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The Role of Cytokine in the Diagnosis and Monitoring of Patients with Systemic Infections

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Abstract: *Sepsis is an immunologic disorder with a high rate of mortality which is caused by the exacerbation of the inflammatory response. The purpose of this study was to characterise the etiologic spectrum and the profile of the most significant pro-inflammatory and anti-inflammatory citokines in patients with sistemic infections, by analysing the relationship between the type of etiologic agents and the intensity of the inflammatory response. The study was carried out on a number of 33 patients with systemic infections who were hospitalized in the "Saint Parascheva "Infectious Diseases Clinic Hospital in Galati, Romania. The intensity of the inflammatory response was higher in the sepsis with Gram-negative Bacilli (BGN) as compared to the systemic infections produced by Gram-positive Cocci (CGP). In the case of the patients with bacterial etiology (CGP and BGN) there was evidence of an increase levels of analysed cytokines (TNF-α, IL-1, IL-4, IL-6, IL-10), while the fungal etiology was correlated with high serum concentrations of TNF-α. The levels of IL-4 and IL-6 were similar in all patients with systemic infections, regardless of their etiology. The Klebsiella pneumoniae sepsis has led to the occurrennce of high serum levels both in the case of pro-inflammatory and anti-inflammatory citokines. The obtained results outline the necessity of monitoring pro-inflammatory and anti- inflammatory cytokines in sepsis, which might provide clues about the intensity of the generalized inflammatory reaction, representing not only an important diagnosis criteria but also a useful guide in the therapeutic methods chosen and the monitoring of these severe infections.*

Keywords: *pro- and anti-inflammatory cytokines, systemic infections, sepsis*

1. Introduction

Sepsis has an ever-growing incidence constituting the main cause of mortality in the Intensive Care wards and the third cause of mortality in the developed countries, being to some extent equal with the number of deaths caused by acute myocardial infarction [1,2]. More than one in four deaths are caused by sepsis every year in the world [3]. Systemic infections are triggered by etiologic agents, most often a bacterial type and their virulence factors (infectivity, aggression, toxigenity) but are maintained and modulated by a multitude of endogenous immunological mediators activated in a flow of complex cellular and humoral actions.

The cells of innate activated immunity induce an acute inflammatory response which is triggered not only by the means of pro-inflammatory cytokines but also of anti-inflammatory ones which regulate the inflammatory answer [4]. Neutrophiles and macrophages are the most efficient producers of pro-inflammatory interleukin, followed by endothelial vascular cells and keratinocytes [5,6]. The synthesis and excessive release of these circular mediators can nevertheless lead to side effects harmful to the body, i.e.: the uncontrolled activation of different leukocyte subpopulations, of the complement system, of the coagulation activation pathways and of the fibrinolytic system which can generate microvascular lesions, tissue ischemia, organ dysfunctions and ultimately to the patient's death [7,8].

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Having a role in intercellular cooperation, cytokines are soluble proteins which play a critical part in the initiation and perpetuation of the sepsis syndrome. As a rule, cytokines include interleukin (IL), interferons (IFN), chemokines and the tumoral necrosis factor (TNF) [4,9,10]; these act *in cascade*, by the means of stimulating synthesis or by the inhibition of another cytokine or by the modulation of the receptors' manifestation for other cytokines [9-11]. Usually, the body's cells are subjected by simultaneous action of several cytokines having a synergic or antagonist effect because they express different receptors for a large number of cytokines the expression of which is quantitatively regulated in time [12].

Pro-inflammatory cytokines (TNF-α, IL-1, IL-2, IL-6, IL-8, IL-12) are responsible for the induction the inflammation [13,14]. The immune system tries to balance the pro-inflammatory effect of cytokines through the production of molecules with anti-inflammatory role of type IL-4, IL-10, IL-13. Their action is more persistent after the first 24 h when the septic shock is triggered [15]. Some cytokines can have both pro-inflammatory and anti-inflammatory properties as well, in depending with the nature of the signals received, of the target cells and of their exposing time as well.

