

Beyond sepsis pathophysiology with cytokines: what is their value as biomarkers for disease severity?

Fernando A Bozza, Patrícia T Bozza*, Hugo C Castro Faria Neto*/+

Unidade de Terapia Intensiva, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil *Laboratório de Imunofarmacologia, Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz-Fiocruz, Av. Brasil 4365, 21040-900 Rio de Janeiro, RJ, Brasil

Sepsis is a major challenge in medicine. It is a common and frequently fatal infectious condition. The incidence continues to increase, with unacceptably high mortality rates, despite the use of specific antibiotics, aggressive operative intervention, nutritional support, and anti-inflammatory therapies. Typically, septic patients exhibit a high degree of heterogeneity due to variables such as age, weight, gender, the presence of secondary disease, the state of the immune system, and the severity of the infection. We are at urgent need for biomarkers and reliable measurements that can be applied to risk stratification of septic patients and that would easily identify those patients at the highest risk of a poor outcome. Such markers would be of fundamental importance to decision making for early intervention therapy or for the design of septic clinical trials. In the present work, we will review current biomarkers for sepsis severity and especially the use of cytokines as biomarkers with important pathophysiological role.

Key words: sepsis - biomarkers - cytokines - stratification

Sepsis is one of the most frequent causes of death in intensive-care patients worldwide. In the United States alone approximately 700,000 people are affected annually and 210,000 deaths are accounted for (Angus & Wax 2001). Accordingly, septic patients are generally hospitalized for extended periods generating a substantial health care burden since care with septic patients may cost as much as US\$ 50,000.00 per patient (Chalfin et al. 1993). Despite new support therapies and more potent antibiotics sepsis is often lethal, killing 30 to 70% of severely affected patients (Wheeler & Bernard 1999) and significantly reducing the quality of life of those who survive (Perl et al. 1995, Heyland et al. 2000). The currently definition for sepsis states that sepsis is a consequence of poorly regulated innate immune response to microbial infection (Glauser 2000). The pathophysiology of sepsis is highly complex and although a large body of knowledge has accumulated over the last decade, several important aspects remain to be fully understood.

Widespread activation of cells responsive to pathogens results in uncontrolled systemic inflammation. The release of an array of inflammatory mediators such as cytokines (e.g. TNF- α , IL-1, MIF, MCP-1, IL-6, IL-10), lipid mediators (e.g. PAF, prostaglandins), and reactive oxygen species will, in combination, induce vascular dilatation and increase in permeability with leakage of plasma components, and extravasation and activation of leukocytes to tissues and organs. In addition, inflam-

matory mediators and pathogen components will also activate the coagulation system causing disseminated intravascular coagulation. Together, those effects will lead to hypoperfusion and tissue hypoxia that apparently are the main cause of organ dysfunction, which represents the often-lethal stage of sepsis (Riedemann et al. 2003, Van Amersfoort et al. 2003).

Controlling or balancing the systemic inflammatory response is thought to be of surmounting importance to the outcome of sepsis. Nevertheless, despite encouraging pre-clinical data, the great majority of clinical trials aiming at neutralization of specific inflammatory mediators showed disappointing results (Polderman & Girbes 2004). One of the possible explanations for the failure of clinical trials in sepsis is that the current definition, although valid and important for clinical purposes, is too broad and do not allow for precise characterization and staging of patients with this condition. A possible approach to this problem is the creation of a staging system that would allow stratification of patients by both their baseline risk of an adverse outcome and their potential to respond to therapy. One of the best examples of disease stratification system has evolved in oncology, the TNM system, developed by Pierre Denoix (PX 1946). In this way, a new system, PIRO, is being proposed that can better characterize sepsis on the basis of predisposing factors and premorbid conditions, the nature of the underlying infection, the characteristics of the host response, and the extent of the resultant organ dysfunction (Levy et al. 2003). Nevertheless, for such a staging system to work adequately, it is essential to identify response profiles for biomarkers able to identify what patients are at risk of developing organ dysfunction, and which interventions are likely to reduce the degree of organ dysfunction. Importantly, assaying for those biomarkers must be timesaving and cost-effective to be useful in the screening of septic patients. In this review, we will focus on current

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*Corresponding author. E-mail: hcastro@ioc.fiocruz.br
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biomarkers for sepsis severity and especially on the use of cytokines as biomarkers with important pathophysiological role.

