

The endothelium in sepsis: Source of and a target for inflammation

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Objective: To discuss a possible role of the endothelium in sepsis.

Data Sources: Studies published in biomedical journals and our own experimental results.

Study Selection: Studies on endothelial mechanisms in the context of sepsis.

Data Extraction and Synthesis: Changes in endothelial cells on activation by inflammatory stimuli are reviewed briefly; potential mechanisms that lead to endothelial damage during sepsis are discussed.

Conclusions: The endothelium is a key organ involved in the pathogenesis of sepsis. Dysfunction of or injury to the endothelium may be involved in the pathogenesis of multiple organ failure and should be discriminated from activation resulting from stimulation with inflammatory stimuli. Identification of the molecular mechanisms that contribute to endothelial dysfunction or damage is likely to provide novel targets for the treatment of sepsis. (Crit Care Med 2001; 29[Suppl.]:S21–S27)

KEY WORDS: endothelium; sepsis; endothelial markers; apoptosis; complement

Sepsis is one of the leading causes of mortality in intensive care units and continues to be a challenge for clinical investigators. Despite advances in intensive care medicine and the availability of a large number of antibiotics, the mortality rate from this condition is high, ranging from 15% to >60% in the case of septic shock. It is now generally accepted that sepsis results from an extensive triggering of the body's defense mechanisms by the invading microorganisms and their products (1, 2). These defense mechanisms include the release of cytokines, the activation of neutrophils and monocytes, and the activation of plasma protein cascade systems such as the complement system, the intrinsic (contact system) and extrinsic pathways of coagulation, and the fibrinolytic system. There is now abundant evidence in the literature that these inflammatory mediator systems all become activated to some extent during sepsis. Activation and release of these mediators occur in the tissues, as well as in the circulation.

Endothelial cells are the predominant lining cells and are in close and intimate contact with blood. These cells are not inert but rather may adapt their function upon interaction with inflammatory mediators—for example, by promoting fibrin formation. In addition, they may generate inflammatory mediators themselves. Presumably, endothelial cells play a pivotal role in the pathogenesis of sepsis, not only because they may influence the inflammatory cascade but also because upon interaction with excessive amounts of inflammatory mediators, the function of these cells may become impaired. It is likely that a general dysfunction of the endothelium is a key event in the pathogenesis of sepsis. In fact, such a dysfunction could explain most of the clinical manifestations and complications of sepsis. This article addresses the role of the endothelium in sepsis, including a summary of basic aspects of endothelial physiology and discussions of the changes in the endothelium resulting from activation, currently available methods to assess endothelial activation in patients, endothelial dysfunction as a key process in sepsis, and a possible process that causes endothelial damage and dysfunction.

Physiologic Role of the Endothelium

The endothelium is the lining between blood and the tissues. In adult humans, the total surface of this cell population has been estimated to be >1000 m² (3). Hence, there is intensive contact of blood cells and

plasma proteins with endothelial cells. Endothelial cells are not inert cells; indeed, under physiologic conditions, they exert a number of functions that are important for normal homeostasis (Fig. 1). These functions include prevention of coagulation, orchestration of the migration of blood cells into the tissues by expression of adhesion molecules, production of chemoattractant compounds, regulation of the microcirculation by dictating the tonus of the arterioles, regulation of blood pressure (via their effects on arterioles), and regulation of vasopermeability.

Under physiologic conditions, endothelial cells inhibit blood coagulation by various endothelial mechanisms: a) they express thrombomodulin, which not only binds thrombin but also shifts the specificity of this clotting enzyme from fibrin to protein C (4); activated protein C in the presence of its cofactor protein S catalytically inactivates activated factors V and VIII of the clotting system; b) they have proteoglycans, such as heparan sulfate, on their surface, which can bind and potentiate the inhibitors antithrombin III and tissue factor pathway inhibitor (5, 6); and c) they release low amounts of the plasminogen activator tissue-type plasminogen activator (tPA). Under normal conditions, the endothelium also inhibits platelet aggregation by producing prostacyclin and nitric oxide (NO) (7) and by expressing a surface-bound adenosine diphosphatase, which hydrolyzes an important agonist of platelets, adenosine

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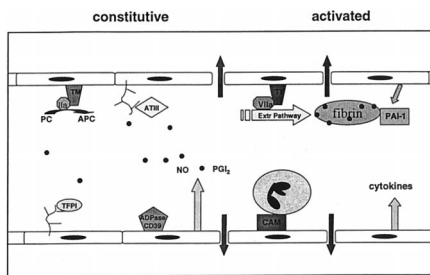


