have included anthrax in their arsenal at some point in their history, and terrorist groups such as Aum Shinrikyo have attempted to use it, fortunately largely without success *(5)*. Most recently, of course, anthrax has also become the first documented disease to be deliberately disseminated through the U.S. mail. However, the handful of cases that resulted pale in comparison to estimates of the potential effect of the intentional dissemination of anthrax from an airplane over an urban area, which could kill hundreds of thousands, or even millions.

Types of Anthrax

Three clinical presentations are seen with anthrax infection: cutaneous, inhalational, and gastrointestinal.

Cutaneous Anthrax

Historically, this has been the most common form of anthrax, representing 95% of anthrax cases seen in the United States in the 20th century *(7)*. Exposed areas of skin such as the face, neck, hands, and forearms are most commonly involved, particularly at the site of prior cuts or abrasions. The initial skin lesion is most often seen 1–6 d after exposure, with reports as late as 12 d out *(6,8)*. Clinically, a pruritic red papule or macule up to 1–2 cm in diameter is usually seen initially and may be mistaken for an insect bite. However, the lesion rapidly evolves so that by the next day, 1–3-mm vesicles containing clear or serosanguinous fluid (**Fig. 1A**) often appear, followed by the development of a painless black eschar (**Fig. 1B**) that falls off within 1–2 wk, most often without even leaving a scar. Edema of the surrounding tissues often accompanies the eschar phase *(8)*. Pathologically, spores germinate at the site of infection, so that the vesicles are filled with bacteria, which produce toxins that cause the surrounding tissue necrosis and edema. In a minority of cases, migration of spores to lymph nodes, with subsequent germination, leads to associated necrosis and edema of the lymph

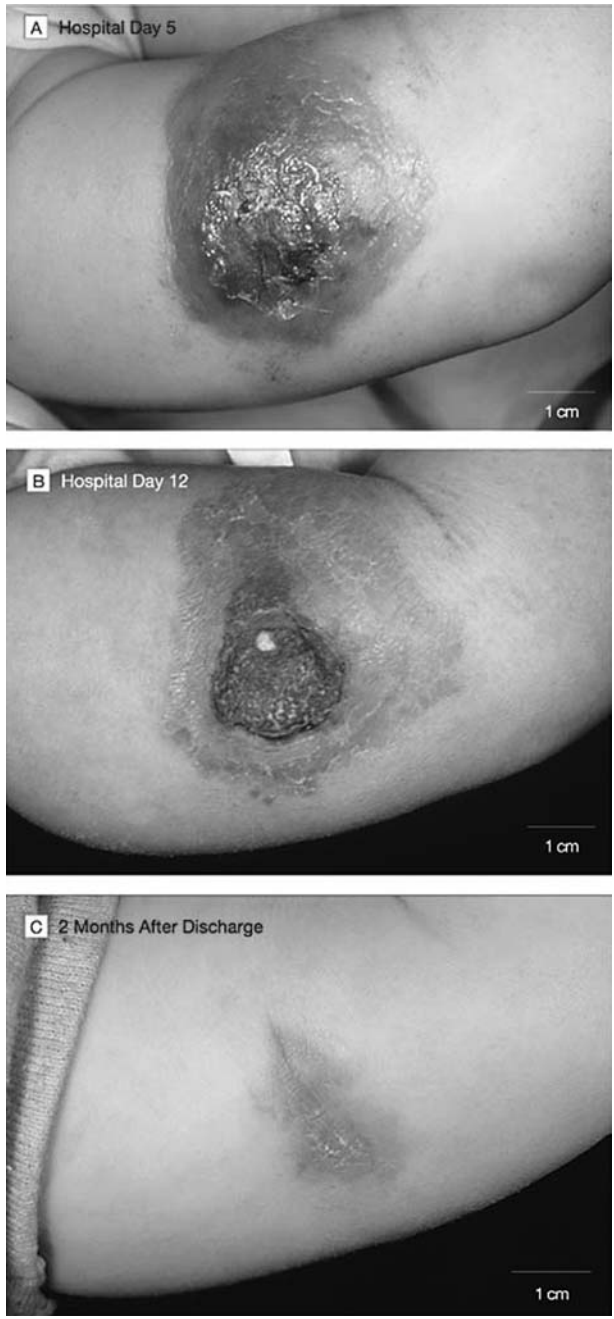

Fig. 1. A 7-mo-old infant with cutaneous anthrax. (From Journal of American Medical Association, 2002, vol. 287, pp. 869–874. Copyright 2002, American Medical Association.)

nodes, which is painful and may consequently lead to fatal systemic infection unless effective treatment has been initiated *(7)*. Recently, bioterrorist-related cutaneous anthrax in an infant was complicated by the development of a severe microangiopathic anemia, thrombocytopenia, and coagulopathy *(9)*.

Inhalational Anthrax

Terrorists are most likely to attempt to aerosolize anthrax spores, resulting primarily in inhalational anthrax, the most fatal form. This is one of the most feared bioterrorism scenarios; even with the administration of appropriate antibiotics, many if not most of those exposed may not be saved, and the medical system would be completely overwhelmed.

Anthrax spores are 1–2μm in diameter, enabling them to pass easily into the alveoli, where they are deposited, taken up by alveolar macrophages, and transported to the mediastinal and peribronchial lymph nodes *(7)*. Spores may germinate immediately, or germination may be delayed for as much as 2–3 mos. Once germination does occur, the rapidly multiplying bacteria release toxins that induce a hemorrhagic mediastinitis, with marked edema and necrosis *(8)*, the hallmark of inhalational anthrax. It is important to remember that the infection is within the lymph nodes; anthrax does not cause a true pneumonia, although there may be a localized region of hemorrhagic necrosis similar to the Ghon complex of tuberculosis. Spores germinate in an environment that is conducive to replication, where there is an abundance of amino acids, nucleosides, and glucose, such as is found in the bloodstream and lymph nodes, whereas spore formation is promoted by exposure to air or environments unsuitable for replication *(8)*. It remains unclear why germination in the lymph nodes is sometimes delayed for weeks.

