Leukocyte Emigration in Normal Calves and Calves with Leukocyte Adhesion
Deficiency

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ABSTRACT. The emigration of leukocytes from calves with β2 integrin deficiency (BLAD) into bronchoalveolar spaces and scraped tissues was compared to that of normal calves. Polymorphonuclear neutrophils were found in bronchoalveolar lavage fluid from BLAD-affected calves showing chronic pneumonia. The neutrophils were complement receptor type 3 (CR3)-negative when characterized by flow cytometric analysis using anti-CD18 monoclonal antibody. Chemiluminescent response mediated by CR3 in neutrophils isolated from bronchoalveolar lavage fluid from BLAD-calves showed similar findings obtained from CR3-deficient neutrophils. Neutrophils from normal calves migrated into scraped tissue which was prepared in an upper gluteal surface area, whereas few leukocytes from calves with BLAD migrated to the scraped tissue, evaluated by skin window (Rebuck) method. These findings confirmed the extravasation of CR3-deficient leukocytes into bronchoalveolar lumen in BLAD calves, and demonstrated in vivo characteristics of extravasating property of normal and CR3-deficient neutrophils into scraped tissues.—KEY WORDS: BLAD, bronchoalveolar lumen, extravasation, neutrophil, skin window method.

Bovine leukocyte adhesion deficiency (BLAD) in Holstein cattle [3, 4, 10, 13, 16, 19] has been characterized as β2 integrin deficiency (CD11a,b,CD18) deficiency in leukocytes [13, 16], and is shown to be highly susceptible to pathogenic bacteria [3, 15, 16]. Complement receptor type 3 (CR3) and Fc receptor for immunoglobulin G (FcR) are key receptors for phagocytes [9, 20]. CR3 and FcR-dependent neutrophil functions in cattle with BLAD have been well characterized [16–18], and in histopathological study, impaired diapedesis of neutrophils from BLAD cattle is commonly seen in inflamed tissues [1, 3, 21].

The extravasation of neutrophils into tissues is initiated by the adherence of circulating neutrophils to activated vascular endothelial cells in the inflamed tissues [6, 20]. Interestingly, the extravasation of CR3-deficient neutrophils into bronchoalveolar lumen in BLAD-affected cattle has been observed by histopathological [21] and morphometric studies [2]. However, in vivo extravasation of leukocytes from cattle with BLAD has not been evaluated yet. The cellularity of bronchoalveolar lavage fluid from BLAD calves showing pneumonia and their functions are still remained to be evaluated. It is of interest to evaluate the in vivo migrating property of CR3-deficient neutrophils into bronchoalveolar lumen or other scraped area in cattle with BLAD. The objective of this study was to describe the difference in extravasating property of CR3-deficient neutrophils into bronchoalveolar lumen and scraped tissues in cattle affected with BLAD.

MATERIALS AND METHODS

Calves: Three female Holstein calves with clinical episodes of fever, pneumonia and severe neutrophilia were referred to the Veterinary Clinical Center Rakuno Gakuen University, and were diagnosed with BLAD based on deficient CD18 expression and a polymerase chain reaction analysis [16]. Major clinical conditions of 3 affected calves at admission were as follows: case 1, 1-month-old, had fever and respiratory distress; case 2, 2-month-old, exhibited emaciation and respiratory distress; case 3, 2-month-old, had diarrhea and respiratory distress, respectively. X-ray photographs of thorax of cases 1 and 2 revealed pneumonia. Cases 1 and 2 were used for the collection of bronchoalveolar lavage fluid, and cases 1 to 3 were for skin window method. Three age- and sex-matched clinically healthy Holstein calves were used as control animals.

Bronchoalveolar lavage: The airways of 2 calves with BLAD were lavaged using essentially the same procedure as described previously [7]. Briefly, a sterile polyethylene tube, 120 cm long and 3 mm in diameter, connected to a 50 ml syringe, was passed via the nose into the trachea and carefully advanced to reach the bronchiole regions. Twenty milliliters of sterile saline solution were carefully infused, then aspirated repeatedly, recovering an average of 65% of the fluid. Cell pellets were recovered by centrifugation (140 × g, 3 min) and resuspended in Hanks’ balanced salt solution (HBSS). Phagocytic cells were isolated from cell suspension using Ficoll-Conray gradients, as described [16]. Cell smears were made, fixed in methanol, and stained with Giemsa solution for morphological evaluation.

