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Original Article

New possible biomarkers for diagnosis of infections and diagnostic distinction between bacterial and viral infections in children

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ABSTRACT

Detailed information about patients with infections is required to ensure appropriate choice of treatment. Although white blood cell (WBC) counts, and C-reactive protein (CRP) levels are useful diagnostic indicators of infections, more rapid and easily assayed indicator(s) could improve diagnosis. Moreover, it is of pivotal importance to distinguish bacteria or viruses as causative pathogens. Overall, TLR2 and TLR4 expression levels in neutrophils derived from individuals (n = 118) with bacterial (n = 37) and viral (n = 34) infections were higher than those in control samples (n = 47). Significant higher levels of TNF-α in patients with both types of the infection were observed, and those of IL-4, IL-8, IL-10, and IL-12 also were observed in the present study. Levels of IL-2, IL-8, and IL-10 on day 1 post-viral infection were significantly higher than those on day 1 post-bacterial infection. Therefore, there is a possibility that IL-4, IL-8, IL-10, IL-12 and TNF-α might be biomarkers for infections, in addition to WBC counts and CRP levels, and that IL-2, IL-8 or IL-10 are potentially able to distinguish between bacterial and viral infections.

1. Introduction

In infections, the host innate immune response is characterized by the initial recognition of invading microbes by host “sentinel” cells via Toll-like receptors (TLRs) or other pattern recognition molecules [1]. The unlimited ability of the innate immune system to recognize a wide range of pathogens is controlled by limited numbers of microbial determinants expressed as pathogen-associated molecular patterns (PAMPs) on infective organisms [2,3]. The innate immune system uses germline-encoded receptors, pattern-recognition receptors (PRRs), which are capable of recognizing PAMPs. TLRs are a major family of PRRs involved in innate immune responses to infectious agents [4,5], and are expressed in various cell types, including circulating immune cells [5]. Among TLRs, TLR2 and TLR4 are expressed on immune cells, including peripheral neutrophils [6]; they are known as bacterial sensors, but are also reported to be involved in the detection of viral infections [7,8]. In addition, the interleukins, together with TNF-α and other chemokines, help to regulate inflammation and the intensity of the immune response, and play a key role in activating the adaptive immune response [9].

Neutrophils are very effective initial phagocytes, whose main function on activation is thought to be the clearance of infecting bacteria. To achieve this, these cells are equipped with a myriad of antimicrobial molecules, grouped into oxidative and non-oxidative systems. The complement system and neutrophil granulocytes are also important for eliminating bacterial or fungal infections [10,11].

Cytokines are produced by the immune system in response to invading pathogens [12]. A network of cytokine signals is essential in modulation of the inflammatory response, clearance of pathogens, and subsequent repair of infected tissues. Cytokines can be classified into two broad groups, based on their predominant functions; IL-1β, IL-1α, IL-6, IL-8, IL-17, MIP-1, and TNF-α are pro-inflammatory cytokines, whereas IL-4, IL-10, and TGF-β are anti-inflammatory cytokines [13–15]. However, this classification is not absolute, as many cytokines are capable of exerting both pro- and anti-inflammatory effects, depending on a variety of factors, such as immunological and clinical contexts [16].

Infections are characterized by signs and symptoms overlapping with other acute critical conditions, such as organ-specific infection syndromes. Laboratory parameters, such as white blood cell (WBC)

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counts, and C-reactive protein (CRP), provide additional diagnostic information. The distinction between bacterial and viral infections is clinically important, but often very difficult. This difficulty often leads to unnecessary treatment with antibiotics, which is unfortunate in the light of the growing problems with antibiotic resistance [17,18]. Currently used methods that may aid in the distinction between bacterial and viral infections are primarily WBC counts and CRP levels [19,20], which have typical clinical performances of 70%–80% sensitivity and specificity, resulting in a high rate of misdiagnosis [21]. Recently, the new bacterial infection markers, presepsin, procalcitonin, CD64 and proADM, have been described [22–25]. Presepsin, procalcitonin, and CD64 are used for the diagnosis of severe sepsis and septic shock, and proADM is used for prediction of the prognosis of bacterial infections; hence, these indicators are not suitable for the diagnosis of mild bacterial and viral infections in outpatients who do not require admission. For those patients, the development of more accurate laboratory methods is warranted.

