Bovine Leukocyte Adhesion Deficiency

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SUMMARY

A disease of young Holstein calves characterized by recurrent pneumonia, ulcerative and granulomatous stomatitis, enteritis with bacterial overgrowth, periodontitis, delayed wound healing, persistent neutrophilia and death at an early age had been originally described in 1983 and again in 1987. Most of these calves had stunted growth and a persistent, progressive neutrophilia (often exceeding 100,000/μl). By investigation of pedigrees, all of the affected calves have now been traced to a common sire. Neutrophils from these calves have several functional deficits and, most importantly, fail to adhere in a β_2-integrin dependent manner. The β_2-integrins represent a family of glycoproteins which participate in various leukocyte adhesion reactions during host defense. The presence or absence of β_2-integrin molecules can be demonstrated on the surface of neutrophils, monocytes and lymphocytes from normal or affected calves using specific monoclonal antibodies and flow cytometry, or by colloidal gold immunolabeling and scanning electron microscopy in backscatter mode. Deficiency of the β_2-integrins in Holstein calves is analogous to leukocyte adhesion deficiency (LAD) seen in humans. Neutrophils in bovine (BLAD) and human LAD patients are unable to adhere to the endothelial lining of the cardiovascular system thus interrupting egression of neutrophils into infected tissues. Both BLAD cattle and LAD children (who do not receive bone marrow transplants) often die at an early age as a result of the failure of neutrophils to extravasate into infected tissues. In 1991, Shuster identified two point mutations within the alleles encoding bovine CD18 in a Holstein calf afflicted with leukocyte adhesion deficiency. One mutation causes an aspartic acid to glycine substitution at amino acid 128 (D128G) in an extracellular region of this adhesion glycoprotein that is highly conserved (>95% identity) between humans, cattle and mice. The other mutation is silent. Numerous calves with clinical symptoms of leukocyte adhesion deficiency have since been tested and all have been found homozygous for the D128G allele. In addition, calves homozygous for the D128G allele have been identified during widespread DNA testing in the United States. All cattle with the mutant allele are related to one bull, who through artificial insemination (A.I.), sired many calves in the 1950's and 1960's. The carrier frequency of the D128G CD18 allele among U.S. Holstein cattle had reached approximately 15% among active A.I. bulls and 8% among cows. The organization of the dairy industry and the diagnostic test developed to genotype cattle, has enabled nearly complete eradication of bovine leukocyte adhesion deficiency among future A.I. bulls.

INTRODUCTION

Calf mortality on dairy farms represents a major economic loss. Overall calf mortality has been reported as 7.7% of all heifer calf births (James, et al., 1984). An etiologic agent is not determined in 36% of dairy calf deaths attributed to diarrhea, a major cause of calf mortality (Morin, et al., 1976). Although seldom diagnosed, a granulocytopathy syndrome was first reported in 1983 and again in 1987 as a cause of death in young Holstein Friesian cattle (Hagemoser, et al., 1983;Nagahata, et al., 1987;Takahashi, et al., 1987). This syndrome was described as a disease of young Holstein cattle characterized by recurrent pneumonia, ulcerative and granulomatous stomatitis, enteritis with bacterial overgrowth, periodontitis, delayed wound healing, persistent neutrophilia and death at an early age (Hagemoser, et al., 1983;Kehrli, et al., 1990;Nagahata, et al., 1987;Takahashi, et al., 1987). Most of

these calves have stunted growth and a persistent, progressive neutrophilia (often exceeding 100,000/μl) and a less dramatic lymphocytosis (Hagemoser, et al., 1983;Kehrli, et al., 1990;Kehrli, et al., 1993;Nagahata, et al., 1987;Takahashi, et al., 1987). Histologically, capillaries, sinusoids and blood vessels throughout the body contain numerous neutrophils although few neutrophils are present subjacent to ulcerated lesions of mucosal surfaces. Large crescents of numerous neutrophils often circumscribe splenic periarteriolar lymphocytic sheaths and increased myeloid/erythroid ratios are present in the bone marrow. Lymph nodes range from hyperplastic, in some reports, to diffuse, hypocellularity with necrosis of secondary follicles (Kehrli, et al., 1990;Nagahata, et al., 1987).