Neutrophils: Ten milliliters of blood were collected from the jugular vein into a tube containing heparin (20 IU/ml). Neutrophils were isolated from the heparinized blood by Ficoll-Conray gradients, as previously described [16]. Isolated neutrophils were resuspended in HBSS to a concentration of 1 × 10⁷ cells. The resulting cell populations
values of $P<0.05$ were regarded as significant.

Flow cytometry: Cells isolated from bronchoalveolar lavage and isolated blood neutrophils were washed twice with PBS, fixed in 1% paraformaldehyde and analyzed with a flow cytometer (Coulter Epics, Hialeah, FI, U.S.A.), as described [17]. Neutrophils were characterized by forward and orthogonal light-scattering (FS and SS) and gated on a flow cytogram. Neutrophils were also incubated with the fluorescein isothiocyanate (FITC) conjugated anti-CD18 monoclonal antibody (Dako MHM23, Glostrup, Denmark) at saturating concentrations. After incubation at $4^\circ C$ for 30 min, cells were washed twice with PBS, fixed in 1% paraformaldehyde, and analyzed with a flow cytometer.

Chemiluminescent (CL) assay: The luminol-dependent CL assay was performed according to the method described [17]. Five hundred microliters of isolated neutrophils (1 $\times 10^6$ cells) in were incubated at $37^\circ C$ for 5 min in a luminometer, and $20 \mu l$ of luminol (final $10^{-4}$ M) were added. This mixture was equilibrated for 5 min, then $20 \mu l$ of OPZ or Agg-IgG were added. The peak response (peak CL, counts per minutes [cpm]) was read from the recorder.

Skin window: In vivo extravasation of leukocytes was evaluated by skin window (Rebuck) method [14]. The left-side gluteal region, upper one-third, of each 3 calves with BLAD and 3 control calves, was clipped of hair and shaved in a square of 40 by 50 mm. Surgical tape in size of 20 by 40 mm with a hole 5 mm in a diameter was banded to the shaved area. The area within the hole in the surgical tape was scraped carefully with the edge of cover glass avoiding pin-point bleeding. The cover glass in size of 20 by 20 mm was set tightly on the scraped area, and then was changed at 1, 3, 6, 12, 24, 36 and 48 hr after setting the cover glass. The cover glasses were fixed in methanol, stained with Giemsa solution, and the number of cells in randomly selected 5 fields was counted in each sample under a microscope at a magnification of 400. The results were expressed as the score as follows based on the number of migrated cells: 0=none; 1=slight, less than 50; 2=slight to moderate, 51–100; 3=moderate, 101–500; 4=severe, 501–1,000; and 5=marked, more than 1,001, respectively.

Statistics: Data was analyzed using Student’s $t$-test, and values of $P<0.05$ were regarded as significant.

RESULTS

The mean number of cells collected from bronchoalveolar lavage fluid from 2 calves with BLAD ranged from $3.6 \times 10^6$ to $1.6 \times 10^7$ cells when $20 \text{ml}$ of sterile physiological saline were infused into the bronchi. Approximately 65% of infused fluid were recovered by repeated suction. Polymorphonuclear neutrophils were clearly observed in bronchoalveolar lavage fluid by morphological evaluation (Fig. 1).

The mean ($\pm$ SD, n=3) percentages of polymorphonuclear neutrophils and mononuclear cells in isolated bronchoalveolar lavage fluid from 2 calves with BLAD were 35.7 ($\pm$ 9.6)% and 59.3 ($\pm$ 10.0)% in case 1, and 40.3 ($\pm$ 7.6)% and 50.7 ($\pm$3.8)% in case 2, respectively.

In a flow cytogram of isolated cells from bronchoalveolar fluid from BLAD-affected calves, the region comprising granulocytes that corresponded to that in blood was clearly monitored and the fluorescence due to CD18 on their cell surfaces was negative (Fig. 2).

