Analysis of Serum Cytokine Profile in a Holstein Heifer with Leukocyte Adhesion Deficiency Which Survived for Long Period

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ABSTRACT. Serum cytokine levels and their expression of mRNA on neutrophils from a bone marrow (BM) transplanted heifer with leukocyte adhesion deficiency were evaluated. The clinical condition of the affected heifer was relatively stable after BM-transplantation. Persistent hyper γ -globulinemia and increased serum immunoglobulin G (IgG) concentrations were monitored longitudinally. The concentration of interleukin (IL)-1 β in serum from the affected heifer ranged from 15.8 to 321.7 ng/ml, and maximum concentration occurred at the time which coincided with peak IL-6. Serum levels of IL-6 ranged from 0.32 to 27.9 ng/ml, and they appeared to be associated with the increment of serum IgG in the affected heifer. mRNAs for IL-1 β , IL-6, IL-8 and granulocyte and macrophage colony stimulating factor (GM-CSF) were increased in neutrophils from the affected heifer compared to controls. Persistent hyper γ -globulinemia of the affected heifer appeared to be associated with enhanced mRNA expression for IL-6 and its serum levels. These findings suggest that humoral immunity of the affected heifer is activated and the production of neutrophils appears to be enhanced under the incapability of β_2 integrin-mediated functions of phagocytic cells.

KEY WORDS: BLAD, IL-1 β , IL-6, IL-8.

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Leukocyte adhesion deficiency in Holstein cattle (BLAD) is a hereditary disease characterized by impaired expression of β_2 integrin on leukocytes [8,12,17]. Profound abnormalities of leukocytes from cattle with BLAD were elucidated [8,13]. Impaired diapedesis of neutrophils was the characteristic finding seen in inflamed tissues of cattle with BLAD. In most cases of the affected cattle with BLAD, animals died at an early age of 1 month to 12 months due to severe pneumonia and/or diarrhea [13]. In our previous study, we applied bone marrow (BM) transplantation to the BLAD heifer aged at 9 months old, and clinical and immunological studies were performed previously during 26 months after BM transplantation [14]. Thereafter the general clinical conditions of this BM treated heifer were relatively stable for 1.6 years. This BM transplanted heifer appeared to be rare because as long as survived up to 4.6 years old. In addition, the finding of systemic κ -amyloidosis in cattle was newly demonstrated in this BLAD heifer by post mortem pathological studies [19]. From these reasons, further information of this heifer appears to be valuable to elucidate the characteristic findings of this affected heifer with leukocyte adhesion deficiency. The aim of this report was to describe the follow up clinical and pathobiological findings and selected cytokine profiles in the BM-transplanted heifer for further understanding of β_2 integrin-deficient host.

MATERIALS AND METHODS

Animals: A 3 year-old-Holstein heifer with BLAD that had been previously BM-transplanted at 9 months old was used [14]. The heifer was housed and fed a 16% protein growing ration, hay, minerals and *ad libitum* water daily.

The clinical picture, rectal temperature, heart and respiratory rates were monitored daily. The body condition of the affected heifer was evaluated based on the routine body condition scores [4].

Blood: Ten millilitres of blood was collected from the jugular vein into a tube containing heparin (20 IU/ml). Total and differential leukocyte counts were performed according to standard hematological methods, as described [13].

Protein assay: Total protein was determined by the routine serum protein assay (Biuret method, Wako). Cellulose acetate electrophoresis of serum proteins was carried out according to the standard procedures. Each fraction of serum proteins was quantified densitometrically.

Immunoglobulins: Concentrations of immunoglobulin (Ig)G, IgA, and IgM were measured by use of single radial immunodiffusion assay kits (Saikin Kagaku Inc., Sendai).

Detection of cytokines: The concentrations of IL-1 β , IL-6 and TNF- α in the sera from the BLAD were determined by a sandwich enzyme-linked immunoassay (ELISA) using bovine IL-1 β , IL-6 and TNF- α specific antibodies. The cytokines used for this assay was a fusion protein of the bovine cytokine sequence with glutathione S-transferase (GST) expressed in *Escherichia coli* as described by Hagiwara *et al.* [6]. Affinity-purified polyclonal antibodies from rabbits immunized with recombinant bovine IL-1 β , IL-6 or TNF- α were used for the capture element of the ELISA. These antibodies were labelled with biotin by a commercially available kit (Amersham) and used for the detection of the cytokine antibodies, as described previously [6]. The concentrations of cytokines were determined by plotted standard curves using these recombinant cytokines.