Figure 1. Endothelial functions under normal conditions (*left*) and upon activation (*right*). *TM*, thrombomodulin; *APC*, activated protein C; *Iia*, thrombin; *TFPI*, tissue factor pathway inhibitor; *NO*, nitric oxide; *PGI₂*, prostaglandin I₂; *ATIII*, antithrombin III; *TF*, tissue factor; *VIIa*, activated clotting factor VII; *extr*, extrinsic; *PAI-1*, plasminogen activator inhibitor-1; *CAM*, cellular adhesion molecule.

diphosphate (8). Under physiologic conditions, the endothelium does not stimulate adherence of peripheral blood cells (except for that in special sites) because the endothelium expresses a small number of so-called adhesion molecules, i.e., molecules that specifically recognize and bind counterstructures on blood cells, and thereby promote adhesion of these cells. By producing the vasodilating agents prostacyclin and NO, the endothelium regulates the tonus of the arterioles, thereby regulating the microcirculation, and decreases blood pressure (9).

The Inflammatory Endothelium

Upon stimulation by various cytokines, including interleukin (IL)-1 α , IL-1 β , and tumor necrosis factor (TNF)- α , and also upon interaction with other inflammatory mediators, such as activated complement, the functions of the endothelium may be grossly altered. These changes are referred to as activation. They encompass a change from an anti- into a procoagulant surface; the expression of adhesion molecules; the production of inflammatory mediators, including chemoattractant agents; and the production of vasoactive compounds. It is notable that although the endothelium generally is said to be activated upon interaction with inflammatory cytokines such as TNF- α , this does not necessarily mean that each individual endothelial cell displays all of the changes described below. Whether the endothelial cells display all or only some of the features discussed below will depend on the time of stimulation and the nature of the inflammatory stimulus and its concentration.

Moreover, the response of the endothelium to inflammatory stimuli may differ greatly at different sites in the body.

Endothelium triggered by inflammatory stimuli loses its anticoagulant properties and becomes a procoagulant surface. The physiologic goal of these changes, which are meant to occur locally and not systemically, is to wall off an infectious process. Some microorganisms produce agents that lyse fibrin, demonstrating that at least these organisms have adapted to this defense mechanism. Upon triggering with such stimuli as TNF- α , IL-1, or endotoxin, endothelial cells lose thrombomodulin and heparan sulfate (10–12) and begin to synthesize tissue factor (TF) (13, 14), which after a couple of hours appears on the surface of the cells. As a consequence, the endothelial cells no longer activate protein C; lose the clotting inhibitors tissue factor pathway inhibitor and antithrombin III; and, via interaction of TF with clotting factor VII, activate the extrinsic pathway of the coagulation system (Fig. 1). It is notable that factors VIIa and Xa and thrombin, which are generated upon extrinsic pathway activation, can further activate the endothelial cells via proteolysis and activation of so-called protease-activatable receptors (15). Four different protease-activatable receptors have been identified. Upon stimulation of protease-activatable receptors, the cells may start to produce a number of inflammatory mediators, including cytokines and chemokines, and to express adhesion molecules (16–20). Thus, protease-activatable receptors may amplify inflammation during coagulation processes.

In addition to the mechanisms described above, endothelial cells may support coagulation by yet another mechanism: the formation of phospholipid microparticles (21). These microparticles are small, vesicle-like structures that often carry the phospholipid phosphatidylserine on the outer leaflet of their membrane (under normal conditions, this phospholipid occurs only in the inner leaflet of cell membranes), which serves as an anchor for vitamin K-dependent clotting factors. Microparticles may support clotting via the extrinsic pathway by exposing TF. Procoagulant microparticles can be generated from platelets, among others, by insertion of the complement membrane-attack complex into the cell membrane (22). This also may occur with endothelial cells. To what extent the formation of these particles

points to irreversible damage to the cells is not clear. However, cells that die of apoptosis may form microparticles that, although not always TF positive, are phosphatidylserine positive. Hence, the presence of endothelium-derived microparticles may point to irreversible damage. Although thrombin-generating microparticles have been described in sepsis patients (23), a thorough evaluation of their role in sepsis has not been done.