Biomarkers are essential for stratification of septic patients

The identification of high-risk patients, the proper share of resources, and early intervention are some of the great challenges in the care of critically ill patients in recent years (Dellinger et al. 2004). Many decisions referring to septic patients are frequently based on clinical and laboratory signs with low sensibility or specificity. The availability of more specific therapies may modify pathophysiologic process involved in the development of sepsis and minimize their consequences. Adequate biomarkers may be useful for the screening of septic patients thus identifying those eligible for specific therapies. Hence it is essential to identify biomarkers able to discriminate more homogeneous patients sub-groups with particular biological abnormalities liable to receive a guided therapy (Marshall et al. 2003).

Several different bioactive molecules have been proposed as severity or outcome biomarkers for patients with sepsis. Among those, bacterial products such as endotoxin and bacterial DNA, acute phase proteins (protein C, procalcitonin, LBP - LPS-binding protein), coagulation factors (fibrin degrading products, antitrombin III, dimer D), membrane cell markers (HLA-DR, CD-64, E-selectin), cellular processes (apoptose), hormones (cortisol, ACTH), soluble receptors (sCD-14, sTNFR1, sTNF-RII) and cytokines (TNF, IL-6, IL-8, IL-10) are the most celebrated ones. Each of those biomarkers is expected to fulfill at least three major functions in the clinical management of septic patients (Marshall et al. 2003): (i) establish or confirm the diagnosis of sepsis in patients with confounding syndromes such as the systemic inflammatory response syndrome (SIRS); (ii) quantify the severity of the disease and identify those patients with a higher risk of an unfavorable outcome; (iii) serve as an easy and reliable way to follow the response of the patient to a certain therapy, and serve as a way to quantify the effect of a certain therapy on the host response.

Nevertheless, only a few biomarkers have ultimately been incorporated into clinical practice, and among those mentioned above C-reactive protein (CRP) and procalcitonin (PCT) are honourable exceptions.

CRP was described in 1930 by Tillet and Francis, as a factor present in the serum of pneumonia patients that was able to precipitate polysaccharide fractions (fraction C) of *S. pneumoniae*. CRP is a cyclic pentamer in which the five subunits are non-covalently bound forming a stable structure that is resistant to proteolysis. Upon binding to polysaccharides present in bacteria, fungus or parasites, CRP is able to activate the classical complement pathway and promote phagocytosis. As an acute phase reactant, CRP is produced by hepatocytes and its transcription is induced by cytokines including IL-6, IL-1 and TGF- β 2. Plasma levels of CRP are below 10 mg/l in 99% of normal individuals and usually raise 4 to 6 h after an infectious stimulus reaching a peak after 36-50 h, which

could be as high as 500 mg/l after intense acute infectious stimulus. However, other inflammatory diseases with non-infectious background such as auto-immune diseases, trauma, major surgeries, burns and malignant diseases, are important causes of high CRP plasma levels. Interestingly enough, viral infections usually do not raise plasma CRP levels significantly (Abbate et al. 2003, Hengst 2003, Willerson & Ridker 2004).

PCT was originally described in 1984 as a 116 aminoacid protein with a molecular weight of 14,5 KDa. PCT gene, *Calc-1*, was localized to chromosome 11p15.4, and its promoter presents binding sites to transcription factors such as NF κ B and AP-1. PCT is expressed in different cells and tissues such as neurons, blood leukocytes, liver and brain after stimulation by cytokines (TNF e IL-6) or LPS. PCT is rapidly secreted and can be measured in the plasma as early as 2 h after the beginning of the infection, peaking within 12-24 h. Normal values are usually below 0.5 ng/ml and can increase up to 2000 fold during severe infections. However, as reported to CRP, PCT is not usually elevated during viral infection (Gattas & Cook 2003, Rau et al. 2004).