Inhalational anthrax often has a biphasic clinical course. The initial phase features nonspecific influenza-like symptoms such as fever, chills, diaphoresis, headache, cough, and malaise, although the presence of two particular symptoms should raise the suspicion for anthrax: dyspnea and chest pain *(7)*. A chest X-ray may show the pathognomonic mediastinal widening induced by anthrax (**Fig. 2**). Of the 11 cases of inhalational anthrax spread through the U.S. mail in October and November of 2001, the initial chest X-ray was interpreted as normal in only three cases, but more careful review by radiologists identified abnormalities in every case *(10,11)*. Two patients had pulmonary infil-

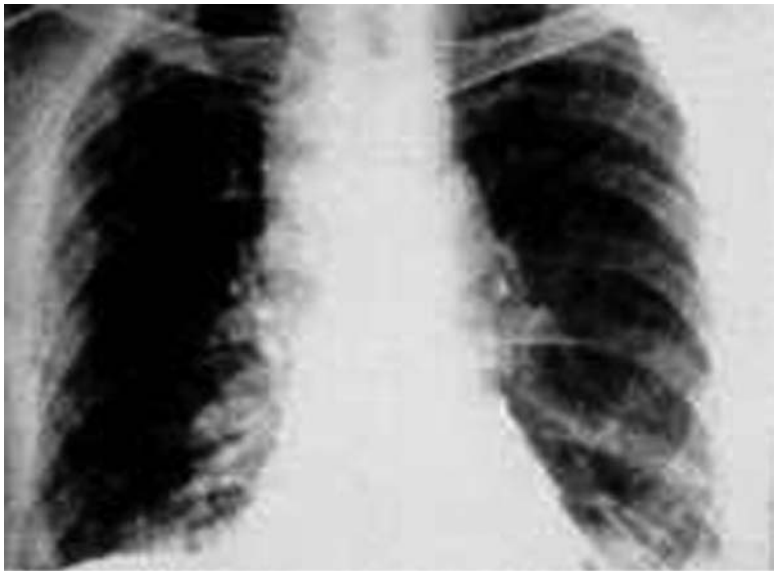

Fig 2. Chest X-ray of inhalational anthrax, demonstrating marked mediastinal widening and a small parenchymal infiltrate. (Reprinted, with permission, from ref. *6*.)

trates without mediastinal widening, with the others having at least hilar or paratracheal fullness, but more commonly frank mediastinal widening, and all 11 had effusions. Although generally not seen in the recent bioterrorism-related cases, historical reports document that some patients may paradoxically have transient improvement in their symptoms after a day or two, but unimpeded progression to severe illness is not uncommon. However, a fulminant downhill course rapidly ensues, consistent with septic shock and manifested by severe dyspnea, hypoxia, hypotension, and death *(8)*.

Meningitis complicates about half the cases of inhalational anthrax, although it also may occur with cutaneous or intestinal anthrax *(6)*. Clinical findings may include nuchal rigidity and other signs of meningismus, as well as delirium and obtundation that progresses to coma and is almost invariably fatal. Pathologic features include widespread edema and hemorrhage into the leptomeninges *(7)*.

Gastrointestinal Anthrax

Classically, gastrointestinal anthrax has been reported after ingestion of infected meat, with deposition and germination of spores in the upper

and/or lower gastrointestinal tract *(6)*. As in other sites of anthrax infection, common features include edema and hemorrhagic necrosis of the bowel wall as well as mesenteric lymph nodes. This form of anthrax has never been reported in the United States *(6)*. However, hematogenous seeding of the gastrointestinal tract may occur with cutaneous anthrax and is especially common with inhalational anthrax. In 39 of the 42 autopsied fatal cases of inhalational anthrax in Sverdlovsk, gastrointestinal lesions were identified *(10)*. As opposed to cases resulting from ingestion, hematogenous dissemination to the gastrointestinal tract typically afflicts the submucosa but not Peyer's patches or mesenteric lymph nodes.

Diagnosis

The symptoms of the initial phase of anthrax infection are nonspecific and are easily mistaken for common viral syndromes. Unfortunately, if treatment is not initiated during this phase, the infection will almost certainly be fatal. Therefore, it is imperative that physicians have a high index of suspicion under the appropriate circumstances, in particular ascertaining the presence of more concerning symptoms such as dyspnea and chest pain, and ordering a chest X-ray promptly when these symptoms are present. Ideally, there would then be a reliable test to test to confirm the diagnosis of anthrax promptly. Blood cultures for *B. anthracis* almost universally provide preliminary positive results within 24 h in patients with inhalational or systemic anthrax infection *(8)*, and they should be obtained promptly before starting antibiotics. However, the patient is unlikely to survive unless appropriate antibiotics have already been obtained prior to the return of results, so cultures should only be used to confirm the presumptive diagnosis. More rapid diagnostic tests using enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) techniques can be helpful but are not yet widely available *(8)*.

Treatment

Inhalational anthrax is unique among potential bioterrorist agents in combining the following features: effective treatment is readily available, it is imperative to initiate treatment immediately, and it is arguably the biologic agent most likely to be disseminated by terrorists.

Although ciprofloxacin is widely believed to be the most efficacious antibiotic against anthrax, it achieved this status largely by chance. When the U.S. military prepared for the possibility that Iraq might use

anthrax as a biologic weapon in the 1990–1991 Gulf War, military planners were concerned that the Iraqis might genetically engineer anthrax strains resistant to common antibiotics such as penicillin and doxycycline. Ciprofloxacin was the new wonder drug at the time, leading to its selection as the treatment of choice in the Gulf War since it was extremely unlikely that the Iraqis could engineer resistance against it. In fact, the anthrax utilized in the 2001 mail-disseminated cases was sensitive to multiple antibiotics, including penicillin, doxycycline, rifampin, and ciprofloxacin. Prompt, aggressive treatment in the mail-related inhalational cases resulted in significantly greater survival (55%) than had been seen historically (<15%) *(10,11)*. It is important to remember that cephalosporins, often used for treatment of pneumonia, are not effective against anthrax, which produces a cephalosporinase.

Patients with suspected inhalational anthrax should be admitted to a hospital and started on multiple intravenous antibiotics *(12)*. (**Table 1**). They may be changed to oral antibiotics upon stabilization, and patients should receive a course totaling at least 60 d *(12)*. Patients who have a credible exposure, especially if identified as positive on nasal swabs, should receive at least 60 d of treatment, perhaps up to 100 d, with consideration given to administration of anthrax vaccine *(13)*. Other actions that should be taken after possible exposure include immediate isolation of any potential source of anthrax spores, removal and isolation of clothing or other items that might have been exposed, and hand washing followed by showering with soap and water. Public health and law enforcement agents should be promptly notified. There is no evidence of person-to-person transmission of anthrax.

