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The immune response after stimulation with wall components of gram-positive bacteria and fungi

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ABSTRACT

Although several components of the microbial wall of gram-positive bacteria and fungi possess immunostimulatory properties, their pathogenetic role remains incompletely evaluated. The purpose of this study was to assess the basic immune status of patients susceptible to infections and their capability for cytokine production after stimulation with wall components of gram-positive bacteria and fungi. We measured serum cytokine levels as well as cytokine production after *ex vivo* lipoteichoic acid (LTA) and mannan stimulation of whole blood. The blood was taken from 10 healthy volunteers, 10 patients with end-stage renal disease (ESRD), 10 patients with diabetes mellitus (DM), and 10 patients on their 2nd day of stay in the Intensive Care Unit (ICU), who suffered from non septic systemic inflammatory response syndrome (SIRS) and had an APACHE II score ≥25. We used 1 μg/ml LTA and 100 μg/ml mannan for an incubation period of 8 h to stimulate 100 μl aliquots of whole blood. All patient groups had higher baseline values of TNF-α, IL-6, IL-1β, and IL-10 compared to the control group, but only for ICU patients the difference was statistically significant. The ratio IL-10/IL-6 was found 0.33, 0.22, and 0.96 in healthy persons, ESRD, and DM patients respectively, and 1.32 in ICU patients. In all examined groups, the levels of cytokines significantly increased after stimulation by LTA and mannan, although in severely ill patients this change was considerably smaller, possibly reflecting a state of monocytes' depression and relative hyporesponsiveness. No significant differences between the LTA and the mannan stimulation were observed.

1. Introduction

Sepsis represents a major health problem, associated with high mortality and morbidity, prolonged length of stay at the hospital, and increased direct and indirect costs. Especially in the Intensive Care Unit (ICU), sepsis is the leading cause of death. According to the findings of the Sepsis Occurrence in Acutely Ill Patients (SOAP) study, 35% of critically ill patients develop sepsis at some point during their stay in the ICU, with lethal complications in 27% of them, a percentage that approaches 50% in patients with septic shock. Gram-positive bacteria have emerged as the commonest cause of infections in the critically ill patients, followed by gram-negative bacteria and fungi; the latter of which are implicated in approximately 18% of cases [1].

Apart from ICU patients, who have the highest risk for septic complications, other patient groups face an increased risk for infections. Chronic kidney disease (CKD) is frequently complicated by infections, especially when it progresses to end-stage renal disease (ESRD) that requires dialysis. The mortality of ESRD patients is approximately 20%, with cardiovascular disease and infections accounting for up to 70% of all deaths. The increased incidence of sepsis in ESRD patients is multifactorial, owing to the uremic milieu, to malnutrition, and to the dialysis procedure, factors that can affect multiple sites of both the innate and the adaptive immunity [2]. Hyperglycemia is a well-known factor that contributes to infectious complications. Altered glucose homeostasis affects many aspects of immune response, including secretion of cytokines, function of immune cells, and endothelial function. Patients with diabetes mellitus (DM) are actually extremely prone to infections [3].

Following a microbial insult, the inflammatory response is initiated after the recognition of components of the microbial invader, called pathogen associated molecular patterns (PAMPs), by the receptors of the innate immune system. PAMPs are connected with the CD14 receptor and with the toll-like receptors (TLRs), which are distinct for each class of bacteria. In macrophages, among the 12 different TLRs which have been identified so far, the TLR4 mediates the signaling by lipopolysaccharide (endotoxin, LPS) and mannan, while the TLR2 by lipoteichoic acid (LTA). Complex

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cytoplasmic interactions result in the release of transcription factors, mainly the NF-kB, which activates genes for the synthesis of inflammatory cytokines [4]. The key pro-inflammatory cytokines are the tumor necrosis factor alpha (TNF-α), and the interleukin 6 (IL-6), 1β (IL-1β), and 8 (IL-8), which promote the inflammatory reaction and the establishment of the systemic inflammatory response syndrome (SIRS). Along with the pro-inflammatory cascade, the anti-inflammatory stimulation is initiated with its main representative, the IL-10. The predominance of the pro- versus the anti-inflammatory profile is associated with both the clinical expression of the infection and the outcome of sepsis [5].

The pathogenesis of gram-negative sepsis has been extensively studied and is almost exclusively attributable to the LPS of bacterial cell wall [6]. The cell wall of gram-positive bacteria contains multiple layers of peptidoglycan (PepG) and molecules of LTA, which protrude from the cytoplasmic membrane as they pass through PepG. LTA makes up about 2% of the dry cell weight and is found in almost all gram-positive bacteria. It has been shown that LTA is a more potent immunostimulant compared to PepG, which is primarily a supportive, inert molecule [7]. The fungus cell wall is constructed by three main groups of polysaccharides; polymers of mannose (mannoproteins, 40% of the cell wall dry mass), polymers of glucose (*b*-glucan, 60% of the cell wall dry mass), and polymers of N-acetylglucosamine (chitin, 2% of the cell wall dry mass). Among them, mannan has special significance since it provides antigenic variability and is a potent immunostimulator [8].