The actual tendency is to identify new fast and efficient evaluation criteria for the diagnosis, treatment and monitoring of the patients affected by sepsis, based on pathogenic mechanisms involved in the systemic response. Up to the present, it has been reported that the levels of several cytokines are correlated with the prognosis and severity of acute inflammatory diseases, and of sepsis implicitly [16-18]. Nonetheless, there have been established no correlations between the cytokine profile, the prognosis or the severity of sepsis. Under these conditions, it is necessary to correlate the paraclinical data with the clinical ones, in order to develop useful norms to optimize the diagnosis, improve the treatment and prognosis, as well as the prophylaxis of these severe illnesses.

2. Materials and methods

The study included a number of 33 patients, admitted in the "St. Parascheva" Infectious Diseases Clinic Hospital in Galati, Romania, between September 2010 to January 2012. The criteria for inclusion in the study were the patients who had two positive blood cultures with the same microorganism and the results of the main cytokines. The diagnosis was divided into four categories: sepsis, meningitis, respiratory diseases and others.

In order to establish a set of characteristics of the etiological spectrum, blood cultures were collected – the microbiologic selection test in the diagnosis of systemic infections [19]. The blood samples were taken in strict asepsic and antisepsic conditions. We used blood culture bottles with special nutrient elements the composition of which favour the development of aerobes, microaerophiles analysed in an automatic system (Bactec9050). The positive samples were used to create Gram-stained smears and subcultures to identify and test antibiotic sensitivity. The biochemical identification of the microbial agents was carried out by the means of classic methods, semi-automatic (RapID™) and automatic methods (Vitek2 Compact) [20]. However, the multiplication of the bacteria depends on a series of factors independent of the growth conditions; the density of the infectious agents and the occurrence of certain inhibiting substances in the blood [21,22].

The concentrations of the main pro-inflammatory (TNF-α, IL-1, IL-2, IL-6) and anti-inflammatory cytokines (IL-4, IL-10) involved in the immune response were determined by the means of the immunoenzymatic method ELISA at the "Ștefan S. Nicolau" Virology Institute in Bucharest. The blood samples were taken from the 33 patients and the vacutainers without anticoagulant, were centrifuged for 10 mins at 1300g. The serum samples were kept at -70°C, and the volume of biological samples used was 100 μL.

The statistics were created by the help of SPSS applications (*Statistical Package for Social Science v.16.0*). The interpretation of data relied both on descriptive statistics and on inferential statistics. Statistical analysis was performed through the use of the one-way analysis of variance (ANOVA) and the Tukey– Kramer multiple-comparisons test with the significance level set at $p < 0.05$.

3. Results and discussions

In this study, 33 bacterial strains were identified in the blood cultures done in the Microbiology Laboratory of the Infectious Disease Hospital in Galati. These strains come from 22 men and 11 women, with a median age of 49 years and suffering from invasive infections [23]. The analysed microbial strains belong to a wide variety of bacterial types and species distributed as follows: CGP (Gram-positive Cocci) –19 cases (57,57%), BGN (Gram-negative Bacilli) –13 cases (39,39%), microfungi –1 case (3,03%). In the group of infections, CGP had the highest rate, and the predominant etiologic agent was *S. aureus* (9 strains).

In the case of these patients, we determined the serum concentration of the main pro-inflammatory (TNF-α, IL-1, IL-2, IL-6) and anti-inflammatory cytokines (IL-4, IL-10) involved in triggering off and regulating the immune response, as well as in the relationship between the etiologic agents and the intensity of the inflammatory response (table1).