OPZ-induced CL response of neutrophils from BLAD calves was characteristically decreased, compared with that of neutrophils stimulated by Agg-IgG with patterns that were typical of those of BLAD calves (Fig. 3). In vivo extravasation of leukocytes from normal and BLAD-calves was evaluated by skin window method (Fig. 4). The migrated cells were found on a cover glass attached to the scraped tissues in normal calves (N) at 3 hr after setting the cover glass. In contrast, few migrated cells were observed in those of BLAD calves (BL). The predominant cell type of migrated cells in normal calves was polymorphonuclear neutrophils from 3 to 6 hr after making a scraped area; however, the proportion of mononuclear phagocytes was increased greatly at 12 hr after setting the cover glass (Fig. 5).

DISCUSSION

To clarify the diapedesis of CR3-deficient neutrophils into bronchoalveolar lumen in cattle affected with BLAD, bronchoalveolar lavage fluid were collected and their selected parameters were evaluated. Leukocytes were clearly found in bronchoalveolar lavage fluid from BLAD calves. Flow cytometric analysis using anti-CD18 monoclonal antibody showed negative fluorescence of CD18 on cells located in the granulocyte region. These results confirmed the previous observation of van Garderen et al. [21] that in BLAD-affected calves with chronic catarrhal pneumonia, numerous extravascularly-located neutrophils were present in the alveoli and bronchiolar lumens, as shown by a histopathological study. In human bronchoalveolar lavage fluid, neutrophils account for up to 2% of lavageable cells [11]. It has been considered that neutrophils migrate
to the alveolar lumen in response to chemotactic factors, most likely secreted by the alveolar macrophages. The OPZ-induced CL response, mediated by CR3, of neutrophils from bronchoalveolar lavage isolated from BLAD calves was clearly diminished as compared to that of neutrophils stimulated with Agg-IgG. This indicates that β2 integrin (CD11/CD18)-dependent neutrophil functions were severely impaired, and was consistent with previous results that were commonly found in blood neutrophils from BLAD cattle [13, 16]. From these results it is concluded that neutrophils from BLAD cattle could migrate into the alveolar lumen by CD18-independent adhesion molecules, but their phagocytic functions appeared to be impaired. In fact, chronic pneumonia has been demonstrated to be commonly associated with calves with BLAD [1, 3, 10, 15, 16]. The mechanisms by which CR3-deficient neutrophils migrate into bronchoalveolar lumen remain to be clarified, it is likely that CD11/CD18-independent mechanisms [5, 8, 12] play a key role for extravasation of CR3-deficient neutrophils. To examine the possible role of extracellular matrices in cell-matrix interaction of neutrophils, adhesive activity of
CR3-deficient neutrophils to collagen type I, type IV, fibronectin, and laminin was evaluated previously [18]. Significantly decreased adhesive activity of neutrophils from cattle with BLAD to collagen type I, type IV, and fibronectin was observed, and expected compensatory effects of adhesive activity of CR3-deficient neutrophils to such matrices were not demonstrated [18]. Although factors contributing to the extravasation of CR3-deficient neutrophils into bronchoalveolar lumen were unclarified in this study, other adhesion molecules such as very late antigens (VLA antigens), L selectin, Sialyl Lewis X, CD15 and CD44 which were commonly expressed on leukocytes might be attributable [5, 6, 8, 12].

Migration is a functional characteristic of leukocytes in response to an inflammatory stimulus. The evaluation of in vivo extravasation of leukocytes from normal and BLAD calves was attempted by skin window method that was developed for evaluation of extravasation and migration of neutrophils from human patients [14]. In the present study, the extravasation of leukocytes from normal calves was clearly observed after preparation of the scraped tissues. In contrast, few migrated cells were observed in similar preparations from BLAD calves. Marked differences were observed in the number of migrated leukocytes between normal calves and calves with BLAD from 3 to 24 hr after making the scraped areas. The predominant cell type of migrated cells in normal calves was polymorphonuclear neutrophils in the early phase after making a tissue scraping, and thereafter the proportion of mononuclear phagocytes increased greatly. This is the first report describing the in vivo extravasation of leukocytes from normal and BLAD-calves by skin window method, and proved to be applicable for this purpose.

In summary, the present study confirmed the differences in extravasating property of leukocytes into bronchoalveolar lumen and scraped tissue in calves with BLAD, and demonstrated in vivo characteristics of extravasation of normal and CR3-deficient neutrophils.

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REFERENCES