Suffredini et al (24) observed that the fibrinolytic system becomes activated in humans who are challenged with a low dose of endotoxin, as evidenced by increased plasmin-antiplasmin complexes. This activation is transient and lasts for up to 3 hrs after the endotoxin challenge. Thereafter, activation is increasingly inhibited by elevating levels of plasminogen activator inhibitor (PAI)-1, the main inhibitor of tPA and urokinase-type plasminogen activator, yet the coagulation system is still activated after this time (25). Thus, a few hours after the endotoxin challenge, a procoagulant state is created, characterized by an imbalance between thrombin and plasmin. Although not conclusively demonstrated, this procoagulant state likely results from the activation of the endothelium. TNF infusion *in vivo* induces the release of tPA from the endothelium, which triggers the activation of plasminogen. It is notable that this *in vivo* effect probably occurs via the release of vasopressin (26), because *in vitro*, this cytokine down-regulates tPA synthesis by the endothelium (27). A few hours after stimulation with TNF, the endothelium also produces and releases PAI-1, which will neutralize tPA and increasingly inhibits the fibrinolytic system, yielding a procoagulant state, because coagulation still proceeds (27, 28). This mechanism seems to be important in sepsis, as high plasma levels of PAI-1 are associated with a poor outcome (29–31).

Under physiologic conditions, the endothelium hardly expresses adhesion molecules. Upon stimulation with a variety of agonists, such as cytokines, this changes dramatically: the cells express, in order of appearance on their membranes, P-selectin (optimal 15 mins after stimulation), E-selectin (optimal at 6 hrs), intercellular adhesion molecule-1 (optimal at 24 hrs), and other adhesion molecules such as vascular cell adhesion molecule-1. As a result, leukocytes interact with these cells. This initially leads to rolling of the leukocytes over the endo-

thelium, followed by strong adherence, and finally transmigration into the tissues. This process requires some activation of the leukocytes as well. This is achieved by production of leukocyte agonists such as platelet-activating factor and chemokines by the endothelium. Studies in knockout mice have confirmed the essential role of adhesion molecules in the process of migration of leukocytes into the tissues. A detailed discussion of the precise role of individual adhesion molecules and their ligands on leukocytes is beyond the scope of this article. The reader is referred to excellent reviews for detailed information on the role of specific adhesion molecules in the interaction of leukocytes with the endothelium (32–34).

The endothelium produces a number of vasoactive compounds that regulate the tonus of the arterioles and hence have a great influence on blood pressure. These compounds include the vasodilating NO and prostacyclin and the vasoconstricting endothelins (7, 35–38). It is notable that NO and prostacyclin also are potent inhibitors of platelet aggregation. The production of these agents is grossly modified upon stimulation with inflammatory mediators. The production of NO can be regulated by two different mechanisms, depending on the type of NO synthase (the key enzyme necessary for the production of NO). Under normal conditions, the endothelial cells constitutively produce one type of NO synthase: constitutive NO-synthase. This enzyme produces NO in a calcium-dependent manner. Inflammatory mediators such as bradykinin, histamine, and also thrombin, by binding to specific receptors, can induce such an intracellular increase of calcium ions (39). Release of these agents *in vivo* causes a rapid decrease in blood pressure. Presumably, hypotension during anaphylactic reactions (e.g., those induced by insect stings) is mediated by this mechanism. It is notable that most of the agents that activate constitutive NO synthase also induce the (rapid) expression of P-selectin. Upon stimulation with cytokines such as TNF- α or IL-1, endothelial cells may produce another type of NO synthase: inducible NO synthase (40). This enzyme produces large quantities of NO in a calcium-independent manner. Expression of inducible NO synthase likely explains the drop in blood pressure in sepsis (41). Indeed, hypotension in patients sepsis can be reversed upon treatment with NO inhibitors (42–44). Be-

cause of other effects, the treatment of sepsis patients with NO inhibitors was not successful despite initial optimistic results.

In addition to generation of vasodilating agents, endothelial cells stimulated with various agonists may form vasoconstricting compounds. These compounds include endothelins. Although increased levels of endothelins have been described in patients with sepsis (45–47), their precise role in the pathogenesis of sepsis is not well known. It is likely that they compensate to some extent for the formation of vasodilating agents.

The activated endothelium further produces an array of inflammatory mediators. For example, it produces lipid mediators such as platelet-activating factor and prostaglandins; cytokines such as IL-6; and chemokines, complement factors, and others. Clearly, the endothelium should not be thought of as an inactive cell layer constituting the vessel wall but rather as a director orchestrating the inflammatory cascade.