Table 1. The serum concentration of the cytokines determined in invasive infections

	IL-1	**IL-2**	**IL-4**	**IL-6**	**IL-10**	**TNF-α**
Biological reference range (N)	<5 pg/ml	<710 U/ml	<3.8 pg/ml	<9.7 pg/ml	<9.1 pg/ml	<8.1 pg/ml
CGP			**Average** (min; max)			
Enterococcus spp.(n=1)	4.4	25.43	55.17	508.43	901	1.43
S. aureus (n=9)	**27.68** (0 -131.23)	**87.46** (0 – 493.72)	**198.77** (0 – 695.17)	**234.71** (20 – 1224.44)	**143.87** (0 – 445.33)	**41.18** (0 -270.6)
Coagulase-negative Staphylococci (n=6)	**28.03** (0 -143.59)	**49.72** (0 – 204.43)	**109.62** (6.34 – 355.94)	**62.57** (9.86– 112.22)	**105.54** (9.41-247.55)	**53** (0 -108.6)
Streptococcus pneumoniae (n=2)	**9.88** (5.32-14.44)	**97.79** (18.29-177.29)	**105.02** (58.5-51.54)	**91.88** (49.86-133.89)	**22.7** (16.07-29.33)	**73.97** (0 -147.93)
Streptococcus pyogenes (n=1)	5.56	11.14	31	235.57	16	0
BGN			**Average** (min; max)			
E. coli (n=8)	**15.76** (0 – 90.32)	**33.01** (0 -188.71)	**153.86** (22.67-663.74)	**157.51** (0 – 485.57)	**113.13** (13.5 – 412.37)	**43.51** (0 – 195.93)
Enterobacter spp. (n=2)	0	**203** (111.57-294.43)	**386.34** (108.94-663.74)	**339.16** (30 – 648.33)	**207.37** (28.3-386.44)	**164.93** (147.27-182.6)
Klebsiella pneumoniae (n=3)	**101.44** (0 - 160)	**848.28** (0 - 2525)	**959.53** (17 -2834.33)	**425.63** (9.44 – 1084.14)	**810.79** (261.25-1834.33)	**212.21** (65.93-331.43)
Microfungi			**Average** (min; max)			
Candida spp.(n=1)	0	0	13.34	32.78	0	130

**N- total number bacterial and microfungi strains (33); n-partial number bacterial and microfungi strains*

For patients with systemic infections IL 6 and IL 4 are synthesized during the infections caused by all three groups of etiologic agents (BGN, CGP, microfungi). With reference to the IL 6 proinflammatory cytokine, a high concentration was noticed during the infections caused by BGN, while the fungic etiology was correlated with a high concentration of TNFα. It is worth mentioning that in the case of the patients with systemic infections of a bacterial etiology, (CGP and BGN) the synthesis of all six analysed cytokines was stimulated (Figure 1).

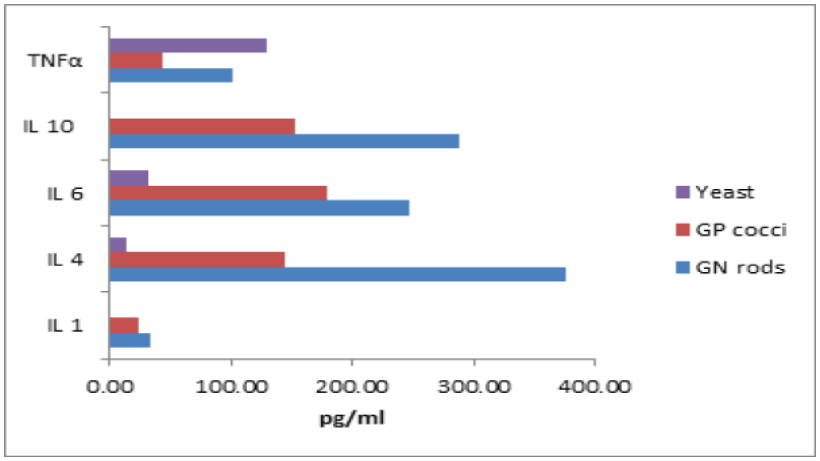

Figure 1. Levels of pro-inflammatory cytokines and anti-inflammatory drugs in patients with systemic infections of various etiology