By investigation of pedigrees, all of the affected calves have been traced to a common sire (Kehrli, et al., 1990). In 1990 (Kehrli, et al., 1990), we reported the molecular definition of the bovine granulocytopathy syndrome as a deficiency of the Mac-1(CD11b/CD18) glycoprotein on the surface of neutrophils. At the time we predicted the probable cause as a deficiency of the CD18 β-subunit of the β_2-integrin family of leukocyte adhesion glycoproteins on all leukocytes in affected cattle. We had based this prediction on the prior knowledge of a similar genetic defect in children called leukocyte adhesion deficiency (LAD)(Anderson, et al., 1984;Springer, et al., 1984) and in Irish Setters (termed canine granulocytopathy syndrome) (Giger, et al., 1987;Renshaw and Davis, 1979). This autosomal recessive trait is clinically characterized by recurrent soft tissue infections, severely impaired pus formation, persistent leukocytosis, and abnormalities of various adhesion-dependent functions of leukocytes in vitro. Leukocytes of human patients with LAD have a deficiency or total lack of a family of structurally and functionally related glycoproteins, including Mac-1, LFA-1 and p150,95 (Sanchez-Madrid, et al., 1983), now termed CD11/CD18 by the World Health Organization (Reinherz, 1986). Each of these molecules contains an α and a β subunit, noncovalently associated in an $\alpha_1\beta_1$ structure. They share an identical β subunit (CD18) and are distinguished by their α subunits designated CD11a, CD11b, and CD11c for LFA-1α, Mac-1α, and p150,95α, respectively. Mac-1 (CD11b/CD18) is the CR3 receptor and binds, most importantly, C3bi and CD54 (ICAM-1). In vivo, Mac-1 mediates tight adherence of neutrophils to activated postcapillary venule, endothelial cells; whereas another adhesion molecule, leukocyte selectin (L-selectin), mediates loose adherence of leukocytes to non-activated endothelial cells (Kishimoto, et al., 1989a). Various distinct mutations of the gene encoding the common β subunit (CD18) have been identified as the basis for disease in all human cases (Dana, et al., 1987;Hibbs, et al., 1990;Kishimoto, et al., 1987;Kishimoto, et al., 1989b;Marlin, et al., 1986).

We have shown that neutrophils from calves with BLAD fail to adhere in a β_2-integrin dependent manner to protein-coated surfaces (Kehrli, et al., 1993). The presence or absence of β_2-integrin molecules can be demonstrated on the surface of neutrophils, monocytes and lymphocytes from normal or affected calves using specific monoclonal antibodies and flow cytometry, or by colloidal gold immunolabeling and scanning electron microscopy in backscatter mode (Ackermann, et al., 1993). β_2-integrin deficiency in Holstein calves is analogous to LAD seen in humans. Neutrophils in LAD patients are unable to adhere to the endothelial lining of the cardiovascular system thus interrupting egression of neutrophils into infected tissues. Calves with BLAD die at an early age as a result of the failure of neutrophil to extravasate into infected tissues as is the case with most untreated human LAD patients. Moreover, severe generalized prepubertal periodontitis, similar to that seen in cattle with LAD, has been reported in children with LAD (Kehrli, et al., 1993;Waldrop, et al., 1987).

