ORIGINAL ARTICLE

# Increased γδ$^+$ and double negative T cell subsets in children with bacteremia of *Salmonella oranienburg*: an early diagnostic marker for bacteremia

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## Abstract

**Bacteremia of Salmonella is a risk factor of death in Salmonella infection. Early diagnosis to initiate intensive care is a key for favorable outcome, yet no clinical marker is available to detect bacteremia of Salmonella earlier than blood culture. T, natural killer (NK), and B cells play an important role against Salmonella infection and expansion of γδ$^+$ T cell subset was shown in the study mainly targeting typhoid Salmonella infection by flow cytometry (FCM) which can analyze immune cell populations within two hours.**

**During outbreak of *Salmonella oranienburg* (SO) infection, we explored clinical marker to detect bacteremia in children with or without bacteremia of SO and those with enteritis due to other Salmonella using FCM. We also measured serum concentration of immunoglobulins (Igs) to evaluate whether immunocompromised or not. Eighteen children (median age, 6.0 years) were studied and divided into four groups; group A: five children with bacteremia of SO, group B: the same patients of group A who recovered 3 months after the onset of the disease, group C: six children with enteritis due to SO, and group D: seven children with enteritis due to other Salmonella but no bacteremia. The percentages of γδ$^+$ and double negative (DN: CD4$^-$CD8$^-$) T cells in CD3$^+$ subset were increased in group A as compared to groups B, C and D. There was no difference between groups in the following variables; the percentages of CD3$^+$ cells, helper (CD4$^+$), and cytotoxic (CD8$^+$) T cells, and NK cells, serum levels of Igs or complements. Our data suggest that expanded γδ$^+$ and DN T cell subsets in SO bacteremia by FCM can help to detect SO bacteremia in early stage of the disease faster than blood culture.**

**Key words**: *Salmonella oranienburg*, bacteremia, γδ$^+$ T cells, double negative T cells, flow cytometry

## Introduction

Salmonella is an intracellular parasitic bacteria, and it's infection is a common and usually self-limiting disease[1, 2]. Although *Salmonella oranienburg* (SO) is a relatively uncommon serotype among the non-typhoid Salmonella (NTS)[1], outbreak of SO infection has been reported in several countries, including Japan[3]. Infection of Salmonella causes enteritis and extraintestinal symptoms, including vertebral osteomyelitis, paravertebral or retroperitoneal abscess, and infection of soft tissues and cartilages. Bacteremia occurs in approximately 5-6% of children with NTS infection[1, 2]. While the outbreak of SO infection[3], we experienced five patients with SO bacteremia among 11 patients with SO enteritis and the prevalence of bacteremia was very high (45%) comparing to other NTS infection.

Mortality rate of Salmonella bacteremia is about 12 to 20% in non-immunocompromized patients[4, 5], therefore, early diagnosis and initiation of intensive antimicrobial therapy targeting Salmonella bacteremia is mandatory. Blood culture, the gold standard of diagnosis of bacteremia, usually takes more than 12 hours to detect[6, 7]. Flow cytometry (FCM) is a rapid diagnostic method and predominant activation and expansion of γδ$^+$ T cells in systemic Salmonellosis[8] using FCM suggested diagnostic usefulness for Salmonella bacteremia. Double negative (DN: CD4$^-$CD8$^-$) T cells act as bacteriocidal against infection by intracellular parasitic microorganisms including Mycobacterium infection[9]. Because Salmonella is also an intracellular parasitic microorganism, DN T cells could also play a protective role against Salmonella infection. Thus, we analyzed DN T cell subset as well as that of γδ$^+$ T cells in children with or without bacteremia of SO and those with enteritis due to other Salmonella to test feasibility whether γδ$^+$ and/or DN T cells can predict NTS bacteremia.

From the view of the immune status as a predisposing factor to systemic infection of Salmonella, T cells play an important role for innate immunity. For example, experimental evidence suggests that CD3$^+$ T cells including γδ$^+$ T cells[10, 11] and DN T cells[12] play a protective role for innate immunity to bacterial infection. In vivo and in vitro evidence suggests that CD4$^+$ and CD8$^+$ T cells play a role for immune response to Salmonella infection[13]. In addition, recent in vivo evidence suggests a role

of natural killer (NK) cells[14], antibody production by B cells and complements[15] for early immune response to Salmonella infection. Therefore, we also examined B and NK cell subsets, and serum levels of Igs and complements in this study.