Methods of Assessing Endothelial Activation in Septic Patients

Several methods are available for assessing activation of endothelial cells in patients. First, von Willebrand factor levels as well as the multimerization of this protein can be assessed (48–51). Upon stimulation, multimers of von Willebrand factor are released from Weibel-Palade bodies by the endothelial cells. Thus, higher levels of von Willebrand factor and increased multimerization in the blood point to endothelial activation. Von Willebrand factor changes have been described in experimental human endotoxemia, upon infusion with TNF- α , and in patients with sepsis.

Second, levels of tPA and PAI-1 provide information about endothelial cells, because upon stimulation of endothelial cells, levels of these fibrinolytic proteins increase in the circulation. It is notable that increases in PAI-1 also may result from platelet activation (52). In experimental human endotoxemia as well as in baboons with sepsis, levels of tPA have been shown to increase before levels of PAI-1 rise, during which interval plasminogen is activated (24, 25, 53). A few hours after tPA levels rise, PAI-1 levels also increase, which coincides with reduced plasminogen activation, presumably because PAI-1 inhibits tPA. In-

creased levels of tPA and, in particular, PAI-1 are common in patients with sepsis and are associated with a worse outcome (29–31).

Third, circulating levels of soluble adhesion molecules may increase upon endothelial activation (54). Upon stimulation, endothelial cells express several adhesion molecules, which can be cleaved off to yield soluble adhesion molecules. Circulating levels of soluble intercellular adhesion molecule-1 and soluble E-selectin are most frequently determined as a variable for endothelial activation. Increased levels of these adhesion molecules have been observed in patients with sepsis (50, 55).

Endothelial cells circulate in very low numbers (56); hence, these numbers as well as their functions could be analyzed. Although these kinds of studies have been done in some viral infections, we are not aware of such studies in sepsis models or patients with sepsis.

Endothelial Activation or Damage in Sepsis?

Although cytokines may be produced by virtually every cell in the body, it can be speculated that the strongly elevated IL-6 levels in sepsis indicate production by the endothelial cells. For example, comparison of IL-6 levels in a sublethal *Escherichia coli* model in baboons with those in the lethal variant of this model reveals that the early rise (after 2–4 hrs) is comparable and likely results from monocyte/macrophage activation. In the sublethal model, levels decrease thereafter; whereas in the lethal model, levels increase further, reaching maximal levels hours thereafter. It is notable that this course of IL-6 explains why this cytokine is one of the best markers for predicting outcome (at least in patients with sepsis on admission to the intensive care unit) (57). Although it is not known under exactly which conditions IL-6 is produced by cells *in vivo*, it has been speculated that IL-6 acts as an alarm hormone, telling the organism that the producing cells are in a dangerous situation (58). Hence, the strong IL-6 response in the lethal sepsis models may point to an alarm situation of the endothelium, i.e., that the cells are injured by the inflammatory and coagulation processes. If correct, this interpretation has several important implications. First, the stage in the lethal *E. coli* model in which IL-6 levels are still rising while in the sublethal models IL-6

levels are diminishing precedes the stage of overt organ dysfunction. This indicates that the alarm situation of the endothelium precedes multiple organ dysfunction, suggesting that this organ dysfunction is triggered by endothelial dysfunction. Second, the variables that indicate endothelial activation discussed in the preceding section do not reflect endothelial damage, because levels of these variables may increase in experimental human endotoxemia. In the latter situation, there is neither a protracted increase in IL-6 nor clinical suspicion of endothelial damage.

The distinction of endothelial activation from endothelial dysfunction or damage is important because it may help us to understand the pathogenesis of sepsis and provide novel targets for therapy. In the case of activation, it is likely that a number of functions of the endothelium, such as permeability, are still intact, whereas in the case of dysfunction, they may be impaired. Indeed, capillary leak may occur as a complication of sepsis. It can be speculated that after endothelial dysfunction, homeostasis is increasingly impaired in organs, leading to organ dysfunction. It is tempting to speculate that the anticoagulant agents that improve outcome in lethal sepsis models, such as anti-TF monoclonal antibody, tissue factor pathway inhibitor, high-dose anti-thrombin, and activated protein C (59), in some way attenuate endothelial dysfunction. In our view, there is no good biochemical variable to discriminate endothelial activation from endothelial dysfunction. Although soluble thrombomodulin is cleaved off from endothelial cells by neutrophil proteinases (60), levels of this variable in plasma also increase during experimental human endotoxemia (61), in which condition, as stated above, severe endothelial dysfunction likely does not occur. Endothelial dysfunction or damage at the end may result in apoptosis of endothelial cells (Fig. 2); hence, apoptosis markers in plasma may provide variables for severe endothelial damage. We recently analyzed some apoptosis markers in plasma from patients with sepsis, such as soluble Fas and nucleosomes (S. Zeerleder et al., manuscripts in preparation). Indeed, levels of circulating nucleosomes seemed to be elevated in the majority of patients with sepsis and multiple organ dysfunction, whereas these levels were lower in patients with systemic inflammatory response syndrome or fever.

