For a period of six months (from November 2001 to March 2002) we have observed 31 patients with typhoid fever who were hospitalized at Communicable Disease Wards, Dr Soetomo Hospital, Surabaya. The objective of this study was to identify correlations between immune response pattern and the predominance of Th1 and Th2 cells role in typhoid patients with and without complications. As parameters of immune response we observed Th1 using measurement of IFN-γ and IL-2, and Th2 by means of IL-4 and IL-10 measurement from PBMC (Peripheral Blood Mononuclear Cells) culture from the patients. It was found that typhoid patients showed increased IL-4 and IL-10 and decreased IFN-γ compared to normal. It indicates that immune response in patients with typhoid fever shifts toward the predominance of Th2 cells.

Keywords: typhoid fever, immune response, cytokine profile, complication

INTRODUCTION

Typhoid fever, which remains a health problem in Indonesia, is induced by the bacteria Salmonella typhi. Clinical symptoms of this disease varies and the symptoms become evident at the second bacteremic period, during which bacteria in the macrophages trigger the activity of macrophage to release various types of mediator or cytokines. These cytokines may induce reactions presenting as the occurrence of systemic inflammatory reaction (de Jong et al, 1998). Clinical signs that may be observed include high fever, chill, blushing, diarrhea or constipation, and sometimes complications, such as convulsion, delirium and bleeding or intestinal perforation, also occur. In general, hospitalized patients with typhoid fever indicate remarkable or severe clinical signs (Mittrucker et al, 2000). It is unclear, however, whether the severity of the symptoms are due to a certain immune response predominated by Th1 cells or Th2 cells. To address this question, the authors had undertaken a study on the cytokine profile of the hospitalized typhoid patients compared to healthy normal individuals with negative results of microbiological examination (blood culture) who served as controls.

MATERIALS AND METHOD

Blood was taken from recent admitted patients suspected of typhoid fever for microbiological examinations, consisting of Gal culture and Widal test, and for lymphocyte separation (PBMC = Peripheral Blood Mononuclear Cell) for the preparation of immunological examination. Supernatant from lymphocyte cultures were kept at -20 degree C up to the accomplishment of cytokine examinations.

As healthy control, normal healthy individuals were enrolled with the criteria of having no febrile history during the last one month and results of either microbiological or Widal serological examinations revealed negative or under threshold values.

As much as 10 ml blood in the syringe was diluted in 1 : 1 using PBS solution. The procedure to separate mononuclear cells by introduction of Histopaque-1077 was undertaken carefully and the results were rotated. Mononuclear cells-containing layer was removed carefully and, after the addition of PBS solution, it was once again rotated and washed twice, and resuspended by means of 1 ml complete RPMI-1640 solution. Whole cells and the lymphocytes were counted by means of Turk solution, Neubauer counting chamber, and leukocyte pipette, and the results were expressed in percentage. Cell concentration was rendered to be 1 x 10^6 by the addition of complete RPMI-1640 solution in the suspension. PBMC was stimulated by means of PHA and lipopolysaccharide (Harbeck, 1991). Suspension of 1 x 10^6 cells/ml in complete RPMI from PBMC was inserted 100 ml into microplate wells (Nunc), specialized for cell
culture. As much as 100 ml LPS solution in the concentration of 10 mg/ml and 100 ml PHA solution in 1 mg/ml was added in RPMI. Microplate was closed and parafilm was embedded to the ridge of the microplate, and the microplate was subsequently put inside the incubator at 37 degree C with 5% CO2 for 4 days. The obtained supernatant was subjected to ELISA examination, and as control, RPMI with unexposed cells suspension was placed to the tubes.

ELISA test for cytokine IFN-γ and IL-4 (Harbeck, 1991). The ELISA kit was complemented by means of Pelikine-Compact Human Cytokine ELISA Kit (CLB, Amsterdam, The Netherlands) (each for IFN-γ and IL-4) and reagent-containing Pelikine Tool Set. The sensitivity of Pelikine Kit for IFN-γ was 1-2 pg/ml and for IL-4 was 0.2 - 0.4 pg/ml.