Anthrax Vaccine

The anthrax vaccine currently used by the U.S. military contains no whole bacteria, dead or alive *(14)*. It is made from a cell-free filtrate of an attenuated strain of *B. anthracis* and is produced by the Bioport Corporation of Michigan. The military administers the vaccine in a series of six shots over 18 mos, and hundreds of thousands of military service members have at least started the series, although production problems delayed plans to vaccinate all military service members fully. Although local reactions at the site of inoculation, such as tenderness, erythema, edema, or pruritis have occurred in 30% of men and 60% of women, serious side effects, such as allergic reactions requiring hospitalization,

Table 1
Pharmacologic Therapy for Agents of Bioterrorism[a]

Agent	*Treatment*	*Postexposure Prophylaxis*
Bacillus anthracis (inhalational) (combination therapy advised for clinical illness)	**i.v. ciprofloxacin** **i.v. doxycycline** i.v. penicillin i.v. levofloxacin i.v. ofloxacin i.v. rifampin i.v. gentamicin i.v. erythromycin i.v. chloramphenicol i.v. tetracycline	**p.o. ciprofloxacin** **p.o. doxycycline** **p.o. amoxicillin** p.o. levofloxacin p.o. ofloxacin
Francisella tularensis or *Yersinia pestis*	**i.v. Streptomycin** **i.v. gentamicin** i.v. ciprofloxacin (other quinolones probably effective too) i.v. doxycycline i.v. chloramphenicol	**p.o. doxycycline** **p.o. ciprofloxacin**
Brucella (various species) (use at least two drugs)	**i.v. doxycycline** **i.v. rifampin** i.v. trimethoprim-sulfamethoxazole i.v. gentamicin	**p.o. doxycycline** **p.o. rifampin**
Coxiella burnetii	**Doxycycline** **Tetracycline** Macrolides Quinolones Rifampin	**Doxycycline** **Tetracycline**
Botulinum toxin	**Antitoxin**	**Toxoid**
T-2 Mycotoxins	Supportive ?Steroids	NA
Stapylococcus enterotoxin B	Supportive	NA
Ricin	Supportive	**Toxoid**
Smallpox	NA	**Vaccination** Cidofovir
Viral hemorrhagic fevers	Ribavirin Antibody for some	NA
Viral encephalitides	Supportive	NA

[a]Treatments of choice appear in bold type.
NA, not available.

have been uncommon *(14)*. Postal workers exposed to anthrax in late 2001 were offered the vaccine, but most elected not to receive it.

SMALLPOX

For many, smallpox is even more frightening as a bioterrorist threat than anthrax. Although the case fatality rate of 25–30% is lower than for anthrax, there are several reasons for particular concern. First, smallpox is the only disease to have been eradicated worldwide, representing public health's greatest victory to date. Vaccination programs were consequently halted by 1980, so that much of the world's population has no immunity. Second, in contrast to anthrax, smallpox is transmitted very easily person to person. Third, those who survive infection are often terribly disfigured by scarring. And finally, there is no antiviral treatment with proven efficacy against smallpox, and the medical infrastructure would probably be overwhelmed in providing supportive care.

Like anthrax, smallpox has historical significance, both for its probable use by the British as a biologic weapon against Native Americans and for Jenner's use of cowpox inoculation to provide protection against smallpox near the end of the 18th century *(2,15)*.

Smallpox is a highly infectious DNA virus that is easily spread by either respiratory secretions or fomites such as clothing or bedding from infected individuals, probably with as little as a few virions *(15)*. The incubation period is typically 12–14 d, after which patients initially experience relatively nonspecific symptoms such as fever, fatigue, headache, backache, and sometimes abdominal pain or delirium *(15)*. These symptoms are usually severe enough to limit patients to bed, fortuitously limiting their exposure to others at the point at which (within a day or two of the onset of symptoms) they first develop a rash and simultaneously become infectious. The rash is initially maculopapular and characteristically involves the oral mucosa, face, and forearms before spreading to the trunk and lower extremities. The rash also evolves, passing through a vesicular stage after 1–2 d, followed by tense, deeply embedded pustules (**Fig. 3**) that crust over after about a week. As the scabs fall off, they leave pitted scarring that tends to be more severe on the face *(15)*.

There are also two variants of smallpox that together account for about 10% of cases *(15)*. In the fatal malignant form, the initial maculopapular skin lesions do not evolve into pustules but remain soft and velvety, lend-

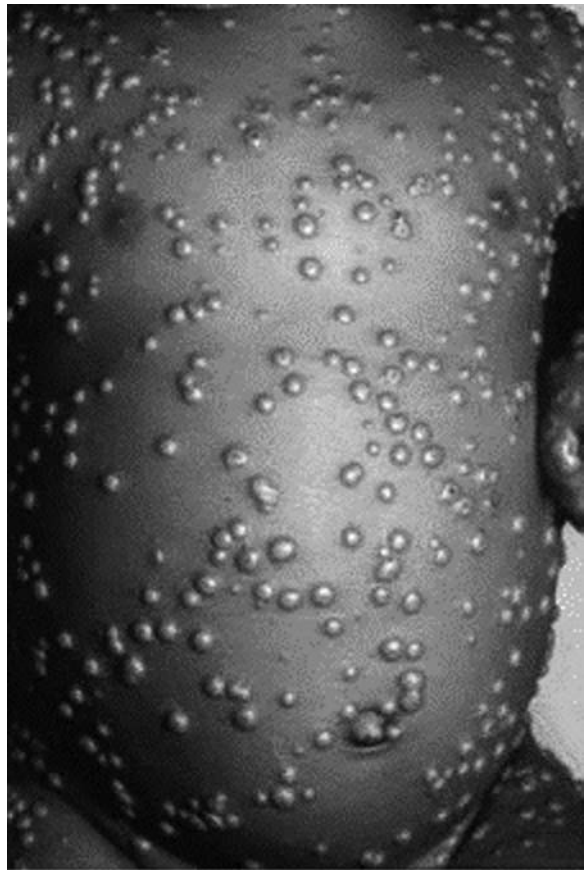

Fig. 3. Deeply embedded pustules of smallpox. Copyright WHO/LD Ladnyi. Reproduced by permission of the World Health Organization from "Smallpox and its Eradication" by Fenner, F, et al., 1988.

ing the skin a reddish hue. Desquamation may occur, and this form is often but not always fatal. On the other hand, the hemorrhagic form is universally fatal; it is heralded by a dusky erythema and then widespread petechiae and hemorrhages. Patients die within 5 or 6 d of the onset of the rash. For unknown reasons, this form is more common in pregnancy.