The common factor underlying the increased susceptibility to infections in the three aforementioned patient groups might be a state of immunosupression which is induced by an exaggerated anti-inflammatory reaction. Immunosupression and its extreme form, immunoparalysis, have mainly been studied in septic patients. However, there is enough evidence that the above situations are responsible for the septic events observed in many clinical states, such as severe trauma and burn, major operation, ischemia-reperfusion injury, and other serious insults. The commonest methods used to detect immunoparalysis include a reduced expression of HLA-DR on monocytes, and the impaired release of pro-inflammatory mediators after *ex-vivo* stimulation of monocytes or whole blood by TLRs stimulants [9].

The main purpose of this study was to concurrently evaluate the immune response of ESRD, DM, and ICU non septic patients after provocation with LTA and mannan. To the best of our knowledge, this is the first reported paper describing this evaluation.

2. Materials and methods

2.1. Patients and definitions

In this observational study, we included 10 healthy volunteers, 10 patients with ESRD before dialysis, 10 patients with DM type II and glycosylated hemoglobin (HbA1c) >6.5%, and 10 ICU patients. Critically ill patients were on their second day of stay, had non-septic SIRS and an Acute Physiology and Chronic Health Evaluation (APACHE) II score ≥25. All volunteers were residents of the urban area of Athens and had normal sleep/wakefulness patterns. General exclusion criteria included age less than 18 or more than 80 years, active infection, a history of recent malignancy, current immunosuppression or corticosteroid therapy, and treatment with non steroid anti-inflammatory drugs. In order to limit overlap among the groups, patients who had impaired renal function (glomerular filtration rate – GFR <89 mL/min/1.73 m^2) were excluded from the DM group, patients who had diabetes as an underlying disease were excluded from the ESRD group, and patients who suffered from pre-existing CKD or DM were excluded from the ICU group. Furthermore, individuals with procalcitonin (PCT) concentration >0.5 mg/L were excluded, while especially for the ICU patients, cultures of blood, bronchial secretions, and urine were collected upon entry in the study, and patients with positive cultures were excluded from the study as well.

Patients with SIRS should manifest two or more of the following criteria according the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: fever (temperature >38 °C) or hypothermia (temperature <35.5 °C), tachycardia (>90 beats per minute), tachypnea (>20 breaths per minute), hypocapnia ($PaCO_2$ <32 mm Hg), and leukocytosis or leukopenia (white blood cell count >12,000/mm^3 or <4,000/mm^3 respectively) [10]. CKD was defined and classified according the National Kidney Foundation criteria: duration >3 months, GFR <60 ml/min/1.73 m^2, and structural or functional abnormalities other than decreased GFR; stage 5 denotes GFR <15 ml/min/1.73 m^2 or need for dialysis [11]. DM was diagnosed according the American Diabetes Association Guidelines: fasting plasma glucose ≥126 mg/dl, 2 h plasma glucose ≥200 mg/dl, glycosylated hemoglobin ≥6.5%, or a random plasma glucose ≥200 mg/dl with classic symptoms of hyperglycemia or hyperglycemic crisis. [12].

The study protocol was approved by the Scientific Council and the Ethics Committee of the "401 General Army Hospital of Athens". Patients and healthy volunteers gave informed consent for blood collection and subsequent laboratory examinations. Demographics, clinical, and laboratory data were also collected.

2.2. Blood collection

Blood samples were collected via a peripheral vein and then placed in tubes containing EDTA and immediately transferred to the lab for further processing. WBC and other cell counts were measured by an automated cell analyzer and then by direct microscopy. Additional blood tests included serum biochemistry including glucose, renal and liver function tests, serum CRP and serum Procalcitonin. An additional blood sample (200 μl) was used for *ex-vivo* LTA and mannan provocation and subsequent measurement of cytokine produced in the supernatants at baseline and 8 h after.

2.3. Reagents

LTA was derived from *staphylococcus aureus* and mannan from *saccharomyces cerevisiae*. Both products were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Both molecules were purified and free of LPS contamination.

2.4. Whole blood assay

Cytokine induction by LTA and mannan was measured as previously described by other investigators and ours [13,14]. Briefly, heparinized blood from examined persons was diluted 1:10 in RPMI 1640 culture medium. Two hundred microliters of blood sample was added to 1800 μL of RPMI 1640 for final volume 2 ml and placed in two plastic culture dishes, 1 ml per dish. Samples were added to wells and maintained at 37 °C in a 5% CO_2 atmosphere, and 1 μg LTA and 100 μg mannan were added at the two aliquots. Incubation period lasted for 8 h. Doses and incubation time were derived from a previous study of our team in 10 healthy volunteers. After the incubation period, the culture plate was briefly centrifuged (1800 rpm, 5 min) and supernatants were collected and stored at −70 °C until the measurements of cytokines.

2.5. Cytokine assays

Levels for TNF-α, IL-6, IL-1β, and IL-10 were determined using commercially available human specific enzyme-linked immunoassay kits (BioVendor R& D Products, Vrno, Czech Republic). All kits for cytokines were human specific with sensitivity of detection