The working procedure in this study was in line with the protocol from Pelikine-Compact Human Cytokine ELISA Kit (each for IFN-γ and IL-4) and for reagent-containing Pelikine Tool Set as a complement of ELISA Kit.

**Working principles**

The test used sandwich-type immunoassay enzyme reaction. IFN-γ or IL-4 cytokines, either from the samples or the standards from manufacturers, were caught by anti-IFN-γ or anti-IL-4 monoclonal antibodies residing at the bottom of the well, rendered by coating that was conducted one day before. After being washed to eliminate material remains unused in the reaction, second monoclonal antibody was added to the cytokines that had been biotin-conjugated with bound cytokines. After subsequent washing, horseradish peroxidase- conjugated streptavidin was added, and the results was once again subjected to washing before the addition of substrate solution, and the emerging colors had intensity proportional to the amount of IFN-γ or IL-4 cytokines that would be examined or to those in the standard. This enzymatic reaction was halted by the administration of stop solution. Change of colors could be read by means of ELISA reader. Curve from the reading obtained from standard serum was made, and the concentration of cytokines from samples could be determined using the curve.

**Working procedure**

1. One day before examination, coating was done in the wells of microtitre plate using 100 ml IFN-γ or IL-4 anti-cytokines monoclonal antibody that has been diluted 100 times using coating buffer. Incubation was done overnight in room temperature.
2. In the following day, microplate was washed with phosphate buffer saline (PBS) five times.
3. As much as 200 ml blocking buffer was added to the wells, and incubation was done for 1 hour in room temperature.
4. Washing was done using washing buffer for five times.
5. 100 ml standard IFN-γ or IL-4 cytokines or IFN-γ or IL-4 cytokines-containing supernatant was added to the wells by emptying them for substrate blank. Plate was covered and incubated for 1 hour in room temperature.
6. Washing was done using washing buffer for five times.
7. 100 ml second biotin-containing antibody conjugate was added to the wells, except those for substrate blank. Plate was covered and incubated for 1 hour in room temperature.
8. Washing was done using washing buffer for five times.
9. 100 ml streptavidin-HRP conjugate was given to all wells, except those for substrate blank. Plate was covered and incubated for 30 minutes in room temperature.
10. Washing was done using washing buffer for five times.
11. 100 ml substrate solution was added to all wells, including those for substrate blank. Plate was covered and incubated for 30 minutes in darkness at room temperature. Blue color emerged.
12. 100 ml stop solution was added to all wells. Blue color changed into yellow. Reading was done using ELISA-reader at the wavelength of 450 nm.
13. IFN-γ or IL-4 cytokines was counted in the samples using standardized curve with predetermined level.

**Quality assurance**

Quality assurance is needed in ELISA examination for cytokine measurement. Laboratory reference standard employed in this study was that from WHO (World Health Organization) through NIBSC (National Institute for Biological Standards and Control) in the United Kingdom. Variations between examinations in ELISA test should not be more than 15%.

**RESULTS**

Result of cytokine examination of PBMC culture supernatant is displayed in the following table:
Table 1. Results of cytokine examination from PBMC

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (pg/ml)</td>
<td>33.84 ± 12.93 (n = 27) *</td>
<td>18.81 ± 13.28 (n = 20)</td>
</tr>
<tr>
<td>IL-2 (pg/ml)</td>
<td>39.87 ± 31.68 (n = 30)</td>
<td>60.02 ± 54.58 (n = 20)</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>19.31 ± 16.32 (n = 31) *</td>
<td>33.37 ± 22.97 (n = 19)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>32.74 ± 29.38 (n = 30) *</td>
<td>14.91 ± 9.14 (n = 19)</td>
</tr>
</tbody>
</table>

* = p < 0.05

In the treatment group it was found that the average of IL-4 cytokines from PBMC supernatant was 33.84 pg/ml, with deviation standard of 12.93, while these values in healthy control group were 18.81 pg/ml and 13.28, respectively. The expression of IL-4 was found to be significantly increased (p < 0.05) among the patients. The concentration of IL-2 in treatment group was averagely 39.87 pg/ml with deviation standard of 31.68, and that in control group was averagely 60.32 pg/ml with deviation standard of 54.58. The expression of IL-2 among the patients was statistically not significant (p > 0.05), thus IL-2 between both groups was not significantly different.

