Inflammatory Cytokines and Antigen-Responsive Mononuclear Cells in Peripheral Blood of Cattle Infected with Salmonella Takoradi

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(Received 11 August 2000/Accepted 16 April 2001)

ABSTRACT. To determine the immunological response in lactating dairy cows infected with Salmonella (S.) Takoradi, the relationships among distributions of peripheral blood mononuclear cell (PBMC) subpopulations, endotoxin concentrations and dynamics of inflammatory cytokines in blood were investigated. The ratio of CD4+ T cells to CD8+ T cells was significantly lower in the affected cattle than in the control cattle (p<0.05) to decrease in the number of CD4+ T cells in the infected cattle. In contrast, the numbers of γδ-T cells, MHC class II-positive cells were significantly higher in the affected cattle than in the control cattle (p<0.01) respectively. Endotoxia was found in all but one of the affected cattle. Serum IL-1 and IL-6 bioactivities were significantly higher in the affected cattle than in the control cattle (IL-1, p<0.05; IL-6, p<0.01). Serum TNF-α activities and levels were not detected in the control and affected cattle. The activities of proinflammatory cytokines determined by the bioassay are important to the relationships between concentration of endotoxin, cytokines and clinical signs, such as leukocytosis, leukopenia, fever or bacterial shedding. Serum IL-2 levels were lower in the affected cattle than in the control cattle. Serum IFN-γ was not detected in the affected cattle except one. These results by the ELISA seemed to reflect the condition of subpopulation in the PBMCs from the shedding cattle. The present results suggest that cellular immunity is suppressed while the humoral immunity is activated in acute bovine salmonellosis.

KEY WORDS: bovine salmonellosis, cytokine, endotoxin, PBMC.

Bovine salmonellosis, caused by infection with Salmonella (S.) Takoradi, is an economically important disease as well as a public health concern in many countries [6, 7, 22]. In adult cattle, salmonellosis causes abortion and severe diarrhea, which can result in death [7, 14]. Clinical manifestations and immunopathological diversities in the course of the disease are dependent on the properties of the pathogen [7, 10, 22, 25]. The most common serotypes in cattle are Salmonella (S.) Typhimurium and S. Dublin [6, 22]. Uncommon serotypes such as S. Infantis and S. Heidelberg in endemic and epizootic outbreaks of bovine salmonellosis have been also recently reported [1, 8, 14].

To date, research on bovine salmonellosis has focused on the biological and molecular bacteriology for vaccine strategies [12, 22, 27, 29]. There have been limited informations on the pathogenesis including the roles of endotoxin and cytokines in this disease [16, 18, 19, 25]. Endotoxin, which is a structural part of the outer cell wall of Salmonellae, induces secretion of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin(IL)-1, and IL-6 by macrophages [7, 16, 18, 25]. It is now accepted that pathodynamic and clinical symptoms caused by intracellular facultative and Gram-negative bacteria depend on interactions between cytokines and immune cells [19, 20, 22, 23, 25, 28]. The pivotal role of immune cells in these responses has been demonstrated most clearly in mouse models [11, 13, 25, 27–29].

However, there is little information on the effects of immunological aspects at the levels of endotoxin, cytokines and subpopulations of mononuclear cells in peripheral blood (PBMCs) in the bovine salmonellosis. The purpose of this study was therefore to elucidate the relationships among the endotoxemia, subpopulations of PBMCs, and cytokine activities or the cytokine levels in the first outbreak case of bovine S. Takoradi.

MATERIALS AND METHODS

Animal preparation: Blood samples were collected from 11 Holstein-Friesian cattle on a dairy farm that had been infected with S. Takoradi on September 16, 1997. All 11 animals showed clinical signs of salmonellosis, including high body temperature, diarrhea and tachypnea. S. Takoradi, which was first reported as a disease of gull (8), was isolated from diarrheal feces of the 11 cattle on September 5, 1997. The blood samples from the 11 cattle were divided clinico-bacteriologically into two groups: one group in which S. Takoradi was identified in the diarrheal feces (group 1, n=5), and the other group in which the organism was not identified in the diarrheal feces (group 2, n=6) on the day of blood sampling. As controls, blood samples col-
lected from 10 healthy cattle in midlactation on a dairy farm in which there had been no outbreak of salmonellosis. The isolated Salmonella serotype was identified by agglutination reactions against somatic (O) antigens (O: 6, 8 DENKA, Tokyo, Japan) and flagella (H) antigens (I: 1, 5, DENKA, Tokyo, Japan). The serum agglutination titers against formalin-inactivated somatic antigen was high (≥52) (Table 1).