Here we report that increased $\gamma\delta^+$ and DN T cell subsets can predict bacteremia of SO by FCM.

## Material and Methods

*Patient population*

We experienced eleven children with SO infection as previously described[3]. All patients have been healthy until admission to Saiseikai Kurihashi Hospital, Saitama, Japan, and none of the patients had underlying diseases including allergic disease or received immunosuppressive agents. Bacteremia occurred in five of eleven patients (45%) infected with SO and other six patients developed enteritis but no bacteremia. During the same period, we had seven children infected with NTS other than SO who developed enteritis but no bacteremia. These eighteen patients (12 males, 6 females, median age, 6.0 years, range 8 months-15 years) were included in the study. This study has been approved by Ethical Committee in Department of Pediatrics, Saiseikai Kurihashi Hospital and written informed consent was obtained from the parents or guardians of each patient.

Patients were divided into four groups; 1) group A: five patients (median age, 6.0 years) with bacteremia of SO in whom bacteria was detected in both blood and stool cultures, 2) group B: the same patients of group A who had bacteremia but recovered from bacteremia 3 months after the onset of the disease, 3) group C: six patients (median age, 5.5 years) with enteritis in whom SO was only detected by stool culture, and 4) group D: seven patients (median age, 6.0 years) who developed enteritis due to other Salmonella but no bacteremia. Lymphocyte subpopulations vary with age[16-18], however median age at the time of the study was similar between groups.

*Assay for populations of T, NK, and B cells in peripheral blood by FCM*

Peripheral blood mononuclear cells (PBMCs) were obtained from each patient and isolated using Ficoll-Hypaque®. PBMCs of each patient were aliquotted to polystyrene tubes and stained with monoclonal antibodies according to staining panel of subsets (ten subsets) as shown in table 1. Percentage of each subset was analyzed by FACScan® with CellQuest3.3® software as previously described[19]. The data for $CD3^+$, $CD3^+\alpha\beta^+$, $CD3^+\gamma\delta^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cell subsets, except for increased variables, are in compatible with the reference values for lymphocyte subpopulations in age-matched healthy children[16-18].

*Assay for serum levels of immunoglobulins and complements*

Serum levels of IgG, IgA, and complements (C3 and CH50) were measured using laser-nephelometry.

*Statistical analysis*

Data are expressed as median and interquartile range. Comparisons of the data between groups were made using Mann-Whitney U-test. Paired data between groups A and B were analyzed using Wilcoxon matched-pairs signed rank test. A p value less than 0.05 was considered significant.

## Results

There was no difference between groups in the percentages of total $CD3^+$cell subset among total lymphocytes in peripheral blood (Table 1). In contrast, the percentage of $CD3^+\gamma\delta^+$ cell subset in $CD3^+$ cell subset was significantly increased in group A ($p<0.05$) as compared to groups B, C and D. However, it did not differ between groups B, C, and D. Similarly, the percentage of DN T cell subset in $CD3^+$ cell subset was significantly increased in group A ($p<0.05$) as compared to groups B, C and D. It did not differ between groups B, C and D.

There was no difference between groups in the percentages of helper T cell ($CD4^+$) in $CD3^+$ cell subset, activated helper T cell ($CD4^+HLA\text{-}DR^+$) in $CD4^+$ cell subset, cytotoxic T cell ($CD8^+$) in $CD3^+$ cell subset, NK cell ($CD2^+CD56^+$) subset, and B cell ($CD19^+$) subset in peripheral blood. No difference was found between groups in the total lymphocyte count of peripheral blood. In contrast, the percentage of $CD8^+HLA\text{-}DR^+$ T cell subset in $CD8^+$ cell subset was increased in group A as compared to group B and D, but did not differ between group A and C. The percentage of $CD3^+\alpha\beta^+$ cell subset in $CD3^+$ cell subset was decreased in group A as compared to groups C, but there was no difference between groups A, B, and D (table 1).

There was no difference between groups in serum levels of IgG and IgA as well as complements, C3 and CH50.