The concentration of IFN-γ in treatment group was lower than that in control group. The average value of IFN-γ in treatment group was 19.31 pg/ml with deviation standard of 16.32, while those in control group was 33.37 pg/ml and deviation standard of 22.97, demonstrating a significant different between both groups.

The increase of IL-10 concentration was found in treatment group, in which the average concentration of IL-10 was 32.74 pg/ml with deviation standard of 29.38, and in control group the average value was 14.91 with deviation standard 9.14. The difference was statistically significant (p < 0.05).

In all of these cytokine measurements, a wide variation was found, so that the deviation standard was also high. The role of quality assurance in these measurements is, therefore, very important.

**DISCUSSION**

Infection with *Salmonella typhi* is an intracellular bacterial infection requiring immune response that presents as cellular immunity and its effectors, i.e., macrophage and T lymphocyte. T lymphocyte population is divided into two groups, T-α-β and T-γ-δ cells. T-α-β cells are furthermore divided into T helper CD4 cells and T suppressor/T cytotoxic CD8 cells. T CD4 cells release various cytokines whose function is also divided into several groups, i.e. T helper 1 (Th1) cells and T helper 2 (Th2) cells. Th1 cells produce IFN-γ, IL-2 and lymphotoxin that increase the microbicidal activity of macrophage and augment the delayed type hypersensitive response.

Th1 cells play an important role in human defense system against intracellular bacteria such as *Salmonella typhi*. Together with IFN-α, IFN-γ strengthens the microbicidal activity through the increase of reactive oxide nitrite metabolite, which may facilitate bacterial elimination. IL-2 induces T cells proliferation and enhances factors that activate local macrophage. Th2 cells produce IL-4, IL-5, IL-6 and IL-10, assist the growth and differentiation of B cells, and strengthen humoral immune response. IL-10, produced by Th2 cells, inhibits cytokine synthesis by Th1 cells, while IL-4 inhibits the growth of Th1 cells and reduces the activity of macrophage.

The severity of a disease may affect cytokine response. In hospitalized patients with typhoid fever, the reduction of Th1 immune response and the increase of Th2 immune response were found, indicating the failure of Th1 cells to increase cellular immune response aimed to eliminate the bacteria, which is subsequently compensated by the increase of humoral immune response that results in antibody excess.

In this study, Th1 immune response is represented by IL-2 and IFN-γ cytokines, while the parameters for Th2 immune response were IL-4 and IL-10 cytokines. Precursor cells CD4+, i.e., Thp and Thpp, also produces IL-2, while some mature Th1 cells only produce IFN-γ, but not IL-2. IFN-γ is mostly produced by the lymphocytes, even though it is recently found that it is also produced by macrophage. IL-4 is also known to be produced by T helper 2 cells, and it possesses dual
important functions for Th2 response, that it is responsible for several functional characteristics of Th2 cell effector, and it plays the role as principal inducer of the subsequent differentiation of precursor cells to become Th2 cells (Seder, 1999).

CONCLUSION

This study found that the increase of IL-4 and IL-10 among the patients demonstrated a Th2-dominated immune response, while the reduction of IFN-γ concentration showed the decrease of Th1 activity. It can be concluded that in patients with severe typhoid fever, the pattern of immune response shifts toward the predominance of Th2 and the reduction of Th1. Keuter et al (1994) found an elevation of TNF-α in typhoid fever, in which TNF-α represented the inflammatory cytokines.

REFERENCES