If smallpox were aerosolized and secretly released as a biologic weapon, infection of as few as several dozen people could easily result in a public health disaster, since it is estimated that the epidemic could expand by a factor of 10–20 with each subsequent generation of cases.

Diagnosis

Diagnosis can be made by electron microscopy identification of the brick-shaped orthopox virus in fluid carefully collected from vesicles or pustules. The agent, variola virus, is a member of the *Orthopox* genus, sharing similar morphology and antigenicity with other members, including vaccinia, monkeypox, and cowpox *(16)*. They are relatively large and complex viruses. However, it is important to establish a presumptive clinical diagnosis. Smallpox can be distinguished from chickenpox (varicella) since the lesions of varicella are superficial, develop rapidly, usually do not involve the palms and soles, are more concentrated on the trunk, and, most important of all, are at different stages of maturation in different areas of the body. Smallpox features lesions that progress gradually at the same pace over the entire body; they are deep and more highly concentrated on the face and extremities, often involving the palms and soles *(15)*. One or two days of prodromal symptoms are also common with varicella, typically fever and fatigue, but these symptoms are usually far milder than the profound fatigue, high fever, and severe headache and backache commonly seen with smallpox.

Treatment

In a combat situation, in which many soldiers are in close quarters, both varicella and smallpox pose a threat since they can be easily transmitted from person to person, rapidly depleting the ranks. Of course, smallpox poses the far greater threat both because most service members are likely to have little or no immunity and because of the severity of the illness and high mortality rate. Any case of smallpox anywhere is an international public health emergency, requiring immediate quarantine with respiratory isolation of all direct contacts of the infected case(s) for 17 d, and vaccination of all potentially exposed individuals *(15)*. This should include revaccination of treating medical personnel and others in close contact with the infected individuals, since there is evidence that immunity is not sustained (most smallpox victims in the 1960s had vaccination scars) *(17)*.

Vaccination should be provided for any individual within 4 d of suspected exposure, when it is likely either to prevent illness, or at least to result in a significantly milder, nonfatal course *(17)*. Emphasis must be placed on vaccination, in part because there is no antiviral therapy that has proven efficacy against smallpox, although there is a nucleoside analog DNA polymerase inhibitor (cidofovir) that may help to prevent

the development of infection if it is provided within 2 d of exposure but that shows no advantage over vaccination and has significant nephrotoxicity *(15)*. The mainstay of therapy if an outbreak of smallpox occurs is likely to be supportive, with prompt treatment of bacterial superinfection if it occurs.

Smallpox Vaccine

The vaccine stores held by the Centers for Disease Control and Prevention (CDC) were produced by Wyeth and feature a live strain of the vaccinia virus derived from the New York City Board of Health virus *(15)*. Administration has been through intradermal inoculation with a bifurcated needle, creating a scar at the site over the shoulder. When vaccination was widespread, most tolerated it well, but on occasion severe, even fatal, side effects occurred, including eczema vaccinatum and Stevens-Johnson syndrome.

TULAREMIA

Francisella tularensis, the causative agent of tularemia, has potential as a bioterrorist agent because it is highly infectious, easily disseminated, and associated with significant morbidity and mortality. Having said that, it is less dangerous than smallpox because there is effective treatment, and less problematic than anthrax, because treatment can still be successful if initiated after patients already have prominent signs and symptoms of infection. Tularemia is a widespread zoonosis, first identified in rodents in the early 20th century and later recognized as a cause of morbidity and mortality in humans in both America and Europe *(18)*. Small mammals serve as a reservoir of infection, with disease transmission occurring through contamination of water, soil, and food, as well as through arthropods such as mosquitoes and ticks *(19)*. Both the United States and the Soviet Union researched its use as a biologic weapon during the Cold War, with the Soviets reportedly weaponizing strains engineered for resistance to antibiotics and vaccines *(18)*. An aerosol form is the one most likely to be used by terrorists; if released in an urban area, it could be expected to result in tens of thousands dead and hundreds of thousands with significant morbidity.

After a 3–5-day interval, aerosol exposure would be expected to result initially in fever and upper respiratory symptoms that are indistinguishable from influenza and flu-like viral syndromes *(18)*. How-

ever, relatively rapid progression to pneumonia and/or pleuritis, and the abrupt onset in a large number of individuals including children and young adults (with life-threatening pulmonary and systemic symptoms in many cases) should lead to consideration of a bioterrorist incident, with tularemia, anthrax, and plague in the differential. The latter two agents would be expected to have more fulminant courses, with anthrax fatally progressing despite antibiotics, and plague also rapidly progressing to septic shock and death in many cases. Tularemia characteristically causes a bronchopneumonia involving one or more lobes, but hilar adenopathy and pleural effusions are not uncommon *(18)* (**Fig. 4**). Although this infection is more severe than a viral syndrome or atypical pneumonia, the rate of progression usually allows for initiation of therapy, and there is usually a good response to antibiotics.

It is unlikely that bioterrorist-disseminated tularemia will be confused with an epidemic of zoonotic origin. Although naturally occurring outbreaks have been reported, they have invariably occurred in rural areas, for example, from aerosolization of the bacteria with movement of hay infected by rodents on a farm or with an inadequately treated water source infected by small animals. Many naturally occurring cases result from contact with infected animals or arthropods, with entrance to the body not through the lungs, but through the mucous membranes, conjunctivae, or breaks in the skin. This is known as the ulceroglandular form of tularemia *(19)*. Typically, the first sign of infection is a papule at the inoculation site, which progresses to a pustule and later ulcerates over several days, with or without an eschar *(19)*. The bacteria spread to the local lymph nodes, which may become fluctuant, and in turn may disseminate the infection systemically. Lymphatic involvement may also occur in the absence of an identifiable inoculation site, which is known as the glandular form. Another form, most common with ingestion of contaminated food or water, is the oropharyngeal form, with prominent exudative pharyngitis or tonsillitis with or without ulcers, cervical adenopathy, and fever *(18)*. Finally, there is a typhoidal form that is characterized by a number of nonspecific symptoms such as fever, chest pain, cough, and abdominal pain, but also commonly includes pneumonia *(18)*. No site of inoculation can be identified in cases of typhoidal tularemia. A variety of organ systems may be involved, resulting in pericarditis, enteritis, appendicitis, peritonitis, meningitis, and erythema nodosum *(19)*.

