The link of biocompatibility to cytokine production

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The link of biocompatibility to cytokine production. Recent studies suggest that chronic inflammation plays a role in the pathogenesis of cardiovascular disease. Cytokines released from jeopardized tissues stimulate the liver to synthesize acute phase proteins, including C-reactive protein (CRP). Baseline levels of CRP in apparently healthy persons or in persons with unstable angina constitute an independent risk factor for cardiovascular events. More recently, it has been suggested that CRP is useful not only as a marker of the acute phase response, but is also involved in the pathogenesis of the disease. CRP may, in fact, directly interact with the atherosclerotic vessels or ischemic myocardium by activation of the complement system, thereby promoting inflammation and thrombosis. Several studies in uremic patients have implicated CRP as a marker of malnutrition, resistance to erythropoietin, and chronic stimulation in hemodialysis. An increased cytokine production secondary to blood interaction with bioincompatible dialysis components has been reported by several studies; interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and mainly IL-6 are the three proinflammatory cytokines involved in the pathogenesis of hemodialysis-related disease. We have provided evidence for the occurrence of high CRP and IL-6 levels in chronic dialytic patients exposed to contaminating dialysate and suggest that backfiltration may induce a chronic, slowly developing inflammatory state that may be abrogated by avoiding backfiltration of contaminated dialysate. Therefore, CRP is implicated as a marker linking bioincompatibility associated with backfiltration and increased cytokine production with a clinical state of chronic inflammation.

The acute phase of the inflammatory response refers to the wide-ranging physiological changes that are initiated immediately after an infection or physical trauma has occurred [1].

The mammalian acute phase response is characterized by fever and changes in vascular permeability, along with profound changes in the biosynthetic profile of liver proteins.

Under normal circumstances, the liver synthesizes a characteristic range of plasma proteins at a steady-state concentration. Many of these proteins have important functions and higher plasma levels of these acute phase reactants (APRs), and the magnitude and rapidity of their induction following an acute phase response, together with their short half-life, suggest a particularly critical requirement in the establishment of host defense [2, 3].

More recently, the acute phase response has been more narrowly defined as the change in concentrations of a number of plasma protein that results largely from a rearrangement of plasma protein synthesis in hepatocytes. The proteins that manifest substantial change in concentrations are referred to as the APRs (Fig. 1) [4, 5].

Ceruloplasmin and the complement component C3 and C4 typically display only a modest acute phase response, while apotoglobin and fibrinogen may increase two- to fivefold.

In contrast, the so-called negative APRs (albumin and transferrin) are decreased in plasma concentration during the acute phase response to allow an increase in the capacity of the liver to synthesize the induced APRs.

More strikingly, plasma levels of the so-called major APRs can increase 1000-fold over normal levels; this group includes serum amyloid A (SAA) and C-reactive protein (CRP) [6].

STRUCTURE AND FUNCTION OF C-REACTIVE PROTEIN

Human CRP is a pentameric protein comprised of five identical, noncovalently bound subunits, arranged in cyclic symmetry in a single plane [7]. The gene for the CRP subunit in humans is encoded on chromosome 1 [8], and each subunit consists of a linear chain of 206 polypeptides with a single intrachain disulfide bond and a calculated molecular weight of 23,017 (Fig. 2) [9, 10].

The protein is nonglycosylated and nonphosphorylated. Neither polymorphic forms nor isolated subunits have been observed in human in vivo.

The precise in vivo function of CRP is still uncertain; nonetheless, several major and potentially important types of biological activity of CRP have been well defined.

First, CRP is able to bind several biological substrates that are widely distributed in nature. It was originally
Fig. 1. The acute phase response. Bacterial-derived fragments stimulate the monocytes/macrophage system to produce cytokines. Interleukin-6 (IL-6) is the major cytokine influencing the hepatic synthesis of acute phase protein. A modest increase for ceruloplasmin, C3, and C4 occurs; haptoglobin and fibrinogen may increase twofold to fivefold. Albumin and transferrin plasma concentration are consistently reduced and are referred as “negative” acute phase protein. The two major human positive, acute, phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA), may increase 1000-fold or more.