**Table 1**. Percentages of T cell, NK cell, and B cell subsets in peripheral blood of children with or without bacteremia of *Salmonella oranienburg* and those with enteritis due to other Salmonella

| Variable | Group A (n=5) | Group B (n=5) | Group C (n=6) | Group D (n=7) |
|---|---|---|---|---|
| **T cell** | | | | |
| $CD3^+$ cell in total lymphocyte | 76.0 (6.2) | 73.0 (8.6) | 66.5 (6.0) | 67.0 (12.0) |
| $CD3^+\alpha\beta^+$ cell in $CD3^+$ subset | 70.6 (18.2)* | 81.2 (6.2) | 93.9 (13.0) | 89.3 (4.0)* |
| $CD3^+\gamma\delta^+$ cell in $CD3^+$ subset | 28.7 (17.6)# | 15.6 (4.2) | 10.0 (6.8) | 10.2 (3.8) |
| helper T ($CD4^+$) cell in $CD3^+$ subset | 42.1 (9.2) | 54.5 (15.0) | 54.8 (15.6) | 60.8 (15.0) |
| $HLA\text{-}DR^+$ cell in $CD4^+$ subset | 8.5 (5.0) | 4.3 (1.6) | 4.6 (4.6) | 4.6 (2.0) |
| cytotoxic T ($CD8^+$) cell in $CD3^+$ subset | 33.3 (9.2) | 39.1 (13.2) | 44.3 (12.4) | 39.1 (14.6) |
| $HLA\text{-}DR^+$ cell in $CD8^+$ subset | 27.0 (3.8) | 7.0 (9.4)** | 16.1 (13.0) | 7.7 (12.8)** |
| $CD3^+CD4^-CD8^-$ cell in $CD3^+$ subset | 40.4 (24.6)# | 19.7 (7.6) | 14.9 (12.2) | 13.3 (5.8) |
| **NK cell** | | | | |
| $CD2^+CD56^+$ in total lymphocyte | 9.4 (4.8) | 5.6 (1.6) | 9.6 (4.2) | 7.0 (8.6) |
| **B cell** | | | | |
| $CD19^+$ in total lymphocyte | 11.0 (9.8) | 20.0 (11.6) | 14.5 (9.0) | 18.5 (16.0) |
| Total lymphocytes (cells/μL) | 2,856.6 (3,003.9) | 3,610.0 (2,899.0) | 3,464.6 (4,597.0) | 3,220.6 (1,906.0) |
| **Serum** | | | | |
| IgG (mg/dl) | 1,110.0 (87.5) | 967.0 (137.5) | 1,100.0 (40.0) | 1,000.0 (9.0) |
| IgA (mg/dl) | 180.0 (25.0) | 160.0 (35.0) | 137.0 (12.0) | 150.0 (15.0) |
| C3 (mg/dl) | 110.0 (10.5) | 119.0 (5.5) | 135.5 (6.0) | 116.0 (19.0) |
| CH50 (U/ml) | 44.0 (1.3) | 40.0 (1.5) | 47.0 (3.0) | 44.0 (5.0) |

Percentages of lymphocytes and NK cells in total lymphocytes are expressed in % as median (interquartile).
***** $p<0.05$, vs. Group C only, # $p<0.05$, vs. Groups B, C, and D, ****** $p<0.05$, vs. Group A only.

**Discussion**

Mammalian $CD3^+$ T cells can be separated into two subsets bearing T-cell receptors (TCR), αβ and γδ chains[10]. Most $\alpha\beta^+$ T cells use αβ TCR as antigen recognition[10, 11] and $\gamma\delta^+$ T cells expressing $\gamma\delta^+$ TCR recognize a variety of proteins including pathogen without antigen processing (20). Accumulating evidence suggests that $\alpha\beta^+$ and $\gamma\delta^+$ T cells play a role for innate immunity to bacterial infection[10, 11, 20]. However, little information is available about an alteration of $\alpha\beta^+$ and $\gamma\delta^+$ T cells in NTS infection in humans, in contrast to $\gamma\delta^+$ T cell expansion in bacteremia of typhoid Salmonella[8].

In the present study, the percentage of $\gamma\delta^+$ T cell subset increased at early phase of bacteremia of SO, which retuned to the basal level after recovery of the disease. Our findings have been supported by previous studies documenting increased percentage of $\gamma\delta^+$ T cell subset in children with systemic infection of various strains of Salmonella as compared to those with enteritis[8, 21] although no change in these cells was reported in septic adult patients with Salmonella infection[22]. $\gamma\delta^+$ T cells can be more activated than $\alpha\beta^+$ T cells at early stage of Salmonella infection and expansion of $\gamma\delta^+$ T cells occurs when cultured with live Salmonella typhimurium[8]. Experimental evidence suggests that $\gamma\delta^+$ T cells bind to lipolysaccharides (LPS) through Toll-like receptors, resulting in production of Th-1 like cytokines, including interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) (10, 11), and accumulation of $\gamma\delta^+$ T cells at the site of infection[23]. In fact, $\gamma\delta^+$ T cells have been shown to express higher levels of INF-γ in patients with Salmonella infection than in healthy controls[24]. $\gamma\delta^+$ T cells can activate macrophages, which allows macrophages to produce proinflammatory cytokines that are cytotoxic to bacteria[11]. In addition, in murine model of *Salmonella typhimurium* infection, depletion of $\gamma\delta^+$ T cells can reduce antimicrobial responses such as production of pro-inflammatory cytokines and neutrophil influx in the intestinal mucosa, leading to increased translocation of bacteria to the liver[25, 26]. Our data, along with these in vivo and in vitro data, suggest a role of $CD3^+\gamma\delta^+$ T cells for innate immunity to bacteremia of Salmonella, regardless of strains, in children.