One interesting clinical finding is that nearly half the patients with

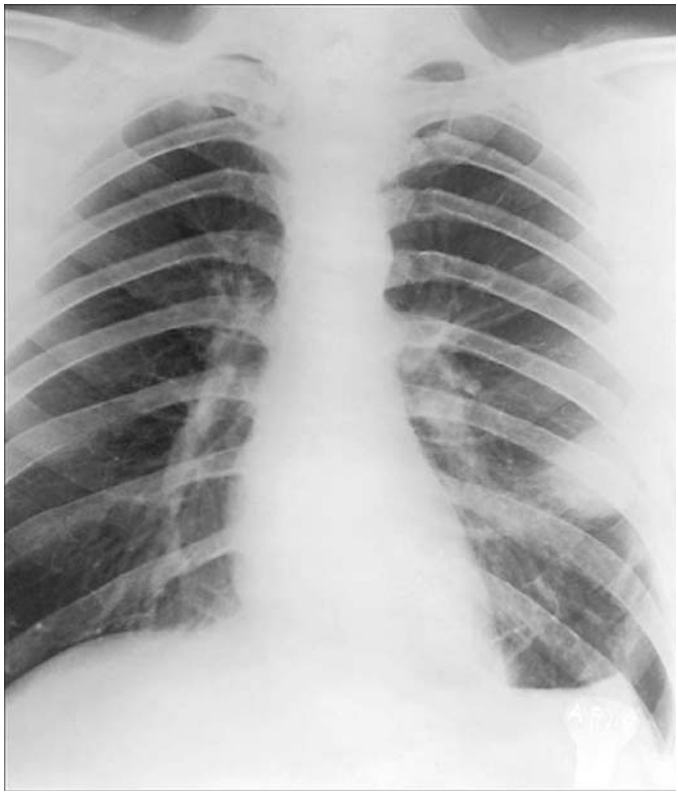

Fig. 4. Chest X-ray of tularemia, with left hilar enlargement, left lower lung field infiltrates, and tenting of left hemidiaphragm. (Reprinted from ref. *18*.)

tularemia may have pulse-temperature dissociation *(20)*, i.e., their heart rate does not rise in proportion to the degree of fever they experience. Sepsis may result with any form of tularemia, but it is much more common with inhalational exposure and with the typhoidal form. If the patient appears septic, prompt treatment is necessary in an attempt to prevent deterioration to stupor and coma, multiorgan failure, acute respiratory distress syndrome, and disseminated intravascular coagulation with bleeding complications.

Diagnosis

F. tularensis is an aerobic Gram-negative coccobacillus *(18)*. It does not form spores like anthrax, but it has a thin lipopolysaccharide-

containing envelope and is able to last for weeks in cool water, damp hay, straw, soil, or dead animals. Routine cultures will not identify the organism. *F. tularensis* can be grown most easily on cysteine heart blood agar, chocolate agar, or buffered yeast agar, and in cysteine-enriched or thioglycollate broth *(18)*. Cultures of sputum or gastric aspirates have a much higher yield than blood in cases of inhalational exposure. Even with the proper media, growth can be delayed, so cultures should be observed for at least 10 d. Clearly, this is not a practical basis for treatment. Research and reference laboratories are able to identify *F. tularensis* within hours using a variety of techniques including PCR, fluorescent-labeled antibodies, antigen detection assays, and enzyme-linked immunoassays *(18)*. Laboratory personnel should be alerted to take special precautions to protect themselves from infection.

TREATMENT

Streptomycin given intramuscularly or gentamicin either intravenously or intramuscularly, for 10 d, is the preferred treatment for tularemia *(18)*. Intravenous ciprofloxacin is also effective. Doxycycline and chloramphenicol are alternatives, but treatment failures and relapses have been reported, so they should be continued for at least 14–21 d **(Table 1)**. For those with probable exposure to tularemia in a bioterrorist incident, without clinical illness, oral administration of either ciprofloxacin or doxycycline is recommended. Human-to-human transmission has not been reported for tularemia, so isolation of individuals is not necessary.

PLAGUE

Yersinia pestis, the causative agent of plague, has profound historical significance and is responsible for the death of millions in several pandemics that afflicted most of the world. The first plague on record began in Egypt in 541 AD, wiping out 50–60% of the population of North Africa, Europe, and parts of Asia *(21)*. The second pandemic cast its dark shadow across Europe in the 14th century, this time felling one-third of the population *(22)*. A third pandemic started in China in the mid-19th century, again killing millions *(21)*. It poses a significant threat as a bioterrorist agent by virtue of its high mortality rate as well as the ease with which the pneumonic form is transmitted from person to person. There is evidence that the Japanese Army utilized it as a biologic weapon in Manchuria in the 1930s *(23)*. The United States and

the Soviet Union both identified methods for aerosolizing plague during the Cold War, and this is likely to be the method of distribution that terrorists would attempt to use. Release over a large city could infect tens of thousands, and as many as 25% of them might die.

Historically, plague has most often been disseminated by bites from infected fleas. Flea bites usually lead to bubonic plague, named for the characteristic buboes that rapidly develop in lymph nodes near the bite *(22)*. The initial symptoms of infection, usually occurring 2–8 d after the bite, are nonspecific (fever, chills, and weakness), but a bubo (a tender, warm, erythematous, edematous, nonfluctuant lymph node) erupts within another day. The bacteria quickly reproduce within the lymph nodes, leading to destruction and necrosis, and in many cases seeding the bloodstream to induce septic shock, disseminated intravascular coagulation, and a rapid downward spiral. Occasionally, sepsis occurs in the absence of clear lymph node involvement. Seeding of the lungs occurs in as many as one in six patients, resulting in a secondary pneumonic plague, characterized by chest pain, dyspnea, cough, and hemoptysis *(22,23)*.

Primary pneumonic plague has rarely occurred naturally but would be the expected presentation after inhalational exposure due to bioterrorism. Symptoms would probably develop more rapidly, 2–4 d after exposure, starting with fever, dyspnea, and cough sometimes productive of clear watery or even purulent sputum and/or hemoptysis *(22)*. Pulmonary manifestations are likely to be accompanied by gastrointestinal symptoms in some cases, with nausea, vomiting, abdominal pain, and diarrhea. Without prompt treatment, progression to septic shock is likely in many cases, and meningitis is an expected complication in some. Findings on chest X-ray will often include infiltrates or consolidation of one or more lobes *(22)* (**Fig. 5**).