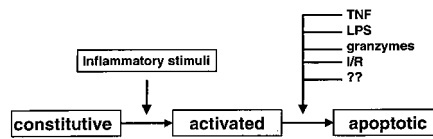


Figure 2. Transition of normal to activated to injured (dysfunctional) endothelium. Triggers for the transition of normal to activated endothelium are discussed in the text and not indicated here. Triggers for the transition of activated to injured endothelium are indicated. *TNF*, tumor necrosis factor; *LPS*, lipopolysaccharide; *I/R*, ischemia followed by reperfusion.

Mechanisms that Cause Endothelial Dysfunction in Sepsis

Several mechanisms may contribute to endothelial damage during sepsis. *In vitro* experiments have shown that activated neutrophils that adhere to the endothelial cells via adhesion molecules are well able to injure the endothelial cells by producing oxygen radicals and proteinases such as elastase (62–65). Also, in perfusion models, neutrophils have been shown to enhance TNF-mediated effects on permeability, e.g., in the lungs (66, 67). This mechanism likely is important in sepsis, as levels of degranulation products of neutrophils such as elastase and lactoferrin are increased in patients and correlate with outcome (68–70). Cytokines such as TNF can themselves induce apoptosis of endothelial cells (71, 72). Although less well known, cytokine-activated natural killer cells or cytotoxic T lymphocytes, together known as lymphokine activated killer cells, similarly can adhere to and injure the endothelium, resulting, for example, in increased permeability (73, 74). It has been claimed that this mechanism causes the severe capillary leak syndrome induced by high-dose IL-2 therapy, which shares many of the manifestations of sepsis (75, 76). We recently developed methods for measuring activation of lymphokine activated killer cells and for assessing plasma levels of soluble granzymes, proteinases that are released during degranulation of these cells and that resemble the neutrophilic elastase (77). Levels of soluble granzymes are markedly elevated in some forms of sepsis, notably, melioidosis (78). A moderate increase in these variables also occurs during experimental endotoxemia and occasionally in patients with sepsis (78, 79).

Another mechanism that may cause severe damage to the endothelium is

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ischemia/reperfusion injury. Reperfusion of ischemic tissues may elicit inflammatory reactions that cause additional damage, as has been shown in animal models such as the hindlimb ischemia model or acute myocardial infarction. This injury is caused by several mechanisms. A number of studies have indicated the importance of cytokines, complement, neutrophils, and adhesion molecules for ischemia/reperfusion injury (80–87). Decreases in intracellular adenosine triphosphate per se are sufficient to induce apoptosis or even necrosis in cells. Decreases in adenosine triphosphate also result in membrane alterations, because the phospholipid asymmetry of normal cell membranes requires energy and hence is lost under ischemic conditions (this phenomenon is called the flip-flop of a membrane). We hypothesized that these membrane alterations may trigger opsonization of cells with the acute phase protein C-reactive protein (CRP) in the presence of a cofactor, the enzyme secretory phospholipase A₂ (88, 89). Subsequently, bound CRP activates the complement system, which in turn stimulates neutrophils and other phagocytes (Fig. 3). Such a mechanism would explain why levels of activated complement correlate with outcome and severity of sepsis (90). Studies in patients with acute myocardial infarction have provided strong evidence for such a mechanism (88, 91). Whether this occurs in sepsis is not known yet. We demonstrated increased levels of complement activation products that are specific for CRP-mediated activation in patients with sepsis (92). Furthermore, in a case report of a patient who was dying of sep-