The concentration of GM-CSF was determined using a

commercially available assay kit (Quantikine kit, R & D systems).

Detection of cytokine mRNA: To detect cytokine mRNA (IL-1 β , IL-6, IL-8 and GM-CSF) in neutrophils from the BLAD heifer, we employed a reverse transcriptase polymerase chain reaction (PCR) method. Total cellular RNAs were extracted from cells using an RNA isolation kit (TRIzol reagent; GIBCO BRL). The RNAs (1 μ g) were reverse-transcribed using ReverTra DashTM (TOYOBO) and random primers (25 pmol/ μ l) for 10 min at 30°C, 20 min at 42°C, and then the reaction was terminated by incubation at 99°C for 5 min. Cytokine specific cDNAs corresponding to the each cytokine sequence were amplified by PCR method [6]. The primers used were the same as described [3, 7, 10, 11]. The PCR products were separated by 1.5% agarose gel electrophoresis and then stained with ethidium bromide.

RESULTS

Clinical course: Changes in the body weight of the heifer from 27 months after BM transplantation showed no increased gain in weight (data not shown). The score of the body condition of the heifer during this period was estimated to be 2.0–2.5, based on the scoring of the routine body condition. Changes in the rectal temperature, and heart and respiratory rates were monitored (Fig. 1). The rectal temperature of the BLAD heifer was generally stable and elevated temperature over 39.5°C was rarely detected from 27 months after BM transplantation until 6 months before death. The heart and respiratory rates were also stable during this period.

Immunoglobulins: Hyper γ -globulinemia gradually developed and persisted (Fig. 2). Levels for serum α - and β -globulins were relatively stable during this period. The concentrations of IgG in serum from the affected heifer were increased gradually from 6 months to 4 years of age (Fig. 3). The mean concentration of IgG in the serum of the affected heifer was 2.4 to 4.5 fold higher when compared to the controls. The concentrations for IgA and IgM in serum from affected heifer were not persistently increased (data not shown).

Cytokines: The concentrations of IL-1 β and IL-6 in serum from the BLAD heifer from 6 months to 4 years old were evaluated. The values of serum IL-1 β in the BLAD heifer during this interval ranged from 69.4 to 321.7 ng/ml, and the peak value was found in serum from the affected animal at 2 years old. IL-1 β levels were undetectable in the controls (Fig. 4). Serum IL-6 in the BLAD heifer ranged from 0.32 to 27.9 ng/ml, and their levels were apparently higher than those of normal controls (Fig. 4). TNF- α in serum from the BLAD heifer, 5 samples from the affected animal, and controls, 5 age-matched normal animals, was undetected.

Cytokine gene expressions: Expression of mRNA for IL-1 β , IL-6, IL-8 and GM-CSF was detected on neutrophils from the BLAD-affected heifer aged from 2 to 4 years old,

Fig. 1. Selective clinical parameters of the BLAD heifer. Rectal temperature, and heart and respiratory rates in the affected heifer aged from 3 to 4 years old are shown.

compared to those of controls which showed undetectable levels (Fig. 5).

DISCUSSION

This BM-transplanted heifer with BLAD survived for 4.6 years. Survival to this age is rare in BLAD-affected cattle. The growth rate of the affected heifer was apparently delayed and was associated with an unthrifty body condition. The general clinical condition was relatively stable during the course of life until 1 month before termination. The heifer developed fever and required systemic treatment

Fig. 2. Changes in serum albumin and α , β and γ globulin concentrations in the BLAD heifer aged from 0.2 to 3.2 years old.

Fig. 3. Changes in IgG concentrations in serum from the BLAD heifer aged from 0.5 to 4 years old. NOR: Values are shown as mean $\pm$ SD of 5 normal heifers.

with antibiotics and fluid therapy during the last month.