Diagnosis

Y. pestis is a Gram-negative rod or coccobacillus that can best be cultured on blood agar or MacConkey agar *(22)*. Culture will usually be positive in 48 h, but the initial diagnosis must be primarily clinical, in the setting of large numbers of previously healthy patients developing severe pneumonia. The initial presentation may be difficult to distinguish from anthrax or even tularemia, but the presence of hemoptysis is more suggestive of plague. Fortunately, there are antibiotics that can cover all three pathogens reasonably well.

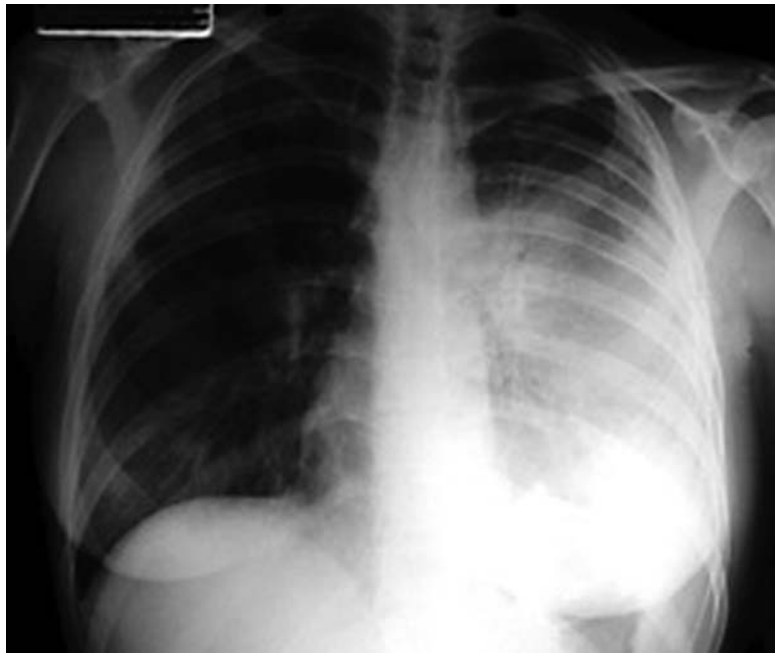

Fig. 5. Chest X-ray of plague, with dense consolidation within left lung. (Reprinted from ref. *22*.)

Specialized laboratories, including the CDC, the military, and some state health departments, have rapid diagnostic tests including enzyme immunoassay, antigen detection, and PCR *(22)*.

Treatment

Intravenous streptomycin or gentamicin is the recommended treatment for active plague infection *(22)* (**Table 1**). Tetracycline and doxycycline have also been effective for naturally occurring plague, but resistance has been identified in some areas of the world. The quinolones, including ciprofloxacin, levofloxacin, and ofloxacin, have been highly effective against experimental pneumonic plague in mice but have not been included in treatment trials of plague in humans, and have not been approved by the Food and Drug Administration (FDA) for this use *(22)* Chloramphenicol is also thought to be effective, although no clinical trials have documented this. If large numbers of individuals are believed to have been exposed, as in a terrorist or battlefield biologic agent release,

doxycycline or ciprofloxacin are the best choices for postexposure prophylaxis *(22)*. In fact, it is fortuitous that either of these well-tolerated antibiotics are effective prophylaxis against plague, tularemia, and anthrax. Unfortunately, one must also keep in mind the possibility that terrorists may genetically engineer resistance to one or more antibiotics, so cultures and sensitivity tests should be performed as early as possible.

There is no vaccine currently available to prevent plague. A vaccine that was previously licensed in the United States afforded protection against bubonic, but not pneumonic, plague *(24)*.

BOTULINUM

Botulinum toxin is not an agent capable of replicating, as are each of those described above. A toxin is not itself a biologic agent but is a chemical that is made by a living organism. However, it is a serious bioterrorism threat because it is relatively easy to produce, it is extremely potent (thought to be the most lethal poison in existence) so only a small amount is required, and most significant of all, it has high morbidity and mortality rates. Japan reportedly killed some prisoners in Manchuria in the 1930s by feeding them *Clostridium botulinum* cultures, and several nations have since explored the potential of botulinum toxin as a biologic weapon *(25)*. Most notably, Iraq weaponized enough botulinum during the Gulf War to kill the entire world's population but did not utilize the weapons *(25)*. Also, bioterrorism attempts by the Japanese cult Aum Shinrikyo in the early 1990s to use botulinum toxin were unsuccessful *(5)*. Ironically, at far lower doses, botulinum toxin also has positive medicinal uses, as effective treatment for neurologic conditions such as blepharospasm and torticollis.

Diagnosis

Botulinum toxin is a dichain polypeptide produced by *C. botulinum*, a spore-forming, obligate anaerobe *(25)*. The CDC and some public health laboratories can perform assays for the toxin in serum (if obtained before antitoxin is given), stool, or gastric contents that are vomited or aspirated *(25)*. Cultures can also be done. However, results are likely to take days to return, so it is important to have high clinical suspicion and to try to make a presumptive diagnosis on that basis. Bioterrorism-related cases of botulism are most likely to be the result of inhalational exposure. The toxin is absorbed and hematologically disseminated to the neuromuscular junction. Irreversible binding to the neuron cell

membrane blocks the release of acetylcholine, causing flaccid paralysis of the muscle. Paralysis may progress very quickly but is characteristically symmetric and involves the cranial nerves first, causing diplopia, dysarthria, dysphonia, dysphagia, ptosis, and blurred vision *(25)*. Paralysis then descends, taking away head control and then resulting in generalized weakness, hypoventilation, and decreased muscle tone and reflexes. A toxin, rather than an infection, causes the illness, so patients characteristically do not have a fever, and since the toxin does not enter the central nervous system, the mental status should not be affected, and analysis of cerebrospinal fluid should be unremarkable *(25)*.

The descending nature of the paralysis is particularly helpful in distinguishing botulism from Guillain-Barré syndrome, which usually manifests by an ascending paralysis with early hypo- or areflexia. The symmetric paralysis, as well as the lack of mental impairment, of botulism is helpful in distinguishing it from a stroke or intracerebral tumor or infection. Finally, electromyography (EMG) can be helpful in differentiating conversion disorder and myasthenia gravis from botulism. The EMG in botulism should show normal nerve conduction velocity, intact sensory nerve function, and a characteristic incremental response to repetitive nerve stimulation at 50 Hz *(25)*.