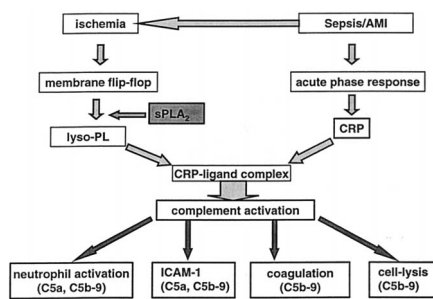


Figure 3. A hypothetical model explaining complement activation by C-reactive protein (CRP) in sepsis. As a result of stimulation by inflammatory mediators or ischemia, cells may produce interleukin-6 as an alarm hormone that, among others, triggers the production of acute-phase proteins such as CRP and secretory phospholipase A₂ (sPLA₂) and may undergo a membrane flip-flop. In the presence of sPLA₂, the flip-flopped membranes in the presence of sPLA₂ provide binding sites for CRP, which then activates complement. Because of the direct effects of complement activation or the indirect effects of neutrophils via activation, the cells become injured. AMI, acute myocardial infarction; lyso-PL, lyso-phospholipid; ICAM-1, intercellular adhesion molecule-1; C5b-9, terminal complement complexes consisting of the complement factors C5b through C9.

sis and multiple organ failure, we described depositions of CRP and activated complement in various involved organs (H.W. Niessen et al., manuscript submitted). Finally, we recently observed some improvement in multiple organ failure scores in patients who had sepsis and were treated with the complement inhibitor C1-inhibitor (C. Chaliezi et al., manuscript submitted). Thus, CRP- and complement-dependent damage to endothelial and other cells likely are relevant for sepsis.

Conclusions

The endothelium is a key organ involved in the pathogenesis of sepsis. Endothelial dysfunction or injury may be the trigger for the development of multiple organ failure, yet the distinction between endothelial activation and endothelial dysfunction or damage is difficult to define in clinical sepsis. Identification of the molecular mechanisms that contribute to endothelial dysfunction or damage likely will provide novel targets for the treatment of sepsis.

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Question and Answer Session After Scientific Review

Jean-François Dhainaut. Erik, C1-Inh also participates in the protection of a procoagulant state in sepsis. In these studies, did you find any modification of D-dimer, TAT complexes, or IL [interleukin]-6 levels?

C. Erik Hack. No, we examined septic patients and patients in postmyocardial infarction situations and did not see an effect on TAT complexes (which we took as a measure of clotting activation), fibrinolytic parameters, or D-dimer levels. We did observe reductions in IL-6 levels, particularly in the myocardial infarction patients. The reductions seen in the septic patients were not statistically significant.

Fletcher Taylor. Some studies have shown that endothelial cells under oxidative stress bind a mannan-binding protein, which can in turn activate complement. Could you comment on this in the context of your own studies?

C. Erik Hack. A recent study (1) has suggested that if you expose endothelial cells to oxidative stress, they start to bind mannan-binding lectin (MBL), which may explain the complement activation seen in these cells. Based on this article,

we looked for MBL binding in our *in vitro* systems but were unable to find any. We know that some sepsis patients have sky high levels of MBL activity; however, most septic patients have only mildly elevated MBL levels. We have also looked at tissue samples from our myocardial infarction patients, and only one of them showed strong MBL binding, but these studies have yet to be completed. We do not understand why this is the case, but it might be related to the fact that there are various allotypes of MBL that may have higher or lower affinity for ligands, etc. Finally, in case of AMI [acute myocardial infarction], we analyzed tissue homogenates from infarcted myocardium and observed good correlations between activation products specific for CRP [C-reactive protein]-mediated activation and overall activation, suggesting CRP is the main activator in AMI.

Konrad Reinhart. If I understand you correctly, you think that CRP may play a role in the pathophysiology of sepsis. How do you explain the fact that we only see elevated CRP levels 24 to 48 hrs after the onset of severe sepsis and septic shock?

C. Erik Hack. I think the timing of the release of CRP is an important factor to

consider, because there are several other acute phase proteins that actually inhibit this mechanism. For example, α -1-acid glycoprotein likely reduces neutrophil adhesion. In addition, we have seen a biphasic activation of complement in myocardial infarction patients similar to that seen by Fletcher Taylor in his baboon studies. Dr. Taylor has shown that the first phase of complement activation is a direct effect of the *E. coli* infection and is independent of CRP. The second phase, which starts after about 8 to 10 hrs, is characterized by the presence of activation products that are only formed during CRP-dependent activation. The same pattern holds for myocardial infarction patients. The first phase is caused by the thrombolytic action of streptokinase, which is a very nice complement activator. We then see a slow increase in CRP-dependent activation products after 6 to 8 hrs and the beginning of the activation mechanism after about 8 to 10 hrs.

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