CRP BIOSYNTHESIS DURING THE ACUTE PHASE RESPONSE: ROLE OF CYTOKINES

Of the many soluble factors that initiate and maintain an inflammatory response, several hormones specifically regulate the transcription of human ARP. These include interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α) leukemia inhibitory factor, transforming growth factor-β, interferon-γ, glucocorticoids, and the more recently identified effector molecule IL-11 [14], oncostatin [15], ciliary neutrophilic factor [16], and retinoic acid [17]. In addition, insulin [18] and okadaic acid [19] have recently been shown to act as inhibitors of the cytokine-driven induction of some ARP.

Some of these mediators are released initially at the site of inflammation by activated mononuclear phagocytes, lymphocytes, or other differentiated cell types and named for its ability to bind the pneumococcal C-poly saccharide [11] and has been shown to have a number of calcium-dependent binding specificities and biological activities related to nonspecific host defense.

Second, it acts as an opsonin for bacteria, parasites, and immune complexes and can activate the classic pathway of complement.

In addition, CRP can bind to chromatin, histones, and small nuclear ribonucleoprotein particles.

The ability to bind to and to effect the clearance of nuclear material released from necrotic tissue during inflammation [12, 13] would provide an efficient means of preventing the initiation of nuclear-antigen-specific autoimmunity, and would explain, at least in part, the need for high levels of circulating pentraxins (such as CRP and SAA) during inflammation.
have potent local and systemic effects. However, IL-6 is felt currently to be the major cytokine influencing acute phase protein changes, while IL-1, TNF-α, transforming growth factor-β1, and interferon-γ are also capable of influencing synthesis of some plasma proteins in liver cells, and interactive effects of combinations of these cytokines have been shown [20–22].

Elevated plasma levels of CRP are detectable as early as four hours following tissue injury, and peak levels are attained within 24 to 72 hours [23]. The major source of plasma CRP is the liver, and progressive recruitment of hepatocytes [24] to CRP synthesis was found following experimental injury in the rabbit [25].

Several in vitro studies of human CRP, using hepatoma cells have shown that CRP mRNA transcription is induced dramatically by IL-6 [26, 27], with protein synthesis being further regulated by translation and post-translation mechanisms. Although no significant changes are elicited by IL-1β alone, when used in combination with IL-6, there is a greatly enhanced response [28, 29]. The promoter of the human CRP gene contains two acute phase response elements (APREs).

These are APRE1, which contains a binding site for the liver-specific transcription factor HNF1, and APRE2, which contains an HNF1 binding site (β site) and an IL-6 binding site (α site) [30].

Nuclear factor (NF)-IL-6 is a recently identified transcription factor that is induced by IL-6 and activated by protein kinase C-dependent phosphorylation at Ser 105. It has the ability to interact with the promoter regions of several IL-6-inducible genes at a consensus sequence of TG/G/A AA [31].

In short, IL-6 activates NF IL-6, which interacts with the α site and enhances binding of NH1 to the β site, resulting in the co-operative liver-specific induction of CRP.

In addition, post-transcriptional events are felt to participate in acute phase protein induction.

Post-translational control of CRP secretion by rabbit hepatocytes becomes more efficient during the acute phase response [32], because of down-regulation of a specific binding site by which CRP is normally retained within the endoplasmic reticulum under nonacute phase conditions [33]. CRP can be further autoregulated by CRP-mediated release from neutrophils of a protease that promote its own degradation [34].

Thus, the dramatic transcriptional induction of CRP by IL-6 is further enhanced by novel translation and post-transitional mechanisms, which eventually lead to a negative feedback loop in which CRP can down-regulate its own levels during an inflammatory response.

Following secretion, CRP appears to display a rapid rate of plasma turnover in the rabbit, with a half-time in the circulation of approximately four to five hours [35]. Studies in humans suggest a half-life of 18 hours [5], but during the inflammatory state, clearance may be slightly more rapid [36]. The histologic observation that CRP is deposited at local inflammatory sites is consistent with the possibility that there is enhanced clearance at the site of inflammation [37].