Treatment

Prompt administration of botulinum antitoxin to patients exhibiting neurologic abnormalities consistent with botulism is the most effective therapy available (**Table 1**). A licensed trivalent equine antitoxin has antibodies to neutralize botulinum toxin types A, B, and E, which are the most common types in naturally occurring cases of botulism (usually related to food poisoning) *(26)*. This is available from the CDC through local health departments. The U.S. Army also has a heptavalent (ABCDEFG) antitoxin that is under investigational status at this point *(26)*. Since the toxin binds irreversibly, antitoxin cannot reverse neurologic abnormalities that have already developed but is effective at preventing further neurologic deterioration.

Aside from administration of antitoxin, therapy is supportive, which can be intensive; in the face of a terrorist attack, it could easily overwhelm medical capabilities. Respiratory failure, often requiring intubation and mechanical ventilation, is the most significant complication. This can in turn lead to additional complications such as pneumonia and other infections. Recovery, by axonal regeneration to reinnervate

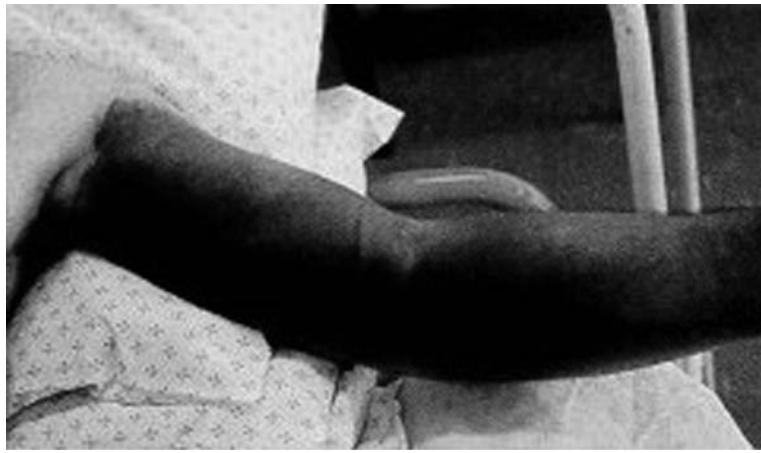

Fig. 6. Marked ecchymosis caused by coagulopathy associated with Crimean-Congo hemorrhagic fever. (Reprinted, with permission, from ref. *27*.)

flaccid muscles, can take weeks to months, creating a long-term demand for intensive medical care.

VIRAL HEMORRHAGIC FEVERS

A wide variety of RNA viruses can be classified together on the basis of their clinical manifestations, including fever, fatigue, malaise, or frank prostration, and increased vascular permeability (**Fig. 6**). Among the myriad viruses that have been identified are the agents of Lassa fever, Rift Valley fever, Ebola, and Crimean-Congo hemorrhagic fever, as well as the hantaviruses. Naturally occurring infections have usually been associated with contact with infected animals or their excreta or with arthropods that have taken blood from the animals. They pose biologic threats owing to their potential for aerosolization and dissemination, high infectivity, and significant morbidity and mortality (as high as 92%—the mortality reported for Ebola in Zaire) *(24)*.

The clinical presentation can vary owing to a wide variety of clinical factors, including the virulence and tendencies of the strain of virus, the dose, the form of exposure, and the immune system of the host. Virally induced hypotension and shock is one of the most problematic manifestations to treat, since intravenous fluids tend to exacerbate pulmonary edema owing to the increased vascular permeability. Ebola hemorrhagic fever, featuring shock, jaundice, and disseminated intravas-

cular coagulation *(27)*, is perhaps the most troublesome in this group, owing to the high mortality occurring in previous small outbreaks. A large number of individuals with fevers and hemorrhage should lead to suspicion of exposure to a viral hemorrhagic fever (VHF). For some of the VHF agents there are rapid identification techniques available, which are more useful than the relatively difficult, delayed viral isolation process. Vaccines are in various stages of investigation for some of the VHF agents, and ribavirin has shown efficacy for some, but the treatment is still largely supportive (**Table 1**) *(24)*.

VIRAL ENCEPHALITIDES

This category includes three alphaviruses from the Togaviridae family: Venezuelan, eastern, and western equine encephalitis viruses (VEE, EEE, and WEE), which are naturally transmitted by mosquitoes *(28)*. They also have significant potential as bioterrorist agents, since they can be manufactured relatively easily and cheaply, can be aerosolized (and are highly infectious in this form), can be genetically modified, and have significant morbidity and mortality. Initial symptoms typically include fever, chills, malaise, myalgias, and headache, sometimes accompanied by photophobia, sore throat, nausea, and vomiting. Nonspecific, prodromal symptoms may persist for days before frank neurologic findings such as cranial nerve palsies, paresis, seizures, and impaired mental status ensue *(28)*. The very young and very old tend to have more severe disease with higher mortality rates, although EEE is more lethal for healthy adults than VEE or WEE *(24)*.

Significant laboratory findings include leukopenia early in the course, followed by leukocytosis. In addition, the cerebrospinal fluid has a high white blood cell count with a lymphocytic predominance *(24)*. Viral isolation is possible early in the prodromal phase, and IgM antibodies to VEE can be measured. Treatment is supportive. Investigational inactivated vaccines exist but have had poor responses *(28)*. On the other hand, a live attenuated vaccine has shown promising efficacy against VEE but has had significant side effects *(24)*.

OTHER POTENTIAL BIOLOGIC WEAPONS

Several other bacteria and toxins have potential as biologic agents but for a variety of reasons are probably less likely to be employed by terrorists.

Bacteria

Brucella

Brucellosis occurs naturally as a result ingestion of inadequately prepared meat or dairy products or exposure to animals infected with one of a variety of species of brucella, which, like tularemia, are Gram-negative aerobic non-spore-forming coccobacilli *(29)*. Although brucella can be aerosolized, is highly infectious in this form, and has been explored by several nations as a biologic weapon, there is effective treatment, and the mortality rate is low even without treatment. However, brucella may involve a number of organ systems, causing organ-specific complications including pneumonia, endocarditis, osteomyelitis, septic arthritis, colitis, and orchitis, in addition to the common nonspecific findings of fever, chills, diaphoresis, weight loss, depression, fatigue, and chest or abdominal pain *(24,29)*. Although mortality may not be high, morbidity certainly is, with symptoms often persisting for months, and relapses possible over years. The onset of symptoms after exposure is likely to be more delayed than for other agents such as plague, anthrax, and tularemia, with an incubation period of 2–3 wk commonly seen *(29)*. Diagnosis is challenging, since cultures may take weeks to grow, and IgM antibodies may sometimes yield false positives with other potential biological agents including those causing tularemia and plague *(24)*. Newer techniques such as PCR and ELISA are in development. Combination therapy is recommended to prevent relapse, with doxycycline and rifampin the favored regimen, trimethoprim-sulfamethoxazole as an alternative to rifampin, and an aminoglycoside as an addition for deep tissue infections (**Table 1**)*(24)*.

