Estimating the Capacity of Nucleated Cells to Produce Cytokines using APACHE II Score for Septic Patients in a Clinical Setting

Tsukasa KUWANA, Kosaku KINOSHITA, Atsushi SAKURAI, Atsunori SUGITA and Junko YAMAGUCHI
Division of Emergency and Critical Care Medicine Department of Acute Medicine, Nihon University School of Medicine

Although immune function is a factor in cases of sepsis with high mortality, it cannot be measured at the bedside. We investigated blood cytokines and the capacity of nucleated blood cells (NBCs) to produce cytokines (IL-6, 8, 10) using clinical data from septic patients, and considered how this was related to the patient outcome.

This study targeted thirty patients suffering from severe sepsis or septic shock. Peripheral whole blood was collected prior to treatment for sepsis, 6 hours after such treatment, and again at 24 hours post-initiation. Residual peripheral blood was placed into laboratory dishes and exogenous lipopolysaccharide (LPS) was added (10 ng/mL). The capacity of NBCs to produce cytokines was calculated using the ratio of each cytokine before (non-LPS) and after (s-LPS) in vitro LPS stimulation (s-LPS/non-LPS).

The ratio of these cytokines exhibited significant negative correlations with a higher APACHE II scores. The APACHE II score was significantly associated with a poor outcome, but there was no correlation with outcome for any other clinical data.

A higher APACHE II score could be used to indirectly estimate immunoparalysis in the clinical setting.

Key words: severe sepsis; lipopolysaccharide; interleukin; APACHE II score

Materials and Methods

This study was approved by the Clinical Research Review Committee of Nihon University School of Medicine (RK-100709-4).

This study targeted adult patients admitted to the intensive care unit (ICU) of Nihon University Itabashi hospital between August 2009 and March 2011, who satisfied the definition of severe sepsis or septic shock and were treated using our sepsis treatment protocol. According to the sepsis treatment protocol, in addition to treatment according to Surviving Sepsis Campaign guidelines, patients diagnosed with severe sepsis or septic shock received direct hemoperfusion using a polymyxin B immobilized fiber (PMX-DHP) therapy at least once.

Outcome was evaluated at 28 ICU days. Peripheral whole blood was collected before sepsis treatment (pre-treatment), 6 hours after treatment, and again at 24 hours. Items recorded include: age, gender, Acute Physiology and Chronic Health Evaluation (APACHE) II score, white blood cell count (WBC), WBC fractions (neutrophils, lymphocytes), procalcitonin (PCT), C-reactive protein.

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Definition of capacity to produce cytokines by nucleated cells and how to measure supernatant cytokines

In this study, peripheral blood was directly stimulated with lipopolysaccharides (LPS) in a small laboratory dish, and then the capacity of the nucleated cells in this peripheral blood to produce cytokines was objectively measured.

First, peripheral blood was collected before sepsis treatment as pre-treatment (on ICU admission), 6 hours after treatment, and again at 24 hours. Second, 2 mL of peripheral blood from each time period was placed in two laboratory dishes (Collagen Type I-Coated Microplate IWAKI, Japan)\(^8\). Exogenous lipopolysaccharide (LPS; 10 ng/mL) (from *Escherichia coli* serotype 055: B5 SIGMA, USA) (s-LPS) was added to one dish and control cell culture medium, Medium199 (non-LPS) (Life Technologies Corporation Grandisland, NY 14072, USA) was added to the other dish. Both dishes were allowed to stand for 5 hours at 37 degrees Celsius. After 5 hours, 1 mL of supernatant was taken from each laboratory dish and centrifuged for 5 minutes at 3000 rpm, then stored at -80 degrees Celsius. IL-6, 8 and 10 in each supernatant were measured. Blood and supernatant cytokines (IL-6, IL-8 and IL-10) were measured twice with enzyme-linked immunosorbent assay (ELISA) (Quantikine Human IL-6, 8, 10 Immunoassay, R & D Systems, Minn., MN, USA) and analyzed spectrophotometrically at 450 nm.

The capacity of the blood to produce cytokines was determined by calculating the amount of each cytokine in the supernatant using the cytokine ratio. The ratio was calculated by dividing the s-LPS value by non-LPS value (s-LPS/non-LPS) at the same time point. This was defined as the “capacity to produce cytokines (IL-6, 8, 10)”.