Persistent and marked leukocytosis was the characteristic feature of the hematological findings of the BLAD-affected heifer, as observed previously [8, 13]. In the analysis of serum proteins, persistent and hyper γ -globulinemia was evident, major parts of the γ -globulin was IgG, indicating that the production of immunoglobulins by B lymphocyte-plasma cell lineage is functioned well. Persistent hyper γ -globulinemia may have contributed to the required environment for the synthesis of amyloid [18, 20]. However, the exact mechanism by which this BM transplanted BLAD heifer synthesized the k -amyloid remains to be elucidated.

It is recognized that inflammatory cytokines, especially TNF- α and IL-1 β contribute to the pathogenesis of acute injury in bovine respiratory diseases and alveolar macrophages are a major source of TNF- α and IL-1 β in response to pathogens [21]. IL-1 β contributes to the defence response against pathogens by promoting the infusion of leukocytes into sites of infection [2]. IL-1 β -mediated up regulation of

Fig. 4. Changes in IL-1 β and IL-6 concentrations in serum from the BLAD heifer aged from 0.5 to 4 years old. NOR= Values are shown as mean $\pm$ SD of 3 normal heifers. * Undetected.

adhesion molecules results in an increase in neutrophil and lymphocyte adhesion on vascular endothelial cells [1, 15, 16]. The higher concentration of IL-1 β in serum from the affected heifer may have contributed to the activation of the host defense mechanisms. IL-1 stimulates both IL-6 and

Fig. 5. IL-1 β , IL-6, IL-8 and GM-CSF mRNA expressions on neutrophils from the BLAD-heifer. Numbers 1, 2 and 3 represent the cell samples from the BLAD heifer aged at 2, 3 and 4 years old. BL: Heifer affected with leukocyte adhesion deficiency. NOR: normal control. GM-CSF: granulocyte and macrophage colony-stimulating factor. β -actin: internal marker.

TNF- α , which participate in the inflammatory process. Several cytokines such as IL-8, GM-CSF and TNF- α appear to have important roles for producing the leukocytosis. The reason why the TNF- α concentration was not elevated remains unclear. Even in calves suffering from natural bronchopneumonia either in the acute phase or during convalescence, absence of circulating TNF- α had been detected by Espinasse *et al.* [5]. The number of leukocytes in peripheral blood in BLAD-cattle ranged from 40 to 110 $\times 10^3/\mu\text{l}$, and 80% of them were segmented neutrophils. These values were 10 to 20 times higher than those of normal controls [13]. It is likely that IL-8 is produced by neutrophils and is the potent factor for inducing neutrophilia. An increase in the level of plasma IL-8 is one of possible parameters in inflammatory conditions [9, 22]. To evaluate the possible cause of marked and persistent neutrophilia in this BLAD heifer, expressions of mRNA for IL-1 β , IL-8 and GM-CSF on leukocytes from this BLAD heifer were determined. IL-8 and GM-CSF mRNA expression on leukocytes from this BLAD heifer were detected in the BM-treated heifer compared to controls. This finding suggests that hematopoiesis of leukocytes in bone marrow has been highly activated, and the production of neutrophils appears to be enhanced. The concentration of GM-CSF in serum from the affected heifer was not determined, and possible role of GM-CSF for the production of bovine neutrophils in bone marrow remains to be evaluated.

IL-6 is a potent cytokine to associate with the production of γ -globulin. In the analysis of the expression of mRNA for IL-6 in neutrophils, enhanced expression of IL-6 was detected in neutrophils from the BLAD heifer when compared to that of control neutrophils. Serum levels of IL-6

were increased in the BLAD heifer from 1.4 to 4 years old when compared to those of the BLAD heifer at 6 months old and normal controls, and they appeared to be associated with the increment of serum IgG. Increased levels of serum IL-6 found in the BLAD heifer may have enhanced differentiation of B-lymphocytes to plasma cells resulting in the persistent hyper γ -globulinemia. This also suggests that humoral immunity of the affected heifer is enhanced under the incapability of β_2 integrin-mediated functions of phagocytic cells in the affected heifer.

In summary, the clinical condition of the affected heifer was relatively stable after BM-transplantation. Serum IL-1 β and IL-6 concentrations in the affected heifer were elevated in association with the increments of γ -globulinemia. Persistent hyper γ -globulinemia of the affected heifer was associated with elevated levels of IL-6 and its mRNA expression on leukocytes. The pathobiological profiles observed in the BLAD heifer demonstrated active humoral immunity in leukocyte β_2 integrin deficiency.

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