In the case of patients with systemic bacterial infections caused by CGP evidence was made of all 6 analysed cytokines with mean circulating levels the highest for IL 6 and the lowest for IL1. By comparing the dynamics of the cytokines produced by different bacterial types and species in the CGP group involved in generalised infections, there were significant differences revealed in the case of infections caused by *Enterococcus spp.* where the average of the concentrations was significantly higher at IL 6 and IL 10, while in the case of infections caused by *S.aureus,* coagulase-negative staphylococcs (CoNS) and *Streptococcus pneumoniae,* even though completely represented, the cytokine profile was characterised bylow serum levels of the analysed cytokines (Figure 2).

Enterococcs represent the third cause of nosocomial bacteremia, which, apart the numerous mechanisms by acquired and constitutive resistance to antibiotics, also presents a series of virulence factors which facilitate the adherence to mucosal surfaces, tissue invasiveness, attachment to the vascular endothelium and the induction of an inflammatory response. It was proven that both in the case of unique as well as in polymicrobial etiology, *Enterococcus* spp. is capable of inducing sepsis, especially in immunodeppressed patients [24].

90,3 % of the studied patients have manifested high concentrations of IL 6. The high levels of IL-6, mediator of the acute phase response, reflect endothelial lesions, which are strongly associated with the evolution of the sepsis [4,25]. Similarly, an increase in IL-6 may enhance the inflammation via endothelial cells as a positive feedback system [26]. Excessive pro-inflammatory cytokines favour the manifestation of the tumoral necrosis factor which is mainly synthesised by the activated monocytes [27, 28], thus leading to coagulation disorders and to the formation of microthrombs [8]. The formation of microthrombs could contribute to dysfunctions of the microcirculation which further lead to a multiple organ failure eventually causing death [29].

Nevertheless, the IL-10 anti-inflammatory cytokine could act by the means of a negative feedback against the inflammatory response [4]. IL-10 is the key cytokine in the anti-inflammatory response and is produced by the stimulated CD4+ T lymphocytes, respectively by the Th2 subpopulation, monocytes and B lymphocytes. IL-10 strongly inhibits the occurrence of IL-2, IFN-γ, TNF-α, IL-1, IL-6, IL-8, IL-12 cytokines by monocytes, macrophages, neutrophils and natural killer (NK) cells [30,31]. Reports show that IL-10 is one of the most critical cytokines in the physiopathology of the sepsis [32]. The high serum levels of IL-10, the high IL10/TNF-α ratio represents an important risk factor given the severity of the sepsis and the number of deaths [33], which shows that patients with sepsis are in a profound immunosuppression.

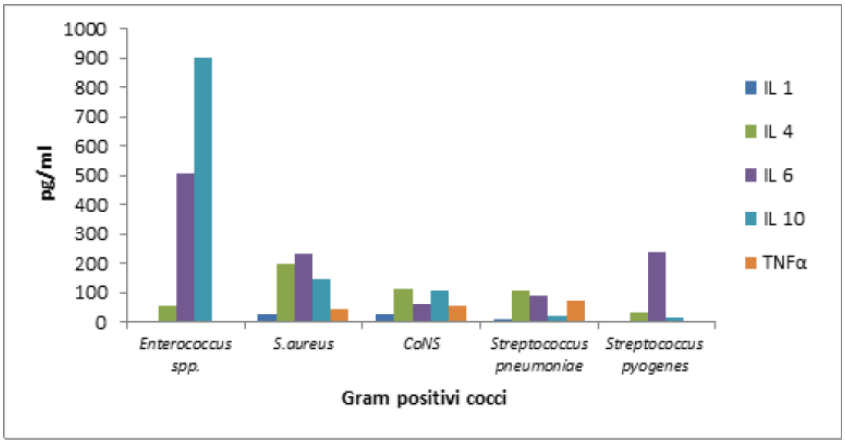

Figure 2. Variations in serum concentrations of cytokines in patients with systemic infections produced by different CGP species

In the case of patients suffering from invasive infections caused by BGN all tested cytokines were highlighted, increased value showing IL-4 and IL-10 anti-inflammatory cytokines (Figure 1).