Q Fever

Q fever is caused by *Coxiella burnetii*, an obligate intracellular rickettsia-like organism that is responsible for a worldwide zoonosis that also afflicts humans in contact with infected animals *(30)*. It poses a bioterrorist risk because it has a spore-like form that is resistant to environmental factors, it is highly infectious, and, like brucella, it has significant morbidity if not mortality. Q fever has a remarkable range of potential clinical presentations, from asymptomatic seroconversion in many cases to the acute onset of fever, chills, diaphoresis, headache, and other less frequent symptoms, such as fatigue, weight loss, myal-

gias, rash, chest pain, and cough *(24)*. Symptomatic presentations may also have a subacute onset and persist for months. Like brucella, Q fever may involve a number of different organisms, with hepatitis and pulmonary involvement (infiltrates and/or effusions, with chest X-ray abnormalities in about half of symptomatic patients) among the most common findings, and endocarditis a far less common but more serious complication *(24)*. Diagnosis of acute infection is challenging, with the most reliable diagnostic method being antibody detection by ELISA or indirect fluorescent techniques, but these do not turn positive until weeks to months after exposure *(24)*. The treatment of choice is doxycycline or tetracycline, with alternatives including quinolines and macrolides, with or without rifampin *(24)*. An effective vaccine is licensed in Australia but the FDA has not yet approved a vaccination in the United States *(24)*.

Toxins

T-2 Mycotoxins

These toxins are fungal in origin and can cause a number of effects depending on the method of exposure *(31)*. Skin contact can cause inflammation, pain, pruritis, and necrosis, eye exposure can damage the cornea, and damage to the mucosa of the upper gastrointestinal tract occurs with ingestion *(31)*. A clinical diagnosis can be made based on the findings plus a suspicious exposure, either a food, smoke, or "yellow rain. There are controversial allegations that mycotoxins were used in Laos, Kampuchea, and Afghanistan in the late 1970s or early 1980s *(31)*. Treatment is supportive, and steroids may be beneficial *(26)*.

Staphylococcal Enterotoxin B

This is one of seven intestinal toxins produced by *Staphylococcus aureus*, which can be pathogenic via either ingestion or inhalation *(32)*. Terrorism or battlefield incidents are more likely to involve inhalation, typically resulting in the acute onset of symptoms within hours of exposure, including cough, dyspnea, chest pain, fever, chills, headache, and myalgias *(26)*. Pulmonary edema may in a minority of cases progress to acute respiratory distress syndrome (ARDS) and hence to death, but persistent cough and fatigue with resolution after several weeks is likely to be more common. Diagnosis may be made by nasal swab within the first 24 h or by ELISA or PCR of respiratory secretions if access to a specialty laboratory is available *(26)*. Terrorist infection of

the food or water supply is also plausible, albeit less likely. This could be expected to result in severe abdominal cramping and diarrhea, sometimes accompanied by fever and headache, developing within a few hours of ingestion, but usually only lasting about 12 hs. In either case, treatment is supportive. Vaccines are in development *(32)*.

Ricin

Ricin has the dubious distinction of having been used in espionage assassination attempts, most famously as the cause of death of Bulgarian defector Georgi Markhov via a ricin-filled pellet fired from an umbrella *(26)*. Ricin is derived from the castor bean, and others have attempted poisoning by mixing it in food *(5)*. It also has potential as a weapon of mass destruction through aerosolization. In such a case, it would be expected to cause fever, cough, dyspnea, and nausea within 24 h of exposure and then progress to a diffuse necrotizing pneumonia with marked pulmonary edema, cyanosis, hypothermia, and death *(33)*. Clinical diagnosis may be supported by antigen detection with ELISA, or PCR, where available. There is a toxoid that can be given if exposure is feared, but treatment is otherwise solely supportive *(26)*.

COMBINATION USE OF WEAPONS OF MASS DESTRUCTION

It is important to keep in mind that terrorists or battlefield enemies may not use a single agent in isolation. Historically, most nations that have experimented with biologic and/or chemical weapons agents have attempted to develop several agents simultaneously. Most recently, during the Gulf War, Iraq is thought to have weaponized multiple biologic and chemical agents. The simultaneous use of multiple agents can be expected to further confound diagnostic efforts that are already likely to be hampered by the large numbers of afflicted individuals that may overwhelm the medical system both in number and severity of illness, clinical features that many of the agents share with each other as well as with common viral illnesses such as influenza, and the more widespread hysteria that will probably be engendered by initial reports of mass casualties. Genetic engineering to enhance virulence and/or resistance to treatment poses another serious concern. Perhaps most alarming of all is the thought of biologic agents used in conjunction with either nuclear weapons or an attack on a nuclear power plant. The combination of

bioterrorism plus the adverse effects of radiation on the gastrointestinal mucosa and hematologic cell lines, not to mention the potential elimination of segments of the medical infrastructure owing to blast damage or lingering radiation, would certainly be our worst nightmare.

On a more optimistic note, a nuclear blast might also destroy a simultaneously released biologic agent. There have been many efforts by terrorists to use biologic or chemical agents in recent years, and the great majority have been foiled for one reason or another. Particularly since September 2001, we have implemented more precautions and security measures. We also have better diagnostic and therapeutic modalities than ever, and the level of awareness of the medical community with regard to biologic and chemical weapons has been considerably heightened. The medical community is also more cognizant of the great significance of psychological sequelae of war and terrorist incidents. Significant somatic and psychological symptoms have been well documented after each war, from the Civil War through the Gulf War, probably attributable to the severe physical and psychological stresses associated with the life-threatening nature of war *(34,35)*. After the sarin attack in the Tokyo subway system, which injured more than 5500 people, the Japanese hospital that treated the largest number of victims documented the persistence of posttraumatic stress disorder in 60% of the victims more than 6 mo later *(36)*. There is no reason to think the psychological sequelae will be less after a bioterrorist incident, and treatment would be neither complete nor truly successful unless the psychological aftermath is also effectively addressed. We have learned much from prior incidents, and we can be confident that the next incident, however terrible it might be, will further expand the capabilities and knowledge of the medical community.

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