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SPIN-TRAPPING AND CHEMILUMINESCENCE STUDIES OF NEUTROPHILS FROM A HOLSTEIN-FRIESIAN CALF WITH BOVINE LEUKOCYTE ADHESION DEFICIENCY

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The ability of neutrophils from a Holstein-Friesian calf with bovine leukocyte adhesion deficiency (the proband with a genetic deficiency of the Mac-1 (CD11b/CD18) glycoprotein corresponding to the receptor of complement iC3b) to generate oxygen radicals was examined using electron spin resonance spectrometry (ESR) combined with a spin-trapping technique and luminol-dependent chemiluminescence spectrometry. When the neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA), an ESR spectrum confirming the generation of superoxide anions (O_2^-) was clearly observed in both healthy and diseased calves. However, when the neutrophils were stimulated by opsonized zymosan, appearance of the ESR spectrum was recognized in the healthy calves but not in the diseased calf. Similar results were obtained from chemiluminescence experiments.

KEY WORDS: neutrophils, superoxide, leukocyte adhesion deficiency, calf, spin trapping, chemiluminescence

INTRODUCTION

At the end of November 1991, a Holstein-Friesian calf (two months old) showing clinical signs of chronic inflammation with severe neutrophilia was referred to the Department of Veterinary Medicine, Rakuno Gakuen University, in Hokkaido, Japan. Recurrent onset of bacterial infection and persistent neutrophilia in the early stage of the disease clearly suggested a dysfunction of granulocytes. This showed not only very similar clinical signs but also hematological and biochemical findings similar to those of calves with bovine granulocytopathy syndrome reported earlier by us,[1,2] Kehrli *et al.*[3], and Stober *et al.*[4] The clinical features and histopathologic findings of the disease in our affected calf were also remarkably similar to those in human patients with either chronic granulomatous disease[5–9] or leukocyte adhesion deficiency.[10–13] It has been reported that the lack of β subunit (CD18) in the CD11/CD18 complex is characteristic of neutrophils from patients with leukocyte adhesion deficiency.[11,12] Shuster *et al.* (1992) have recently reported that the subunit deficient in the CD11/CD18 complex of neutrophils from calves with granulocytopathy syndrome was the β subunit.[14] Our preliminary experiments have also showed that the β subunit was deficient in the CD11/CD18 complex of neutrophils

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from our affected calf (unpublished results). These results suggest that our affected calf is diagnosed as having bovine leukocyte adhesion deficiency, though we previously reported the similar calves to be bovine granulocytopathy syndrome.[1,2]

Until now the functional abnormalities of neutrophils from calves with granulocytopathy syndrome[3], from human patients with chronic granulomatous disease[5,7,9] or leukocyte adhesion deficiency were reported.[10,11,13] ESR spectrometry combined with the spin-trapping method has been reported to be quite useful to detect and identify oxygen radicals which are liberated from human neutrophils when stimulated by opsonized zymosan, PMA and other stimuli,[15-17] suggesting that ESR spectrometry is an useful method to diagnose animal diseases as well as human diseases related to the ability of oxygen-radical generation. In the present study we examined the ability of neutrophils from our diseased calf to generate reactive oxygens. For this purpose, electron spin resonance (ESR) spectrometry combined with a spin-trapping method was employed. Opsonized zymosan and PMA were used as stimuli. Since chemiluminescence with luminol or other fluorescent reagents has also been proved to be an useful method, especially for measuring quantitatively reactive oxygens,[18,19] we also applied this method to observe oxygen radicals liberated from neutrophils of the diseased calf.

MATERIALS AND METHODS

Animals

A Holstein-Friesian calf (two months old) referred to the Veterinary Teaching Hospital of Rakuno Gakuen University and first diagnosed as suffering from bovine granulocytopathy syndrome was used for experiments. The main clinical signs at the first observation were emaciation, pyrexia, anorexia, diarrhea, chronic pneumonia, enlargement of superficial lymph nodes, ulcer and granulomatous inflammation in the oral membrane and gingivitis. In addition, a severe defect in chemotaxis and adhesion, and a mild impairment in phagocytosis were observed. As hematological findings at the admission and terminal stages, marked neutrophilia ($10 \pm 4 \times 10^4/\mu L$) was observed. Since this calf was found to belong to the same family line as that of calves reported earlier by us,[1,2] this syndrome was regarded as an inherited, congenital disorder. Treatment with various antibiotics was not effective at all and the calf died three months later. Two healthy cows (three years old) were used for establishing normal response. Blood was collected from either the jugular or tail vein into evacuated tubes (Terumo Co., Tokyo, Japan) containing heparin (20 units/mL of blood) as an anticoagulant.