Research experimented on rodents and primates proved that, if endogenously produced and exogenously administered, IL-10 can reduce the extent of the inflammatory reaction and improve the result, mainly in case of the endotoxin shock and bacteremia models. Nonetheless, the IL-10 endogenous production and systemic administration can exacerbate the dysfunction of the T lymphocytes, reduce the cellular apoptosis and the antimicrobial function and can even increase the rate of mortality in other bacteremia models which are less acute than sepsis caused or thermic lesions. The IL-10 transmission targeting certain tissues might eliminate the side effects produced by the systemic administration. The potential anti-inflammatory properties of IL-10 need to be carefully considered in keeping with its immunosuppresive properties when administered to patients with sepsis [34].

Sepsis caused by *Klebsiella pneumoniae* leads to the occurrence of high serum levels of cytokines, both in the case of the pro-inflammatorial and of the anti-inflammatorial ones (Figure 3). Research on experimental sepsis models prove the capacity of the *Klebsiella pneumonia* strain to induce high values of the inflammatorial mediators especially due to an intense activation of the macrophages [35].

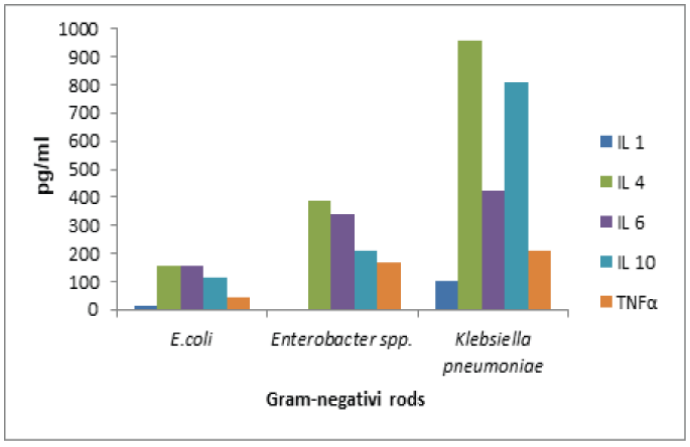

Figure 3. Variation in serum concentrations of cytokines in patients with systemic infections produced by different species of BGN

Recently, the concomitant occurrence of both mediators, i.e. pro- and anti-inflammatory, has been marked as early as the beginning of sepsis [36]. The results of the present study for all groups of etiological agents were similar to this report and suggest that both pro-inflammatory and anti-inflammatory responses correlate with the pathogenesis of sepsis in a mutual relationship[37].

In the case of systemic infections caused by microfungi, the zymosan (Zym) initiates intracellular signalling and activates macrophages via tool-like receptor 6 (TLR 6) [12]. The patient suffering from a generalized infection of fungal etiology was detected to have only four cytokines, IL 4, IL 6, IL 10 and TNFα. The sepsis caused by a fungal infection has caused higher circulating levels for IL 6 and TNF α (Figure 1). It is supposed that an early mediator of the systemic inflammatory response would be TNF-α, which is released just a few minutes after the infection, and in 3-4 hours it becomes untraceable in the patient's serum. When produced within normal limits, TNF-α has a protective role, and when produced in excess, it has a negative role [38, 39].

The IL-1 was the lowest in all studied groups, regardless of the etiology of the invasive infections. IL-1 is produced by several celular types as response to lesions, infection or other antigenic stimulation and it influences many categories of cells and physiologic processes, but the main IL-1 source is represented by mononuclear phagocites.