**CRP AS A MARKER OF CARDIOVASCULAR RISK**

Normally the acute phase response lasts only a few days; however, in the case of chronic or recurring inflammation, an aberrant continuation of some aspects of the acute phase response may contribute to the underlying tissue damage that accompanies the disease and may also lead to further complications such as cardiovascular diseases or reactive amyloidosis.

Laboratory-based evidence and epidemiological studies have investigated the association between coronary heart disease and plasma protein involved in the acute phase response [38].
Two previous cross-sectional studies with detailed CRP values and cardiovascular risk factors are one in 300 British men [39] and the other in 400 older Americans [40]. They made conflicting claims, particularly with respect to the possible association between CRP and smoking status or serum lipids; however, the samples were relatively small, and no attempts were made to avoid spurious association arising from a multiple comparison.

The prospective data of Ridker et al (Physician Health Study) provide convincing evidence that the baseline plasma concentrations of CRP in apparently healthy men are predictive of first myocardial infarction and ischemic stroke, but not of venous thrombosis [41]. In addition, the risk of arterial thrombosis associated with the level of CRP was stable over long periods and was not modified by other factors, including smoking status, body mass index, blood pressure, plasma concentration of total or high-density lipoprotein cholesterol, triglyceride, lipoprotein, fibrinogen, or homocysteine. In contrast, the benefit of aspirin in reducing the risk of a first myocardial infarction diminished significantly with decreasing CRP concentrations. Finally, the study of Ridker et al also suggests that CRP is not simply a short-term marker of risk, as has previously been demonstrated in patients with unstable angina, but is also a long-term marker of risk, even for events occurring six or more years later.

This observation made in men with favorable coronary risk factor profiles expands on the results of previous reports showing the long-term prognostic values of CRP levels in people with multiple risk factors and in patients with chronic angina [42].

The mechanisms that related the levels of CRP to atherothrombosis are unclear. Previous infection with Chlamydia pneumoniae, Helicobacter pylori [43], herpes, or cytomegalovirus may be a source of the chronic inflammation detected by CRP. However, this seems unlikely because the risk association was found for values obtained in 90% of normal people and was sustained over several years. In addition, Maseri found no evidence of replicating cytomegalovirus in endarterectomy specimens from unstable coronary plaques, but found an abnormal immunologic response in patients with unstable angina and elevated CRP levels that was consistent with previous report of leukocyte activation [44]. In fact, inflammatory cells are commonly found in chronic atherosclerosis, and evidence of immunologic activation in plaques can be found in both acute and chronic ischemic syndromes [45].

CRP AS A CARDIOVASCULAR RISK FACTOR: MORE THAN AN EPIPHENOMENON?

The link between CRP and cardiovascular risk is thought to be indirect reflecting circulating CRP only the extent of the acute phase reaction in response to nonspecific stimuli such as confounding risk factors, atherosclerosis, vascular injury, ischemia, and necrosis.

However, several arguments are against this explanation that increased plasma levels of CRP are merely an epiphenomenon; in fact, there is increasing evidence that CRP is not only a marker of the acute phase response, but is also involved in the pathogenesis of the disease.

First, chronic infections that cause a rise in circulating CRP also yield a higher risk for cardiovascular disease [46–48]. Second, CRP is a cardiovascular risk factor even after correction for other risk factors [41]. Finally, CRP and activated complement factors can be found localized in inflamed tissues [49], including atherosclerotic vessels [50] and infarcted myocardium [51].

Recently, Lagrand et al postulated the “flip-flop” hypothesis. These authors suggested that membrane lyphospholipids in ischemic myocardium may constitute ligands for CRP [52].

The inner and outer leaflet of the cell membrane of normal cell differs in phospholipid composition: sphingomyelin and phosphatidylethanolamine mainly in the outer leaflet and phosphatidylserine and phosphatidylethanolamine in the inner leaflet. During apoptosis or ischemia, the asymmetry is lost, and the various phospholipids of outer and inner leaflets exchange (flip-flop of the membrane).