Chemicals

Phorbol 12-myristate 13-acetate (PMA) and zymosan A (from *S. cerevisiae*) were purchased from Sigma Chemical Company (St. Louis). Percoll was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Dimethyl sulfoxide (Me_2SO) and luminol (5-amino-2,3,dihydro-1,4,phthalazinedione) were from Nacalai Tesque, Inc. (Kyoto, Japan). Diethylenetriamine-N,N,N′,N″,N‴-pentaacetic acid (DTPA) was from Dojindo Laboratories (Kumamoto, Japan). The spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), was from Aldrich Chemical Company (Milwaukee). The buffer system used in this work was Hanks' balanced salt solu-

tion (HBSS), pH 7.4, which is free from Ca^{++} and Mg^{++}. PMA was dissolved in Me_2SO(1 mg/mL, final concentration).

Isolation of Neutrophils from Peripheral Blood

After peripheral blood (40 mL) was diluted twice with HBSS, the solution was layered onto 40 mL of Percoll solution (density: 1.077) and centrifuged at 1600 g for 20 min at 20 °C. The upper layer was discarded and the cells in the lower layer were treated by adding 0.85% Tris-NH_4Cl solution and incubating for 5 min to lyse the red cells. The solution was again centrifuged at 500 g for 5 min at 4°C. The supernatant was removed and the remaining cells were washed twice with cold HBSS and resuspended in the same solution at a concentration of about 7×10^7 polymorphonuclear cells per mL. This procedure yielded a polymorphonuclear cell preparation of more than 94% purity.

Preparation of Opsonized Zymosan

Zymosan A from *S. cerevisiae* was suspended in physiological saline and boiled at 100°C for 30 min. After centrifugation at 500 g for 20 min, the zymosan A was washed twice with physiological saline and resuspended in HBSS at a concentration of 10 mg/mL. Opsonization of the zymosan A was carried out by adding three parts of cow fresh serum to one part of the suspension followed by incubation at 37°C for 30 min with shaking. The opsonized zymosan A was once recovered and then resuspended in HBSS at a concentration of 10 mg/mL.

Spin Trapping and ESR Spectrometry

The same experimental conditions were applied to the spin-trapping and ESR spectrometry for neutrophils from both the healthy and diseased calves. The experiments were carried out by modifying the method of Cohen and his colleagues originally developed for experiments with human neutrophils.[15-17] Aliquots of HBSSs containing the spin-trapping reagent, DMPO, and the metal-chelating reagent, DTPA, at concentrations of 100 mM and 0.1 mM, respectively, were added to 300 μL of HBSS containing 2×10^7 neutrophils. The solution was incubated for 2 min at 37°C. Then 30 μL of Me_2SO containing PMA or 50 μL of HBSS containing opsonized zymosan was added to the solution followed by a further incubation for 1 min. The reaction mixture was subsequently transferred to a flat ESR cell ($10 \times 40 \times 0.2$ mm), fitted into the cavity of the ESR spectrometer (JEOL ME-1X ESR spectrometer, Akishima, Japan) and the spectrum was obtained at 25°C. Control experiments were done in the absence of stimulant. Neither DMPO nor DTPA induced the ESR spectrum.