Statistical analysis

All analyses were conducted using SPSS (IBM Statistics Version 22, Chicago, IL, USA). The data were presented as its mean (SD) or as the number of cases (%). Continuous variables were compared using the Mann-Whitney U test as appropriate. The \( r^2 \) test was used to compare categorical variables and the Friedman test was used to compare changes over time within the same group. Multiple logistic regression (odds ratios and 95% confidence interval [CI]) were used to examine whether capacity of the blood to produce cytokines (cytokine ratio: s-LPS/non-LPS ratio) was an independent factor for outcome in septic patients. Predicted patient outcomes were studied using single regression analysis on the calculated APACHE II score. Finally, the relationship between the APACHE II score, blood cytokines levels (pg/ml) and cytokines ratio (IL-6, 8, and 10), as represented by the fraction s-LPS/non-LPS, was estimated using the Spearman rank test, with \( P < 0.05 \) set as statistical significance.

Results

Patients background

During the 20-month study period, of the 62 patients who satisfied the diagnostic criteria of severe sepsis, 32 patients were excluded following the exclusion criteria (15 patients underwent other continuous hemofiltration dialysis to remove cytokines using a polymethylmethacrylate membrane hemofilter, a catheter for dialysis could not be inserted in 11 patients at the time of diagnosis, five patients had undergone two courses of PMX-DHP, and one patient was suspected of allergy to the salt nafamostat mesylate). As a result, a total of thirty patients were included in this study. Outcome after 28 ICU days was survival in 20 cases (survival group) and mortality in 10 cases (non-survival group). Patient background of these two groups (survival and non-survival group) is shown in Table 1. No significant difference was observed between the two groups regarding gender, WBC, WBC count fraction (neutrophil, lymphocyte count), PCT, CRP, platelet count, endotoxin levels, mean blood pressure and core body temperature, except for age, APACHE II score, heart rate and respiratory rate. A high incidence (50%) of septic shock, criteria for inclusion of sepsis patients in the study, was observed in the non-survival group.

Cytokine ratio as capacity to produce cytokines

IL-6 cytokine ratios (s-LPS/non-LPS) at each point in time were associated with outcome (Fig. 1).

A weak negative correlation between APACHE II score and individual cytokine ratio was observed (Fig. 2).

In addition, a negative correlation between blood cytokine levels and individual cytokine ratio was observed (Fig. 3). A high serum cytokine level indicated a significant negative correlation with the cytokine ratio, although there was no reference data for the cytokine ratio in patients with sepsis or other critical illnesses.

Outcome prediction using APACHE II score

Single logistic regression analysis was performed to identify outcome based on APACHE II score. From the logistic-regression analysis, the APACHE II score (pre-treatment: adjusted odds ratio per 1 change; 1.24; 95% CI, 1.0609–1.4446, \( P = 0.0006 \), 6 hours after treatment: adjusted odds ratio per 1 change; 1.31; 95% CI, 1.0864–1.5788, \( P < 0.0001 \), at 24 hours; adjusted odds ratio per 1
Table 1  Characteristics of patients with severe sepsis or septic shock in the survival and non-survival groups at admission

| Characteristics                  | All          | Survival     | Non-survival | P value |
|----------------------------------|--------------|--------------|--------------|---------|
| Number (n) total (n = 30)        | (n = 30)     | (n = 20)     | (n = 10)     |         |
| Age (years)                      | 70.0 ± 11.4  | 64.3 ± 10.6  | 76.0 ± 7.5   | 0.0163  |
| Gender (male; %)                 | 19 (63%)     | 12 (60%)     | 7 (70%)      | 0.0514  |
| Severe sepsis/septic shock, n    | 22/8         | 17/3         | 5/5          | 0.0410  |
| APACHE II score                  | 19.4 ± 8.3   | 16.5 ± 6.8   | 27.3 ± 3.6   | 0.0020  |
| WBC (× 10^3/µL)                  | 14.3 ± 8.2   | 12.0 ± 7.5   | 19.3 ± 9.7   | 0.0502  |
| Neutrophil (× 10^3/µL)           | 13.4 ± 8.0   | 11.2 ± 7.3   | 18.2 ± 9.4   | 0.0529  |
| Lymphocyte (× 10^3/µL)           | 0.5 ± 0.31   | 0.41 ± 0.31  | 0.42 ± 0.30  | 0.8950  |
| Platelet (× 10^4/µL)             | 12.7 ± 9.9   | 13.6 ± 11.0  | 6.1 ± 4.46   | 0.0556  |
| PCT (ng/mL)*                     | 48.1 ± 80.9  | 36.8 ± 77.6  | 27.4 ± 32.7  | 0.4794  |
| CRP (mg/dL)                      | 16.5 ± 9.1   | 19.6 ± 9.46  | 14.0 ± 6.41  | 0.4815  |
| Endotoxin (pg/mL)**              | 4.3 ± 10.5   | 5.4 ± 12.3   | 1.4 ± 0.00   | 0.8388  |
| Blood IL-6 (pg/mL)               | 9381.4 ± 33647.6 | 470.9 ± 821.1 | 27202.3 ± 55833.1 | 0.0311 |
| Blood IL-8 (pg/mL)               | 2704.8 ± 12913.0 | 201.8 ± 351.5 | 7710.9 ± 22254.6 | 0.0678 |
| Blood IL-10 (pg/mL)              | 3266.8 ± 862.3 | 58.2 ± 31.7  | 863.3 ± 1383.4 | 0.0197 |
| MAP (mmHg)                       | 83.2 ± 17.4  | 80.6 ± 17.4  | 79.1 ± 15.6  | 0.8949  |
| HR (/min)                        | 104.3 ± 20.6 | 101.1 ± 15.6 | 116.8 ± 22.4 | 0.0426  |
| RR (/min)                        | 22.3 ± 7.8   | 19.9 ± 7.5   | 27.8 ± 7.7   | 0.0162  |
| Temperature (degree)             | 37.0 ± 1.08  | 37.3 ± 0.76  | 36.6 ± 1.6   | 0.3900  |