Thus, ligands for CRP may be generated in flip-flop cells with activation of the classic pathway of complement, and this activation subsequently enhances inflammation and contributes to myocardial tissue damage or dysfunction.

This hypothesis predicts that high CRP levels will lead to more intense CRP deposition and inflammatory reactions and hence tissue damages in the jeopardized myocardium. It also explains why high-normal or slightly increased baseline levels of plasma CRP constitute and predict an increased risk for cardiovascular events with the presence of phospholipids and membrane flip-flop in the coronary vessels, which may result from short-term ischemic periods.

UREMIA AS A CHRONIC INFLAMMATORY STATE

Cardiovascular diseases still represent the largest single cause of mortality in chronic renal failure patients, and several risk factors are involved in the development of atherogenesis in these patients.

Strong and independent predictors of mortality or cardiovascular diseases are low levels of serum albumin, high plasma CRP, and lipoprotein (a), and all of these plasma proteins, described to function as negative or positive APRs, are modulated by IL-6 [53].

The study by Kaizu et al demonstrated that the serum
levels of IL-6 were significantly correlated with the major parameters indicating malnutrition, such as body weight loss and serum albumin level [54]. Even the more rapid and sensitive index of malnutrition, such as prealbumin and insulin-like growth factor-1, tended to be lower in patients with high IL-6 levels.

The impact of protein intake and inflammation as separate independent factors in predicting serum albumin levels in chronic hemodialytic patients was investigated by Kayser et al, who found that the high concentration of acute phase proteins have a greater impact on serum albumin than low normalized polymerase chain reaction (PCRn) [55]. This suggests the activity of APRs as an important predictor of mortality independent of nutritional factors.

Bergström et al stated that plasma CRP was the most powerful predictor of serum albumin levels, and that it was a better predictor of death within the first year of hemodialysis than was serum albumin (abstract, Bergström et al, *J Am Soc Nephrol* 6:573, 1995) [56]. Furthermore, proinflammatory cytokines that stimulate CRP synthesis also induce muscle catabolism with the liberation of amino acids, inhibition of muscle protein synthesis [57], and anorexia [58].

In conclusion, malnutrition is associated with mortality and morbidity of chronic uremic patients; the strong correlation between chronic inflammation and protein content among dialysis patients is mediated by the cytokine-induced acute phase response.

In addition, in chronic renal failure, plasma levels of Lp(a) are elevated, especially in patients with low plasma albumin levels. A close relationship has been demonstrated between high Lp(a) and CRP, sialic acid, and IL-6 in hemodialysis patients [59]. These findings implicate that Lp(a) is elevated following an activation of the acute phase response. In fact, seven IL-6-responsive elements sequence can be identified in the flanking regulatory region of the Apo(a) gene of chromosome 6 [60].

Stenvinkel et al recently demonstrated that the rapidly developing atherosclerosis, studied by carotid B-mode ultrasonography, in advanced predialytic chronic renal failure appears to be caused by a synergism of different mechanisms, such as malnutrition, oxidative stress, genetic factors, and chronic inflammation [61]. Apart from classic risk factors, CRP levels are associated with an increased intima-media area, whereas increased levels of oxidized low-density lipoprotein are associated with the presence of carotid plaques.

Patients with CRP levels over 10 mg/L had a significant increase of mean cross-sectional calculated intima-media area compared with patients with CRP < 10 mg/L. Also, the mean lumen diameter was slightly but significantly elevated in patients with elevated CRP.

In this study, predialysis patients with carotid plaques had significantly elevated plasma levels of TNF-α, and the authors postulated a role for this cytokine in atherosclerosis.

Personal data (abstract; Panichi et al, *Nephrol Dial Transplant* 13:A221, 1998) suggest that 25% of a population of 102 chronic predialytic patients had CRP values over 5 mg/L taken as the upper limit in normal human subjects (Fig. 3). These data could be even more dramatic when we consider that in the study of Ridker et al, the risk of experiencing a cardiovascular accident was clearly increased for CRP values over 3.1 mg/L [41]. CRP and IL-6 were significantly related, and both were related to renal function as plasma creatinine and creatinine clearance.