Chemiluminescence Spectrometry

As a chemiluminescence probe, luminol was used. Luminol was added to 2 mL of HBSS containing 5×10^6 neutrophils to a concentration of 10 μM. The solution was transferred to a thermostatically controlled cuvette for chemiluminescence measurements and kept at 37°C until the background chemiluminescence became constant. Then 10 μL of Me_2SO solution containing PMA or 50 μL of HBSS containing opsonized zymosan was added to the cuvette and chemiluminescence was

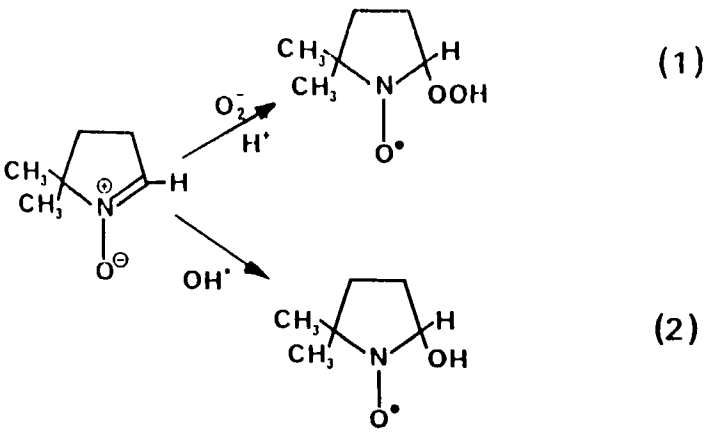

FIGURE 1 Spin-trapping reactions of O_2^- (1) and OH radical (2) by DMPO.

subsequently observed by the use of a luminometer (Biolumat LB9500C, Berthold, Wildbad, Germany).

RESULTS

Observation and Identification of Reactive Oxygens from The Stimulated Neutrophils by ESR Spectrometry

When the spin trap, DMPO, is present in an aqueous solution in which reactive oxygens such as OH radicals and/or O_2^- are generated, the reactions shown in Figure 1 take place to form the spin adducts (so-called spin-trapping reactions).[15-17] Figures 2a and 2b show the ESR spectra obtained when neutrophils from healthy and diseased calves were stimulated by PMA, respectively. Both spectra were qualitatively and quantitatively similar to each other and consisted mainly of a hyperfine structure with twelve lines (3 × 2 × 2). Computer simulation of these spectra indicated that the splitting width of the first triplet hyperfine structure due to a nitrogen of the nitroxide group, a_N, was 1.44 mT, the splitting width of the second doublet hyperfine structure due to a hydrogen at the β position, a_H^β, was 1.12 mT, and the splitting width of the third doublet hyperfine structure due to a hydrogen at the γ position, a_H^γ, was 0.14 mT, indicating that these spectra could be assigned to the spin adduct between DMPO and O_2^- (the formation of DMPO-OOH through reaction 1 in Figure 1).[15] This means that the reactive oxygen species which PMA-stimulated neutrophils produced was O_2^- irrespective of the difference between healthy and diseased calves. Since the ESR spectra were recorded under the similar experimental conditions for both calves, Figure 2 shows that neutrophils from both calves had almost the same ability to produce O_2^- when stimulated by PMA.

Figures 3a and 3b show the ESR spectra obtained when neutrophils from healthy and diseased calves, respectively, were stimulated by opsonized zymosan. In the case of healthy calves an ESR spectrum was clearly observed as shown in Figure 3a, but little ESR intensity, as shown in Figure 3b, was observed in the case of neutrophils obtained from the diseased calf. In contrast to the result obtained with

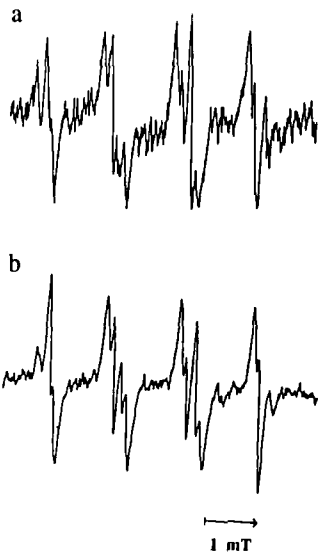

FIGURE 2 ESR spectra obtained from PMA-stimulated neutrophils from (a) healthy and (b) diseased calves. HBSS containing 100 mM of DMPO and 0.1 mM of DTPA was added to 300 μL of HBSS containing 2×10^7 cells, followed by incubation for 2 min. Then 30 μL of Me_2SO containing PMA at a concentration 1 mg/mL was added to the solution and incubated for 1 min. Immediately after incubation, the solution was transferred to a flat ESR cell which was designed for the ESR measurement of aqueous solutions. ESR measurements were performed at 25 °C. The ESR conditions were as follows. Microwave power was 20 mW, modulation frequency was 100 KHz with an amplitude of 0.1 mT, sweep time was 1.0 mT/min, and the receiver gain was 5×10^3 with response times of 0.5 sec for (a) and 1.0 sec for (b).