In contrast, the percentage of $\alpha\beta^+$ T cell in $CD3^+$ subset of group A was decreased as compared to groups C and D but it did not differ between groups A and B. These findings suggest a minor value of $\alpha\beta^+$ T cell subset for detection of bacteremia in human NTS infection.

DN T cells play a role for regulation of immune responses in bacterial infection[12]. However, no information is available about an alteration of these T cells in human Salmonella infection. We found increased DN T cell subset in peripheral blood at early phase of bacteremia due to SO, which returned to the basal level after recovery of the disease. Similar increase in the DN T cell subset has been shown in infection with *Leishmania major*[27], *Mycobacterium tuberculosis*, and *Francisella tularensis*[9, 28]. DN T cells can be activated after the recognition of bacterial antigen through the major histocompatibility complex class Ib molecule, CD1b[29]. After being activated, DN T cells can proliferate and regulate immune response by producing Th1 and Th2 type cytokines that activate macrophages[12, 30]. In fact, experimental evidence suggests a protective role of DN T cells in infection due to *Listeria monocytegenes*[30] or malaria[31]. Our data, together with these *in vivo* and *in vitro* data, suggest that DN T cells could play a protective role for innate immunity to bacteremia of SO in humans.

Experimental evidence suggests a role of $CD4^+$ and $CD8^+$ T cells for acquired immunity to Salmonella infection[13]. However, we found no difference in the percentage of $CD4^+$ and $CD8^+$ T cell subsets in PBMCs of patients with or without bacteremia of Salmonella, suggesting a minor predictive value of these cells for bacteremia in human Salmonella infection. The percentage of $CD8^+$HLA-$DR^+$ T cell subset was increased at early phase of bacteremia in our patients as compared to that during recovery phase of the disease and to other NTS. However, it did not differ from that of patients with enteritis due to SO, suggesting a minor predictive value for early diagnosis of SO bacteremia.

NK cells play a role for innate immunity to Salmonella infection[14]. Proliferation and maturation of NK cells, promoted by interleukin-15, can reduce bacterial colonization at intestine and systemic tissues in Salmonella infection[14]. Antibody production by B cells or complement-dependent killing of bacteria[15] also play a role for innate immunity to Salmonella infection. Despite these experimental data, we found no difference between patients with or without bacteremia of Salmonella in the percentages of NK or B cell subset in peripheral blood as well as serum levels of IgG, IgA, C3 or CH50. Our data suggest a minor predicting value of these cells, Igs, or complements for bacteremia in human Salmonella infection.

Leukocytopenia occurs commonly in patients with typhoid fever[32], however, there was no difference in total lymphocytes count in children with or without bacteremia of SO and those with enteritis due to other NTS. This suggests total lymphocyte count has less clinical value for identifying SO bacteremia.

We have analyzed ten subsets of immune cells in PBMCs by FCM and were able to obtain analyzed data sets within two hours using 4 ml of whole blood. According to our result, FCM could distinguish SO bacteremia from SO enteritis prior to obtaining of blood culture. Though not all hospitals can equip this simplest flow cytometer comparing to automated blood culture system, flow cytometer is a powerful tool for investigating immune system to provide quantified data.

In summary, our study showed increased percentages of both $\gamma\delta^+$ and DN T cell subsets in PBMCs at early phase of bacteremia in children infected with SO, which returned to the basal level after recovery of the disease. These data suggest that both $\gamma\delta^+$ and DN T cells could contribute to the prevention of bacteremia in human SO infection and increased population of $\gamma\delta^+$ and DN T cell subsets can predict bacteremia of SO in early phase of the disease by FCM.

**Conflicts of interest: None**

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