Research has shown that in sepsis there takes place the suppression of the macrophages that produce pro-inflammatory mediators, a mechanism known as immunoparalysis, a protection mechanism against the endotoxin shock, which, even though stimulated by the presence of the bacterial components, no longer produces high quantities of pro-inflammatory cytokines [12, 40- 42].

In this study, the intensity of the inflammatory response was higher in sepsis with BGN, as compared to the cases generated by CGP. Regardless of the etiology, IL-4 and IL-6 were constantly synthetized. Microfungs produce the highest level of TNF-α as compared to the other two etiologic agents.

The evolution of patients was favorable in 78.8% of cases and unfavorable in 21.2% of cases (with only one lethal case being registered). The deceased patient had sepsis with *S.aureus* and showing increased serum levels of pro-inflammatory and anti-inflammatory cytokines, as well as comorbidities. Host dependent factors, as well as strain aggression, have been shown to contribute to unfavorable evolution.

The individual response to infection is determined by several factors, among which we mention: the virulence of the pathogen agent, the size of the inoculum, the patient's preexistent comorbidities, the age and the genetic polymorphism of the receivers, cytokines and enzymes involved in the inflammatory process [43]. A key role in the immune response in sepsis is played by macrophages.

During infections, certain parietal microbial components like lipopolysaccharides *(*LPS) from the Gram-negative bacteria, lipoteichoic acids (LTA) from the Gram-positive bacteria and Zym from microfungi connect themselves to different receivers TLR and activate macrophages. The production of pro-inflammatory cytokines (TNF-α, IL-1, IL2, IL-6), as well as the secretion of chemokines (IL-8) which accumulate neutrophils to the site of infection, play a very important role in stopping infections. Similarly, macrophages also produce anti-inflammatory mediators (IL-4, IL-10) in order to balance the production of pro-inflammatory mediators. However, just as an excessive pro-inflammatory reaction can be harmful by producing major cellular destruction, an exaggerated anti-inflammatory response can lead to a state of immunosuppression [12,44].

Recent researches have proven that the suppression of macrophages post-sepsis can be determined not only by LPS but by LTA or Zym as well. These stimuli activate macrophages via different ways of signalling, LTA via TLR2 and Zym via TLR6 [40]. The diminished response of the macrophages in the LTA or Zym stimulation show that immunosuppression is a complex phenomenon which can be due to a tolerance crossed between LPS and other microbial stimuli. Accordingly, human monocytes tolerant to LPS have developed a crossed tolerance and have not responded to subsequent stimulation via LTA. Nonetheless, the cells tolerant to LTA had a diminished response only when restimulated via LTA, maintaining the response for a new stimulation via LPS [41].

4. Conclusions

The high occurrence and the gravity of the invasive infections justify the necessity and the importance of the researches focused on the optimization of the diagnosis methods, all the more since sepsis is a complex clinic entity which needs to be further investigated in the dynamics of molecular disorders. The results of these study outline the necessity to combine paraclinic data with the clinic ones with a view to establishing a correct and early diagnosis, thus having a positive impact on the prognosis of the patients with systemic infections.

The pattern of the cytokine release during sepsis defines the early as well as the late mediators of the systemic inflammation. In order to characterise the multitude of clinic symptoms occurring in different progressing stages of the inflammatory process, it is necessary to establish a pattern of the cytokines produced during sepsis, one similar to molecular patterns, which will enable a group of cytokines to be associated with a specific group of clinic symptoms, characteristic of a certain stage in the evolution of the syndrome. Such a pattern would have major implications in the development of efficient treatment methods against sepsis and would manifest an appropriate modulating influence on the cytokine pattern.

Likewise, clinical and experimental studies are also necessary to identify new biomarkers, as well as a description of their contribution to the pathogenesis of systemic infections and a monitoring of the treatment both during infectious and non-infectious processes which are, as a rule, associated to sepsis.

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