Several clinical studies aimed to establish the usefulness of CRP and PCT in confirming the diagnosis and in predicting outcome of septic patients. A recent meta-analysis (Simon et al. 2004) evaluating 12 studies that simultaneously compared CRP and PCT levels for the diagnosis of bacterial infection in hospitalized patients, showed that PCT levels were more sensitive (88 vs 75%) and also more specific (81 vs 67%) than CRP levels to distinguish bacterial from non-bacterial inflammation. However, few studies evaluated the prognostic accuracy of these markers, and when this was done the discriminative performance for prognosis of PCT and PCR was poor (Clec'h et al. 2004). As a general conclusion, these studies show that CRP and PCT are useful biomarkers for diagnosis in septic patients but, although they are regarded as markers of systemic inflammation, higher levels are usually seen in patients with bacterial infection than inpatients with SIRS or viral infection. Between CRP and PCT, the latter shows better sensibility and specificity in differentiating septic from non-septic patients. Moreover, PCT seems also to be a good severity marker in sepsis and may be useful in accessing the effectiveness of therapeutic measures in septic patients.

Identifying biomarkers with key pathophysiological roles

In recent years, there has been a growing interest in identifying biomarkers with pathophysiologic roles in sepsis. Although CRP and PCT are considered useful biomarkers in sepsis (see above), their precise role in the pathophysiology of sepsis and organ dysfunction, if present, is still unclear. Cytokines are key mediators in the host response to infection and increased plasma and tissue levels of those mediators are associated with the intensity of the inflammatory response. Nevertheless, the usefulness of individual cytokines as prognostic biomarkers is controversial, at the best.

Our group has been working in the identification of new biomarkers for severity and outcome of critically ill patients. As our mainstream strategy, we are looking for

biomarkers with potential role in the pathophysiology of generalized and unbalanced inflammation, and that could therefore be targeted for new therapeutic interventions. Using a new technology for cytokine quantification based on fluorescently dyed microspheres associated with a two-laser flow cytometry system (Luminex), that allows multiple analyses simultaneously in a single sample, we have been able to simultaneously measure the levels of 17 different cytokines in the plasma of patients with sepsis (Bozza et al. unpublished data). Our data show that among those 17 cytokines, 9 (IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IFN γ , G-CSF, and MCP-1) were able to discriminate survivors from non-survivors. In contrast, TNF, IL-5, IL-7, IL-12, IL-13, IL-17, MIP-1, and GM-CSF levels were not different in both groups. IL-8 and IL-1 were the cytokines with the best performance in predicting outcome. In this respect, it is worth mentioning that the predictive value of those cytokines were even better than the prototype prognostic clinical score used in intensive care units, the Acute Physiology and Chronic Health Evaluation Score (APACHE II). Interestingly, the same approach was used to study patients with severe Dengue virus infection. In this case, cytokines such as IL-1 β , IL-2, IL-4, IL-10, IL-13 and GM-CSF were significantly increased as compared to septic patients or healthy controls (Bozza et al. unpublished data).

Classically, IL-6 is a cytokine with important prognostic value in sepsis. Although the pathophysiologic role of IL-6 in this syndrome is still controversial, IL-6 has been proposed as an important cytokine biomarker in sepsis due to its slow and stable plasma kinetics, allowing its easy detection in blood samples, and its good correlation with the intensity of the inflammatory response. Different studies have confirmed that the majority (64-100%) of septic patients have increased circulating levels of IL-6, and that levels are correlated with severity and outcome (Gogos et al. 2000, Kox et al. 2000). Persistently elevated IL-6 levels are associated with both multiple organ failure (Pinsky et al. 1993) and death (Tanaka et al. 1996). Accordingly, in our population, IL-6 was a valuable outcome predictor in patients with sepsis and septic shock. All patients had detectable levels and high levels were found in most of them, but as mentioned above, IL-8 and IL-1 were even better as outcome predictor in our hands.