the PMA-stimulated neutrophils, this result clearly indicated that neutrophils from the diseased calf produced few reactive oxygens when stimulated by opsonized zymosan. Figure 3a consisted mainly of a 1:2:2:1 quartet hyperfine structure with hyperfine couplings of $a_N = a_H^\beta = 1.48$ mT. This is a typical ESR spectrum that could be assigned to the spin adduct between DMPO and the OH radical (DMPO-OH).[15-17] Therefore, this might have been interpreted as indicating that the stimulation of neutrophils with opsonized zymosan resulted in the production of OH radicals as shown in reaction (2) in Figure 1. However, when Me_2SO (10 mM) was added as an OH radical scavenger to the spin-trapping reaction system, no effects of Me_2SO were observed on the ESR spectrum shown in Figure 3a and, in addition, no ESR spectrum due to the DMPO-CH_3 adduct was observed (data not shown). This means that the reaction between OH radicals and Me_2SO to produce CH_3 radicals rarely took place, indicating that few OH radicals were induced in opsonized zymosan-stimulated neutrophils. Observation of the DMPO-OH adduct in opsonized zymosan-stimulated neutrophils was interpreted to mean that the DMPO-OOH adduct was transformed to a DMPO-OH adduct.[15] Similar experiments were repeated using neutrophils obtained from different healthy calves. While the ESR spectrum due to the DMPO-OOH adduct was stably observed in the case of PMA, the ESR spectrum observed in the case of opsonized zymosan consisted mainly of that of the DMPO-OH adduct. Ueno *et al.* (1989) reported that the formation of DMPO-OH instead of DMPO-OOH was sometimes observed

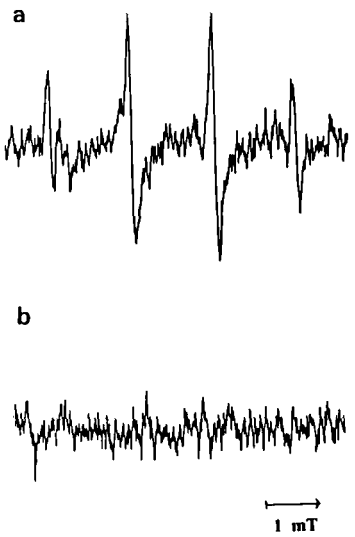

FIGURE 3 ESR spectra obtained from opsonized zymosan-stimulated neutrophils from (a) healthy and (b) diseased calves. HBSS containing 100 mM of DMPO and 0.1 mM of DTPA was added to 300 μL of HBSS containing 2×10^7 cells, followed by incubation for 2 min. Then 50 μL of HBSS containing opsonized zymosan was added to the solution and incubated for 1 min. Immediately after incubation, the solution was transferred to a flat ESR cell. ESR measurements were performed at 25 °C. The ESR conditions were as follows: Microwave power was 20 mW, modulation frequency was 100 KHz with an amplitude of 0.1 mT, sweep time was 1.0 mT/min, and the receiver gains were 3.6×10^3 for (a) and 10×10^3 for (b) with response times of 0.5 sec.

when neutrophils were stimulated with opsonized zymosan.[20] This was explained by the fact that a relatively high concentration of DMPO injured neutrophils and, thus, the DMPO-OOH was transformed to the DMPO-OH due to the action of enzymes and/or factors which were released from the lysed cells. In spite of the same concentration of DMPO, little effects of DMPO were observed in the ESR spectrum in the case of PMA (Figure 2a). Opsonized zymosan interacts with neutrophils in a manner different from PMA. The interaction of opsonized zymosan with neutrophils may potentiate the ability of DMPO to induce cell injury. But the detailed mechanisms are not known. In conclusion, the reactive oxygen was mainly O_2^- when neutrophils from healthy calves were simulated with opsonized zymosan, and that the ability of neutrophils from diseased calf to generate O_2^- was quite low when stimulated with opsonized zymosan.