* Measurement of PCT was performed in 20 cases in the survival group and 9 cases in the non-survival group.
** Measurement of endotoxins was performed in 20 cases in the survival group and 8 cases in the non-survival group.

P value; survival group versus non-survival group.

Results are shown in mean ± standard deviation.

n: number, APACHE II: Acute Physiology and Chronic Health Evaluation II, PCT: procalcitonin, CRP: C-reactive protein, IL: Interleukin, MAP: mean arterial pressure, HR: heart rate, RR: respiratory rate, WBC: white blood cell.

Cytokine ratio Odds ratio and 95% CI

| Cytokine ratio | Poor outcome | Good outcome | Odds   | 95% CI   | P value |
|----------------|--------------|--------------|--------|----------|---------|
| Pre-treatment  |              |              |        |          |         |
| IL-6           |              |              | 0.80   | 0.572-0.978 | 0.0133  |
| IL-8           |              |              | 0.99   | 0.929-1.062 | 0.8815  |
| IL-10          |              |              | 1.05   | 0.860-1.299 | 0.5742  |
| 6 hours after treatment |              |              |        |          |         |
| IL-6           |              |              | 0.87   | 0.644-0.983 | 0.0114  |
| IL-8           |              |              | 0.98   | 0.907-1.019 | 0.2878  |
| IL-10          |              |              | 1.11   | 0.325-4.951 | 0.8663  |
| at 24 hours    |              |              |        |          |         |
| IL-6           |              |              | 0.74   | 0.482-0.951 | 0.0014  |
| IL-8           |              |              | 1.02   | 0.938-1.081 | 0.4689  |
| IL-10          |              |              | 0.91   | 0.483-1.031 | 0.1757  |

Fig. 1  Cytokine production response to LPS related to outcome in patients with severe sepsis or septic shock.

Multiple logistic regression analysis showed that a decreased level of IL-6 ratio was significantly associated with poor outcome (adjusted odds ratio 0.74–0.87; per 1 decrease).

Pre-treatment: before sepsis treatment (n = 30), 6 hours after treatment: 6 hours after sepsis treatment was started (n = 30), at 24 hours: 24h after sepsis treatment was started (n = 30).

Cytokine ratio: cytokine production response to LPS; s-LPS/non-LPS ratio.

We based the cytokine production capacity of the supernatant to produce cytokines on the ratio of each cytokine at the same point in time. This is calculated by dividing the value of s-LPS by non-LPS (s-LPS / non-LPS).

IL: Interleukin, CI: Confidence interval, Odds ratio: Adjusted odds ratio per 1 change in s-LPS/non-LPS ratio.
There was a weak negative correlation between APACHE II score and each cytokine ratio (Figure 2-A:IL-6 ratio, Figure 2-B:IL-8 ratio, Figure 2-C:IL-10 ratio) based on all data points obtained from pre-treatment (n = 30), 6 hours after treatment (n = 30), and at 24 hours (n = 30).

A: IL-6 ratio: $r = -0.5825, P < 0.0001$ (n = 90)
B: IL-8 ratio: $r = -0.5552, P < 0.0001$ (n = 90)
C: IL-10 ratio: $r = -0.4925, P < 0.0001$ (n = 90)

Cytokine ratio: cytokine production response to LPS; s-LPS/non-LPS ratio.