Blood sampling and analysis: Blood samples for analysis were collected from the external jugular vein. Erythrocytes and leukocytes, and hemoglobin concentrations were measured by a blood cell counter. Hematocrit values were determined by the microhematocrit method, and total serum protein was determined using a refractometer. Blood films stained with the Giemsa's solution were used for the hemograms.

Flow cytometric analysis of PBMCs: Blood samples for flow cytometric analysis were centrifuged at 250 g for 45 min in order to collect PBMCs.

The separated PBMCs were diluted 1:1 with phosphate-buffered saline (PBS) and layered over Ficoll Conray (1.078 g/mL, Japan). After centrifugation at 900 g for 30 min, the serum was then aspirated. The gelation times were measured using an ET-208 Toxicometer (Wako Chemical Co.) for 90 min as previously described [18].

Bioassay for cytokines: TNF-α in sera was assayed on the basis of cytolytic activity against WEHI 164 clone 28–4 cells using the 3–2,5-diphenyltetrazolium bromide (MTT) method as described previously [3]. Recombinant human (rh) TNF-α was used as a standard to calculate bovine TNF-α activity. Serum IL-1 activity was assayed on the basis of growth inhibition using A-375 cells as described previously [9]. The specificity of IL-1 was confirmed according to the inhibition or reduction of its activity by goat anti-human IL-1 antibody. For the IL-6 assay, serum samples were treated at 56°C for 30 min and then assayed for proliferative activity on IL-6-dependent MH60/BSF2 cells using rhIL-6 as a standard [17].

ELISA for cytokines: If the sandwich enzyme-linked immunosorbent assay (ELISA) developed with immunoasays kit for measurement of human cytokines would be available for the assay of the bovine cytokines, this can be used as a means of assessing cytokine for the diseased cows [24]. Levels of TNF-α, IL-1, IL-2, IL-4, IL-6, IL-8 and interferon-γ (IFN-γ) in serum were determined using the ELISA. These: TNF-α (human TNF-α, #KHC3010-SB; Toyobo, Japan), IL-1β (human IL-1β, #KHC0010-SB; Toyobo, Tokyo, Japan), IL-2 (human IL-2, #KHC0020-SB; Toyobo, Japan), IL-4 (human IL-4, #KHC0040-SB; Toyobo), IL-6 (human IL-6, #KHC0060-SB; Toyobo), IL-8 (human IL-8, #KHC0080-SB; Toyobo) and IFN-γ (human IFN-γ, #KHC4020-SB; Toyobo). Assays were performed according to the manufacturer’s instructions.

Statistical analysis: The mean values and standard errors of the clinical and laboratory data were calculated. The data were evaluated by one-way variance (ANOVA), and p-values < 0.01 or < 0.05 were regarded as significant. Statistical analysis was performed using the statistical program InStat (Graph Pad Software, San Diego, CA, U.S.A.).

RESULTS

Blood analysis: The overall clinical results of hematology are summarized in Table 2. The number of erythrocytes and the levels of hematocrit and hemoglobin were significantly (p<0.05) less in the cattle with salmonellosis than in the control cattle.

FACS analysis of PBMCs: The results of FACSs analysis
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of PBMC are summarized in Fig. 1 (Fig. 1). The numbers of CD4-positive lymphocytes (CD4+ T cells) in groups 1 and 2 cattle were remarkably low (group 1, p<0.05; group 2, p<0.01) compared with those in the control group. No significant intergroup differences in the number of CD8-positive lymphocytes (CD8+ T cells) were observed (Fig.1). Therefore, the ratios of CD4+ T cells to CD8+ T cells in cattle with clinical signs in group 1 (1.26 ± 0.21) and group 2 (1.64 ± 0.25) were lower than the ratio in the control group (2.76 ± 0.30) (p<0.01). The number of CD3-positive lymphocytes (CD3+ T cells) in group 1 (15.03 ± 3.01 × 10^3/µl) and group 2 (13.23 ± 2.77 × 10^3/µl) were also lower than that of in the control group (19.46 ± 2.66 × 10^3/µl). The number of CD14-positive cells in group 2 (4.40 ± 0.78 × 10^3/µl) was lower than that in the control group (6.64 ± 0.97 × 10^3/µl).

In contrast, the number of γδ-T lymphocytes (γδ-T cells), MHC class II-positive cells and IgM-positive cells in both groups 1 and 2 were higher than those in the control group. The immuno-cell counts in group 1 cows were notably higher than those in the healthy cattle (p<0.01) (Fig. 1).