Measurements of Reactive Oxygens from Stimulated Neutrophils by Luminol-Dependent Chemiluminescence Spectrometry

To confirm more quantitatively the results described above, and to examine the time-course of O_2^- generation after adding PMA or opsonized zymosan, we employed luminol-dependent chemiluminescence spectrometry in the present experimental systems. Figure 4 shows the results obtained when neutrophils from healthy (a) and diseased calves (b) were stimulated by PMA. After 10 μL of luminol was added to the solution containing 5×10^6 neutrophils, the solution was stored at 37°C in the cuvette until the background became constant. Then 10 μL of PMA was added to the solution and subsequently the measurement of chemiluminescence

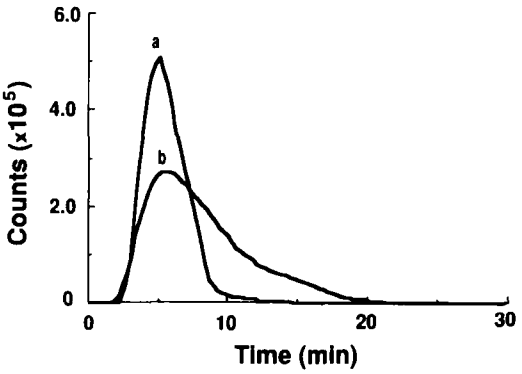

FIGURE 4 Chemiluminescence spectra obtained from PMA-stimulated neutrophils from (a) healthy and (b) diseased calves. Luminol was added to 2 mL of HBSS containing 5×10^6 cells to a concentration of 10 μM. The solution was transferred to a thermostatically controlled cuvette and kept at 37 °C until the background chemiluminescence became constant. Then 10 μL of Me_2SO containing PMA was added to the solution and chemiluminescence spectra were recorded with a luminometer. The integrated counts per sec are plotted in the Y-axis.

was started. The counts integrated per second were plotted against the time (minutes) after PMA was added. For neutrophils from healthy calves, the luminescence counts rapidly increased, reached the maximum at 6 min after addition of PMA, and then approached zero within 10 min (curve a). In the case of neutrophils from diseased calf, the luminescence counts reached the maximum at 6 min after addition of PMA, but decreased slowly and it took 20 min for the luminescence counts to reach zero (curve b). Since the areas under curves a and b, which correspond to total counts of chemiluminescence, were almost equal to each other, the ability of neutrophils to generate reactive oxygens seemed to be almost same in both calves. Figure 5 shows the results obtained when neutrophils from the healthy (a) and diseased calves (b) were stimulated by opsonized zymosan. After 10 μL of luminol was added to the solution containing 5×10^6 neutrophils, 20 μL of HBSS containing opsonized zymosan was added to the reaction solution and the measurement of chemiluminescence was started. Neutrophils from healthy calves showed that the luminescence counts slowly increased, and reached the maximum at 13 min after addition of opsonized zymosan, and decreased slowly for 30 min without dropping zero (curve a). On the other hand, little chemiluminescence was observed in the case of neutrophils from diseased calf. This means that while neutrophils from healthy calves showed a normal response to opsonized zymosan, neutrophils from diseased calf have little ability to generate reactive oxygens when exposed to opsonized zymosan. The results obtained from chemiluminescence experiments clearly supported those obtained from ESR experiments.

Both ESR-spin-trapping and luminol-dependent chemiluminescence experiments proved that NADPH-dependent enzymatic systems generating superoxides were intact in both calves, though there was some abnormality in the response of neutrophils from diseased calves to PMA as observed in Figure 4. The essential abnormality seems to be present in the binding ability of complements deposited on the surface of zymosan to receptors of neutrophils.