We based the cytokine production capacity of the supernatant to produce cytokines on the ratio of each cytokine at the same point in time. This is calculated by dividing the value of s-LPS by non-LPS (s-LPS/non-LPS).

APACHE: Acute Physiology and Chronic Health Evaluation.

A negative correlation between blood cytokine levels and individual cytokine ratio (Figure 3-A:IL-6 ratio, Figure 3-B:IL-8 ratio, Figure 3-C:IL-10 ratio) based on all data points obtained from pre-treatment (n = 30), 6 hours after treatment (n = 30), and at 24 hours (n = 30).

A: IL-6 ratio: $r = -0.7399, P < 0.0001$ (n = 90)
B: IL-8 ratio: $r = -0.6653, P < 0.0001$ (n = 90)
C: IL-10 ratio: $r = -0.6802, P < 0.0001$ (n = 90)

Cytokine ratio: cytokine production response to LPS, ratio of s-LPS to non-LPS.

Cytokine production capacity: capacity of the supernatant to produce cytokines was calculated using the ratio of each cytokine at the same point time. The ratio is calculated by dividing the value of s-LPS by non-LPS (s-LPS/non-LPS).

Blood cytokine levels (pg/mL).

Figures were constructed using a semilog graph.
change; 1.34; 95% CI, 1.073–1.679, P < 0.0001) was associated with non-survival. In ROC analysis, APACHE II score at pre-treatment, 6 hours after treatment or at 24 hours yielded maximum sensitivity at 0.90, 0.90, or 0.90, and specificity at 0.75, 0.80, or 0.85, respectively (Fig. 4).

Discussion

Main results of this study indicate that a decreased level of capacity of blood nucleated cells to produce cytokines after LPS stimulation was an independent factor for poor outcome. In fact, this phenomenon significantly correlates with poor outcome among APACHE II scores, accompanied with lower cytokine ratio. A higher APACHE II score may be used as a parameter to indirectly estimate whether a septic patient has a decreased level of capacity to produce cytokines from blood nucleated cells. However, the question remains as to whether this phenomenon is the result of immunosuppression, or if cytokines were already excessively released from blood cells. Greater cytokine production from blood nucleated cells might be no longer be possible as high blood cytokine levels were indicated at the points with a lower cytokine ratio.

It has been reported that the capacity of leukocytes isolated from peripheral blood to produce cytokines and superoxides can be suppressed, causing severe stress response, such as septic shock. The reason for this suppression is that various substances (e.g. various proteases, steroids, catecholamines, nitric oxide, endotoxin) released into the blood due to sepsis suppress mediator production from nucleated cells in the blood. In severe sepsis, immunodeficiency has been characterized by inactivation of monocyte function, so-called immunoparalysis. Although severe sepsis is characterized by hypercytokinemia, physicians cannot always identify the markers of immunoparalysis, such as a decreased capacity to produce cytokine from nucleated cells in patients who fall under the definition of severe sepsis or septic shock at the time of hospitalization. APACHE II score has been shown to predict the outcome for sepsis, but is still not used as a method to rate these conditions in septic patients. It may be clinically significant to determine the appropriate therapeutic strategy by using the APACHE II score to estimate the relationship between the cytokine production suppression ratio and sepsis-related outcome. Rapid detection of these sepsis-induced immune changes could lead to early identification of an immunoparalytic state and timelier therapy.

We were not able to determine the origins of serum cytokines, and the severity of the clinical classification of severe sepsis or septic shock might not always be in agreement with the immunoparalysis etiology for sepsis patients. In fact, many reports indicate that there is a dissociation of immunosuppression of organ cells and hypercytokinemia in cases of severe sepsis or septic shock.

Limitations

There are some limitations in this study. First, the
amount of LPS was fixed. We did not measure how varying the amount of LPS would affect cytokines, so whether the cytokine value changes corresponding to a different amount of LPS was not considered. In addition, we could not determine the limit of the capacity to produce cytokine when stimulated by LPS. Moreover, there is no basic data for LPS dose dependent IL responses by nucleated cells. Other additional relevant factors need to be examined.