**CAN DIALYTIC MODALITIES INFLUENCE THIS CHRONIC INFLAMMATORY STATE?**

As stated earlier in this article, the role of inflammation as a general pathway in the progression of vascular and tissue damage in chronic uremic patients—associated to anemia, cardiovascular disease, and malnutrition—is well established.

Furthermore, hemodialysis may induce a chronic stimulation on the monocyte macrophage cellular components via the exposure of the patient to a generally bioincompatible hemodialytic system.

In recent years, several studies have reported an increased cytokine production secondary to blood interaction with bioincompatible dialysis components. IL-1, TNF-α and mainly IL-6 are the three proinflammatory cytokines that are involved in the pathogenetical aspects of hemodialysis-related disease [62–66]. The proposed mechanisms include blood interaction with the hemodialysis membrane and back-leakage of endotoxins from the contaminated dialysate through a high-flux dialysis membrane.

In fact, backfiltration and backdiffusion of contaminated dialysate are the driving forces for the transmembrane passage of endotoxins or other cytokine-stimulating substances (CIS) during dialysis [67].

In a multicenter study, we measured the intracellular IL-1Ra and IL-1β concentrations in patients undergoing low-volume exchange hemodiafiltration, which is known to be associated with backfiltration, in comparison to those found in patients treated with a modified hemodiafiltration modality without backfiltration (paired filtration dialysis) [68]. Hemodiafiltration with backfiltration was associated with a significant increase of IL-1Ra and IL-1β.

Nevertheless, conflicting results are present regarding the real impact of monocyte activation in several clinical problems related to dialysis treatment. What is the clinical relevance of these observations in the clinical outcome of chronic uremic patients?

In a search for a clinical link with biocompatibility-
related cytokines production, we analyzed the APRs in the same patients. The increased production of IL-1 and IL-6 in patients exposed to backfiltration was associated with a chronic inflammatory state, as reflected by increased plasma proteins of acute phase as CRP [69].

To verify these findings in a larger population, we have planned a multicenter cross-sectional and longitudinal trial in over 300 clinically stable patients [70]. Patients with clinical conditions known to be associated with chronic inflammation and high CRP values such as amyloidosis, malnutrition, resistance to erythropoietin, and/or clinically evident illness were excluded from the study.

Patients were treated with conventional hemodialysis, hemodiafiltration with low exchange volume and high exchange volume, and double chamber hemodiafiltration (paired filtration dialysis).

C-reactive protein and IL-6 were measured at the beginning of the study and after six months of follow-up and resulted highly related.

A large proportion (50%) of the global population had CRP values over 5 mg/L, taken as the upper limit in normal human subjects (Fig. 3). In particular, 43% of the patients were between 5 and 20 mg/L, and 7% were over 20 mg/L.

By analyzing these patients according to their different dialytic modalities, a striking difference was present between low-volume (less than 10 L per session) hemodiafiltration versus standard hemodialysis, high-volume hemodiafiltration, and paired filtration dialysis (Fig. 4).

After six months of follow-up, CRP values in patients in hemodiafiltration with low exchange volume were higher in regard to the values in the same patients determined at the enrollment (Fig. 4).

We suggest that the increase of CRP in clinically stable dialytic patients may be due to the stimulation of the monocyte/macrophages system by backfiltration of dialysate contaminants [Limulus Amebocyte Lisate (LAL)-negative CIS]. There was, in fact, a significant increase of CRP in low-volume but not in high-volume hemodiafiltration.

Ronco et al suggested that backfiltration may, in fact, be dependent on ultrafiltration rate and dialyzer geometry: Increasing the ultrafiltration rate may drastically reduce the risk of backfiltration that otherwise occurs with low ultrafiltration rate as 2 L/hour [71]. Double-chamber hemodiafiltration, which eliminates backfiltration, was associated with CRP values indistinguishable from those
recorded in high-volume hemodiafiltration and conventional hemodialysis performed with low-flux membranes.

Evidence has been provided for the occurrence of a state of chronic inflammation in predialytic and dialytic uremic patients; furthermore, we have suggested that 1 and 2A, inhibits induction of acute phase proteins by interleukin

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