Serum endotoxin concentrations: Serum endotoxin was detected in almost all cattle in groups 1 and 2 (10 of 11 the cattle). The endotoxin level in group 1 (6.77 ± 2.27 pg/ml) was higher than that in group 2 cattle (2.92 ± 1.15 pg/ml) (Fig. 2). Endotoxin was not detected in the control cattle.

Serum cytokine activity estimated by bioassay: Serum TNF-α was not detected in all three groups (data not

Table 2. Clinical hematology in cattle

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (µl)</th>
<th>Group 2 (µl)</th>
<th>Controls (µl)</th>
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</thead>
<tbody>
<tr>
<td>Erythrocyte</td>
<td>463.0 ± 12.0*</td>
<td>482.2 ± 22.0*</td>
<td>542.9 ± 16.6</td>
</tr>
<tr>
<td>Leukocyte</td>
<td>76.0 ± 5.6</td>
<td>59.0 ± 1.2</td>
<td>75.3 ± 4.0</td>
</tr>
<tr>
<td>PBMCs</td>
<td>44.5 ± 7.4</td>
<td>37.7 ± 8.2</td>
<td>37.7 ± 2.0</td>
</tr>
<tr>
<td>Band neutrophil</td>
<td>3.6 ± 1.1</td>
<td>2.2 ± 0.9</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>Segmented neutrophil</td>
<td>27.9 ± 4.0</td>
<td>19.1 ± 3.8</td>
<td>28.8 ± 8.5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>26.6 ± 2.5*</td>
<td>26.2 ± 0.6*</td>
<td>33.7 ± 0.7</td>
</tr>
<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>8.3 ± 0.5*</td>
<td>6.7 ± 0.1*</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>Total serum protein (g/dl)</td>
<td>7.1 ± 0.2</td>
<td>7.2 ± 0.1</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>Albumin/Globulin</td>
<td>0.91 ± 0.08</td>
<td>0.91 ± 0.06</td>
<td>1.18 ± 0.06</td>
</tr>
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</table>

* The value is significantly different from that of the control cattle in the healthy herd. (p< 0.05).

** The value is significantly different from that of the control cattle (p<0.01).

Fig. 1. Subpopulation of PBMCs determined by FACS analysis. The numbers of CD3+ and CD4+ cells in the affected cattle were remarkably low. The numbers of WC-1, MHC class II and IgM cells in the affected cattle were high. Each bar represents the mean and standard deviation. * The value is significantly different from that of the control cattle (p<0.05). ** The value is significantly different from that of the control cattle (p<0.01).
IL-1 activities were shown by bioassay to be higher in the affected cows (9.7 ± 3.3 U/ml in group 1 and 7.1 ± 2.2 U/ml in group 2) than that in the control cattle (1.7 ± 0.1 U/ml) (p<0.05) (Fig. 3). Serum IL-6 activities were higher in the affected cattle (1.06 ± 0.11 U/ml in group 1 and 0.79 ± 0.14 U/ml in group 2) than the activity in the control cattle (0.06 ± 0.06 U/ml) (p<0.01) (Fig. 3).

Serum cytokine levels estimated by ELISA: Serum IL-1β level (Fig. 4) in the group 1 cattle (2355.2 ± 818.8 pg/ml) were higher than that in the group 2 cattle (567.8 ± 368.3 pg/ml) and the control cattle (522.8 ± 156.1 pg/ml) (p<0.01). Serum IL-6 level (Fig. 4) was significantly (p<0.05) higher in the group 1 cattle (1811.0 ± 594.2 pg/ml) than in the group 2 cattle (1001.5 ± 273.9 pg/ml) and the control animals (957.3 ± 502.9 pg/ml) (p<0.05). There were no differences in the levels of IL-1β or the levels of IL-6 between the group 2 cattle and the control animals. Serum IL-2 levels in the groups 1 and 2 were lower than the level in the control group (Fig. 5). Serum IFN-γ was detected in only one cattle of group 2 (53.7 pg/ml) and in the control cattle (35.7 ± 21.9 pg/ml) (data not shown). TNF-α, IL-4, and IL-8 were not detected in the sera of all animals.