Although there is accumulating evidence concerning the relationships among PAMPs, TLRs and levels of various cytokines in infections, differences in the roles of TLRs and levels of cytokines depending on whether an infection is bacterial or viral are not clear. The aim of this study was to analyze these in patients. Our studies revealed, for the first time, differences in TLR2 and TLR4 expression levels in peripheral neutrophils and variation in the pattern of plasma cytokine production between bacterial and viral infections.

2. Patients and methods

2.1. Study population

This study was reviewed and approved by the Toho University Ethics Committee. (permissions No. 19032 and No. 24003). Patients (n = 118) were enrolled at first visit to the pediatric outpatient department at Toho University Omori Medical Center and received medical treatment without being admitted to a hospital. Written informed consent was obtained from their parents. Patients were classified into three groups; 37 with bacterial infections, 34 with viral infections, and 47 without infections as controls, after diagnosis by a pediatrician. Patients in control group were clinically diagnosed with non-infectious and non-inflammatory diseases (e.g., umbilicus and inguinal hernia). No patients in any group developed immunological disorder. Patients in the bacterial and viral infection groups were further classified into those with samples taken on day 1 (range, ≥day 1 − <day 2), day 3 (≥day 3 − <day 4), or day 5 (≥day 5 − <day 6) after initial fever symptoms. These patients were not continuously examined and were all different individuals (i.e., more than one sample was not taken from any patient). All patients in both infection groups were judged as having “mild infection”, because they had recovered at the time of subsequent visits. Patient characteristics and clinical laboratory data are presented in Table 1 and causative microorganisms data are presented in Table 2.

2.2. Isolation of neutrophils

Whole blood samples (2 mL) were obtained from all patients before medical treatment. Each sample was centrifuged for separation of cells and plasma, and the plasma was stored at −80 °C until analysis of cytokines. Red blood cells in the cellular fraction were lysed with VersaLyse reagent (Beckman Coulter, Inc., CA, USA) for 10 min. The remaining cells were washed with PBS and neutrophils isolated using a Human CD66abce MicroBead kit (Miltenyi Biotec Inc., CA, USA). The isolation procedure was carried out following a published method [26] and isolated neutrophils were used immediately for quantitative analysis of TLR2 and TLR4 expression. The purity of the neutrophils was >99%.

2.3. Quantitative analysis of TLR2 and TLR4 expression

Neutrophils were stained with monoclonal antibodies CD282/TLR2-PE or CD284/TLR4-PE for 30 min at 4 °C.

The fluorescence staining of neutrophils was measured by FACSCalibur flow cytometry (Becton Dickinson, NJ, USA). Data were analyzed using FCS express 4 (De Novo Software, CA, USA).

2.4. Cytokine assays

Plasma IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, and TNF-α levels were measured using a Q-Plex Cytokine assay kit (Quansys Biosciences, NJ, USA), following the manufacturer's instructions. When cytokine levels were lower than the detectable limit, values were recorded as half of the Lower Limit of Detection.

2.5. Statistical analyses

Direct comparisons between any two groups were performed using the Mann–Whitney *U* test. P values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, CA, USA).

3. Results

3.1. Clinical laboratory data in patients with bacterial or viral infection

In patients with bacterial infections, total white blood cell and neutrophil counts, and CRP levels were significantly higher than those in controls (Table 1). For patients with viral infections, white blood cell and neutrophil counts were significantly higher than those of controls in the early period of infection (day 1 post-infection); however, after day 3 post-infection counts decreased and were not significantly different to those of controls. CRP values in patients with viral infections were significantly higher than those of controls at all time points.

3.2. TLR2 and TLR4 expression levels in neutrophils from infected patients

TLR2 and TLR4 expression levels were significantly higher in neutrophils from infected patients compared to those in neutrophils of controls until day 5 post-infected outpatients with bacterial infections (Fig. 1). In patients with viral infections, expression levels of both TLR2 and TLR4 gradually increased over the course of the infections.

There was a significant difference in the level of TLR2 between bacterial and viral infection groups on day 3 post-infection (Fig. 1).

3.3. Patterns of cytokine production in infected patients

The level of IL-1α was significantly higher in samples from patients with bacterial infections than in those from controls until day 5 post-infection (Fig. 2). IL-1α, IL-1β and IL-2 cytokine levels were significantly elevated compared to those of controls on day 1 post-viral infection and a significantly higher level of IL-1β was also observed on day 3 post-viral infection. IL-4, IL-8, IL-10, IL-12, IL-17, and TNF-α levels were significantly higher in samples from patients with both bacterial and viral infections than in those from controls until day 5 post-infection, except for that of IL-17 on day 5 post-viral infection. IL-6 levels were significantly higher than those of