Second, the relationship between immunoparalysis and cytokine ratio is still unclear because of no previous clinical data regarding the cytokine ratio in physiological condition or critical illness are available. This suggests that estimating the immunosuppressed state of septic patients has clinical significance. We did not measure the number of nucleated cells directly contained in whole blood in which supernatant cytokines were present. The next step is to analyze the pathogenesis of these details, by culturing monocytes, neutrophils and lymphocytes to confirm the inhibitory effect of cytokine production as a whole rather than by peripheral blood nucleated cells as used in this study. To measure the number of nucleated cells, performing flow cytometry is usually needed, but it was not carried out at this time. This is because we wanted to study the response of whole blood to the organism, rather than the reaction of each cellular component (neutrophils, lymphocytes and monocytes).

In this study, WBC and WBC fractions were measured. A significant difference between survival group and non-survival group was not observed. Therefore, reference on how the number of nucleated cells in an individual case may affect the capacity to produce cytokines was not observed.

Clinical significance of the minimal change in the capacity of nucleated cells to produce cytokine in cases of severe sepsis or septic shock is still unclear. However, these phenomena may influence the secondary inflammatory process, which can contribute to the development and progression of multiple organ dysfunction, leading to a worsened clinical condition and ultimately, poor outcome. If a higher APACHE II score could be used to estimate patient immunoparalysis indirectly, it may be a useful tool to devise strategies for the next treatment step in cases of severe sepsis. The number of patients in this study was limited but these results may be useful in developing a hypothesis for future trials.

**Conclusion**

Main results of this study indicate that a decreased level of capacity to produce cytokines, especially IL-6, from blood nucleated cells following LPS stimulation was an independent factor for poor outcome. In fact, this phenomenon significantly correlated with poor outcome among APACHE II scores.

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**Conflict of Interest**

The authors declare that there are no conflicts of interest.

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**Supplementary Table**  APACHE II Severity of Diseases Classification System

| Physiologic Variable | +4 | +3 | +2 | +1 | +0 | +1 | +2 | +3 | +4 |
|----------------------|----|----|----|----|----|----|----|----|----|
| Temperature (°C)     | ≥ 41 | 39–40.9 | 38.5–38.9 | 36–38.4 | 34–35.9 | 32–33.9 | 30–31.9 | ≤ 29.9 |
| MAP (mmHg)           | ≥ 160 | 130–159 | 110–129 | 70–109 | 50–69 | ≤ 49 |
| HR (1/min)           | ≥ 180 | 140–179 | 110–139 | 70–109 | 55–69 | 40–54 | ≤ 39 |
| RR (1/min)           | ≥ 50 | 35–49 | 25–34 | 12–24 | 10–11 | 3–9 | ≤ 5 |
| Oxygenation (mmHg)   | a. FIO2 > 0.5 use A-aDO2 a. FIO2 < 0.5 use PaO2 | (a) ≥ 500 | 350–499 | 200–349 | < 200 | 61–70 | 55–65 | ≤ 65 |
| Arterial pH          | ≥ 7.7 | 7.6–7.69 | 7.5–7.59 | 7.33–7.49 | 7.25–7.32 | 7.15–7.24 | < 7.15 |
| Serum Sodium (mmol/l)| ≥ 180 | 160–179 | 155–159 | 150–154 | 130–149 | 120–129 | 111–119 | ≤ 110 |
| Serum Potassium (mmol/l)| ≥ 7 | 6–6.9 | 5.5–5.9 | 3.5–5.4 | 2.5–2.9 | < 2.5 |
| Serum Creatinine (mg/dl) | ≥ 3.5 | 2–3.4 | 1.5–1.9 | 0.6–1.4 | < 0.6 |
| Hematocrit (%)       | ≥ 60 | 50–59.9 | 46–49.9 | 30–45.9 | 20–29.9 | < 20 |
| WBC (in 1000/mm³)    | ≥ 40 | 20–39.9 | 15–19.9 | 3–14.9 | 1–2.9 | < 1 |
| GCS                  | Score = 15 – GCS |
| Serum HCO₃ (mmol/l)  | ≥ 52 | 41–51.9 | 32–40.9 | 22–31.9 | 18–21.9 | 15–17.9 | < 15 |

A = Total APS Sum of the 12 individual variable points
B = Age Points ≤ 44 years 0 points 45–54 years 2 points 55–64 years 3 points 65–74 years 5 points ≥ 75 years 6 points
C = Chronic Health Points If the patients has a history of severe organ system insufficiency or is immunocompromised assign points as follows:

a. For nonoperative or emergency postoperative patients – 5 points
b. For elective postoperative patients – 2 points

APACHE II Score = Sum of A (APS points) + B (Age points) + C (Chronic Health Points)

MAP: mean blood pressure, HR: heart rate, RR: respiratory rate, WBC: white blood count, GCS: Glasgow Coma Scale, APS: Acute Physiology Score