DISCUSSION

CD4+ T cells are important for resistance to infection with S. Typhimurium, though the antigen specificity of this T cell response is unknown [23, 25–27], and there has been much reported evidence showing their roles as anti-cytozoic bacteria [20–22, 25, 28, 29] by secreting INF-γ and IL-2 from helper T (Th)-1 cells in CD4+ T cells [21, 23, 27]. In cases of salmonellosis, it has been reported that the number of CD4+ T cells in peripheral blood increases and that the ratio of CD4+ T cells to CD8+ T cells is increased [21, 25, 27]. On the other hand, in experimental oral infection in mice with S. Typhimurium, it was found that the number of γδ-T cells increases in association with the immune response [11, 13]. These lymphocytes play a critical role in mucosal immune responses to intracellular facultative bacterial infection [11, 13, 25, 27]. Among the lymphocytes in ruminants, there are large numbers of γδ-T cells in peripheral blood, jejunal and ileal Peyer’s patches. The γδ-T cells play important roles in the immune response to intracellular bacteria [4, 5, 22] and immune regulation for the participation of MHC class I-
encoded molecules [11, 13, 25].

The low number of CD4+ T cells in affected cattle of the present results on PBMCs by FACS analysis may be in close accord predictor of dysfunction of the Th cells. Especially low level of IL-2 and dismissed INF-γ in the affected cattle estimated by the ELISA suggests that some CD 4+ T cells perform a functional phase as subset Th-2 cells [11, 20, 25, 27].

In contrast, the increased numbers of γδ-T cells, MHC class II-positive cells and IgM-positive cells in affected cattle show that these cells are associated with the immune response, particularly humoral immunity, against salmonel-lae [13, 24, 26, 27]. The increased number of MHC class II-positive cells to activate the innate system by the mononuclear phagocyte system and IgM-positive cells to activate the B-lymphocyte system suggest that some CD 4+ T cells perform a functional phase as subset Th-2 cells [7, 25]. The increased number of γδ-T cells in the group 1 suggests that these cells are also associated with the cytotoxic role for infected enterocytes with the antigen and immune regulatory function in the infected mucous membranes [4, 5, 25, 28].

In the present study, there are differences between levels of cytokines obtained by ELISA and activities of cytokines estimated by the bioassays in details. This may explain to some extent bioactivities and the amount of proteins of cytokines. The activities of the proinflammatory cytokines in the sera of affected cattle with both shedding and non-shedding salmonellosis were significantly higher than the activity in the control cattle. The IL-1β and IL-6 levels in the sera of the affected cattle with shedding salmonellosis were higher than their levels in non-shedding and control groups. To evaluate data obtained by ELISA, the tendency for belonging to β-sheet based folds to cross react should be taken into account [26]. The important points in present results obtained by the cytokine activity assay are in accorded with clinical signs such as leukocytosis or leukopenia, fever during septic shock syndromes [2, 18]. These results by the ELISA seemed to reflect the condition of cytokine activity and subpopulation in the PBMCs from the group 1 cattle (shedding cattle).

Our data suggest that the protective immunity by CD4+ T cells against S. Takoradi has suppressed by the activated
CD8+ T cells to modulate the regulatory activity of the γδ-T cells [4, 5, 13, 25, 27, 28].

Endotoxemia was confirmed in all but one in the present study. Endotoxin, which is a structural component in cell wall of Gram-negative bacteria such as Salmonella [12, 18, 19, 22, 24], elicits a variety of reactions through the induction of proinflammatory cytokines such as TNF-α, IL-1 and IL-6 [3, 13, 18, 19, 22, 25]. TNF-α, in particular, plays a critical role in the pathogenesis of septic shock syndrome induced by endotoxemia [2, 18]. During the course of septicemia and experimental endotoxemia, initiation of the cytokine network by TNF-α is followed by activation of other mediator systems, which is thought to be a major mediator to the development of septic shock syndromes, i.e., systemic inflammatory response syndromes (SIRS) [2, 16–18, 25]. The undetectable TNF-α in the affected cattle in the present study might be attributed to low concentration of serum endotoxin or tolerance condition against endotoxin [2, 19, 25]. Clinical signs of systematic inflammatory responses such as pyretic reaction and tachypnea might be caused by elevations in IL-1 and IL-6 [2] induced by endotoxemia during salmonellosis.

In conclusion, the present results suggest that cellular immunity is suppressed while humoral immunity is activated in acute bovine salmonellosis. The suppression of cellular immune response might impair the ability for bacterial clearance, leading to persistent shedding of salmonellae or chronic manifestations. Clinical signs in the cattle infected with S. Takoradi might depend on the serum levels of proinflammatory cytokines such as IL-1 and IL-6 [24].

Acknowledgments. This work was supported in part by Grants-in-Aid to Cooperative Research from Rakuno Gakuen University, 1996-1, Grant-in-Aid for Recombinant Cytokines Project from the Ministry of Agriculture, Forestry and Fisheries, Japan (RCP1998-2210, RCP-1999-2210), and Gakujutsu-Frontier Cooperative Research in Rakuno Gakuen University (1999).

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