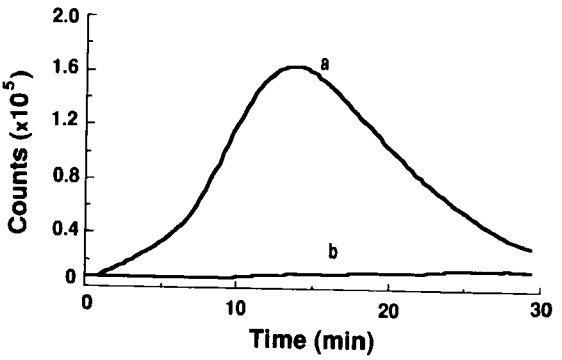

FIGURE 5 Chemiluminescence spectra obtained from opsonized zymosan-stimulated neutrophils from (a) healthy and (b) diseased calves. Luminol was added to 2 mL of HBSS containing 5×10^6 cells to a concentration of 10 μM. The solution was transferred to a thermostatically controlled cuvette and kept at 37 °C until the background chemiluminescence became constant. Then 50 μL of HBSS containing opsonized zymosan was added to the solution and chemiluminescence spectra were recorded with a luminometer. The integrated counts per sec are plotted in the Y-axis.

DISCUSSION

The abnormality of neutrophils from diseased calf reported in the present experiments was similar to that of neutrophils from human patients with leukocyte adhesion deficiency.[10,13] As another disease similar to leukocyte adhesion deficiency, chronic granulomatous disease has been reported in humans.[5-9] Neutrophils from patients with chronic granulomatous disease did not manifest a respiratory burst under the conditions in which PMA and/or opsonized zymosan were used as stimuli, suggesting the functional abnormality of NADPH-dependent O_2^- generating enzymes[5-7,9] or cytosol factors closely related to these enzymes.[8] In the present study, examination of the functional abnormality of neutrophils from diseased calf with ESR-spin-trapping and luminol-dependent chemiluminescence spectrometries clearly proved that NADPH-dependent O_2^- generating enzymes were normal. Human leukocyte adhesion deficiency is known as a disease with a genetic deficiency of the Mac-1 (CD11b/CD18) in granulocytes and macrophages. In particular, the β subunit (CD18) of the CD11/CD18 complex is deficient in this disease.[11,12] A recent report by Shuster *et al.* (1992) also showed a deficiency of the β subunit (CD18) of the Mac-1 in neutrophils from diseased calves,[14] though Kehrli *et al.* (1990) first reported a deficiency of the α subunit (CD11b) of Mac-1 in those from calf with granulocytopathy syndrome.[3] The calf examined in the present study showed a deficiency of the β subunit and, in addition, a severe defect in chemotaxis and adhesion, and a mild impairment in phagocytosis were observed (unpublished results). Therefore, our affected calf may be diagnosed as having bovine leukocyte adhesion deficiency. Since Mac-1 corresponds to CR3, which is a receptor of complement iC3b, the deficiency of Mac-1 in the neutrophil is responsible for its lack of binding to complement iC3b deposited on the surface of zymosan. The binding of the iC3b fragment on the surface of zymosan to the CR3 receptor in neutrophils may play an important role as a trigger to generate O_2^-.

Until now, many papers concerning neutrophils from human patients with chronic granulomatous disease or leukocyte adhesion deficiency and from calves with bovine granulocytopathy syndrome have mainly determined their ability to generate reactive oxygens by measuring oxygen consumption with polarography,[10] H_2O_2 production with the fluorometric assay method,[3,13] and O_2^- production with the reduction of cytochrome c or nitroblue tetrazolium.[1,2,3,6,9] In the present study we employed ESR spectrometry combined with spin-trapping and luminol-dependent chemiluminescence spectrometry. ESR spectrometry is the only method able to directly identify the species of reactive oxygens. However, the stability of the spin adduct between DMPO and O_2^- or OH is known to be strongly affected by the surrounding oxidative or reductive substances such as transient metals.[21] Chemiluminescence spectrometry is more sensitive and a more precise method to measure the reactive oxygens than ESR unless identification of reactive oxygen species is required. In the present study both methods gave similar results, proving that ESR spectrometry was useful not only as a method to qualitatively measure reactive oxygens but also as a method to quantitatively measure reactive oxygens. However, the latter revealed a characteristic of neutrophils from diseased calf which may not be recognized by ESR as shown in Figure 4. In this Figure a lag in the time-course of superoxide generation was observed in neutrophils from diseased calf. The deficiency of Mac-1 glycoprotein may have some effects on the structural and functional integrity of NADPH-dependent O_2^--generating enzymes or the NADPH-dependent O_2^--generating enzymes themselves may have some intrinsic abnormality, though it has little effect on their ability to generate O_2^-. Further studies will be continued when we again obtain calves with this syndrome.

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