More recently, an important role for another cytokine, macrophage migration inhibitory factor (MIF), was established in sepsis. MIF is a pre-formed protein that is present in the pituitary gland, in T-cells and in macrophages and is released in response to different stimuli, including infection and stress (Bernhagen et al. 1993, Calandra et al. 1994, Bacher et al. 1996). LPS induces the expression of MIF in several tissues and also the release of significant amounts in the circulation (Bernhagen et al. 1993, Bacher et al. 1997), and the co-injection of MIF with LPS exacerbates the lethality in mice (Bacher et al. 1997). A unique property of MIF is its secretion from immune cells in response to physiological increases in glucocorticoid levels, and once released, MIF can counter-regulate the anti-inflammatory effects of steroids on cytokine production (Calandra & Bucala 1995). The paramount role played by endogenous MIF in the host response to gram-

negative and gram-positive toxins was underscored by the observation that treatment with neutralizing anti-MIF antibodies or targeted disruption of the MIF gene protected mice from LPS and superantigen-induced death (Calandra et al. 1998, Bozza et al. 1999). Elevated concentrations of MIF have been detected in the alveolar airspaces of patients with acute respiratory distress syndrome (ARDS) (Donnelly et al. 1997, Lai et al. 2003) and we have demonstrated that higher circulating MIF levels 6 h post-cardiopulmonary bypass surgery were associated with worse postoperative pulmonary short course outcome (Mendonça Filho et al. 2004). More recently, we have also shown that circulating levels of MIF can early detect sepsis with positive culture in patients submitted to cardiac surgeries (Mendonça Filho et al. 2004).

Despite its prominent role as a pro-inflammatory cytokine, MIF gene deficiency increased the ability of mice to clear *Pseudomonas aeruginosa* instilled in the lungs (Bozza et al. 1999). This result was confirmed and extended with the finding that MIF neutralization with antibodies led to better survival in a mouse model of lethal septic shock induced by cecal ligation and puncture (CLP) and peritoneal infection with *E. coli* (Calandra et al. 2000). Recently, it has been shown that MIF regulates the expression of Toll-like receptor 4 (TLR4), the signal-transducing molecule of the LPS receptor complex (Roger et al. 2001). The reduced expression of TLR-4 in MIF deficient macrophages is related to a reduced TNF production by these cells when stimulated by LPS.

Previous clinical studies have shown an increase in MIF levels in the sera of patients with SIRS, sepsis and septic shock (Calandra et al. 2000, Gando et al. 2001). MIF levels were not indicative of severity of an acute critical illness in the study by Lehman et al. (2001), but two different studies correlated high levels of MIF with poor outcome in patients with SIRS (Gando et al. 2001) and in patients with septic shock (Beishuizen et al. 2001). In addition, we have recently shown that, in septic patients, both MIF and IL-6 levels are significant different between survivors and non-survivors, and that MIF levels show a better discriminative power in prediction of sepsis related mortality than IL-6, as judged by receiver operating characteristic curves analysis. Therefore, elevated MIF concentrations appear to be an early indicator of poor outcome of septic patients in intensive care (Bozza et al. 2004). The observed detrimental role of endogenous MIF in systemic bacterial infections suggests that anti-MIF treatment may represent an important therapeutic strategy for patients with sepsis and septic shock (Riedemann & Ward 2003). Thus, bedside documentation of elevated MIF levels may be entry criteria for a future study of therapeutic intervention aiming at MIF neutralization.

Concluding remarks

Today the basic pathophysiology of sepsis is understood. Nevertheless, sepsis research has reached a critical point. To integrate our knowledge towards a consistent theory of the disease that would provide adequate prognosis and effective therapies is a task to be achieved. Attempts to derive clinical applications from the results of research in the basic mechanisms of the

disease have failed dramatically. After many single-agent anti-mediator trials failed, it has become widely accepted that sepsis is a complex and non-linear process. The present sepsis definition favors the heterogeneous nature of the patient populations in clinical studies that are not mimicked by experimental models. The development of new diagnostic tools and more clinically relevant models would allow more precise determination of the immune/inflammatory status of a septic patient, and may significantly contribute to stratification of patients that may benefit from a certain therapeutic strategy. The use of new technologies such as Luminex may enable the simultaneous detection of multiple biomarkers, such as cytokines, providing a "biomarker profile" for each patient. Such a profile could be useful for prediction of prognosis and for stratification aiming clinical trial entry or specific therapy. Significantly, the search for biomarkers with significant pathophysiologic role in sepsis might have important implications in the patient care and in trials of new compounds in clinical development.

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