In 1991 Shuster et al., identified two point mutations within the alleles encoding bovine CD18 in a Holstein calf afflicted with leukocyte adhesion deficiency (Shuster, et al., 1992b). One mutation causes an aspartic acid to glycine substitution at amino acid 128 (D128G) in an extracellular region of this adhesion glycoprotein that is highly conserved (>95% identity) between humans, cattle and mice. The other mutation is silent. A DNA-PCR-Restriction Fragment Length Polymorphism technique was reported that can be utilized to unequivocally identify the genotype of cattle at the CD18 locus. Numerous calves with clinical symptoms of leukocyte adhesion deficiency have since been tested and

all have been found homozygous for the D128G allele. All cattle with the mutant allele are related to one bull, who through artificial insemination (A.I.), sired many calves in the 1950's and 1960's. The carrier frequency of the D128G CD18 allele among U.S. Holstein cattle had reached approximately 15% among active A.I. bulls and 8% among cows. The organization of the dairy industry and the diagnostic test developed to genotype cattle, has enabled nearly complete eradication of bovine leukocyte adhesion deficiency among future A.I. bulls. BLAD, is the acronym chosen for *B*ovine *L*eukocyte *A*dhesion *D*eficiency which has an inheritance pattern of an autosomal recessive trait (Takahashi, et al., 1987). Cattle that have been tested for this genetic condition and found free of the defective allele are designated *TL, carrier animals are designated *BL. To date, well over 15,000 cattle around the world have since genotyped in various laboratories using this information. Although, the heterozygotic condition has not been completely studied with regard to effects on health, at this time it appears there are no detrimental effects on cattle carrying the defective allele. Factors contributing to low frequency of diagnosis of this syndrome undoubtedly relate to its overall low prevalence on individual dairies and to the impracticality of extensive diagnostic laboratory studies in food producing animal species. We summarize here this work and some of the clinical findings of cattle afflicted with BLAD. We also point out some of the problems encountered in testing such a larger number of cattle in a short period of time.

MATERIALS AND METHODS

The discovery of BLAD in 1989 was serendipitous and occurred in conjunction with a study on a new method to prevent mastitis in periparturient dairy cows (Kehrli, et al., 1991;Stabel, et al., 1991). An apparently healthy, Holstein heifer born to a cow randomly assigned to the placebo-injected control group, exhibited a leukocytosis of 34,600 cells/μl at seven days of age after having counts of 14,000 and 17,000/μl at birth and two days of age, respectively. The calf was immediately examined more closely and a mild fever (39.6 C) was detected. Analysis of blood samples obtained over the next 41 days revealed chronic progressive neutrophilia, which peaked at >85% neutrophils and exceeded 100,000 leukocytes/μl. In vitro assessment of isolated blood neutrophils obtained from the heifer at 38 and 45 days of age revealed selected functional abnormalities. Similar in vitro assessment of neutrophils obtained from the calf's dam revealed no functional abnormalities. The calf died at 48 days of age, with persistent fever and chronic diarrhea, despite administration of antibiotics. Histologic examination at necropsy revealed large numbers of intravascular neutrophils in most tissues, including massive neutrophil sequestration in the spleen. However, a striking lack of extravascular neutrophils was evident in inflamed submucosa adjacent to intestinal ulcers heavily contaminated with enteric microorganisms. Bone marrow examination revealed diffuse myeloid hyperplasia, but no other abnormalities. The proband, a Holstein heifer, was the fourth calf born to its dam and was the result of artificial insemination. Health information on previous calves of the dam was unavailable.

Eight months after the death of the proband, we were able to demonstrate a deficiency of the α-subunit of the Mac-1 β_2-integrin heterodimer (Kehrli, et al., 1990). Flow cytometric analysis of neutrophils from the sire and dam of the proband using a monoclonal antibody specific for canine CD18 indicated that the parents of the proband expressed 65% to 70% of what was an apparently normal level of CD18 expression on other cattle (Kehrli, et al., 1990). In our early studies, it was also apparent that if the reduced expression of CD18 was indicative of a carrier state for an allele producing a defective CD18 protein, that random testing of Holsteins would often find other cattle with similarly reduced levels of CD18 expression.

Based upon the apparent deficiency of the Mac-1 β_2-integrin heterodimer on neutrophils of the proband, we proceeded to sequence a normal allele for bovine CD18 (Shuster, et al., 1992a). The 2833 nucleotide bovine sequence coded for a protein with 769 amino acids. When compared to human and murine sequences, portions of the 5' and 3' noncoding regions of the cDNA sequence were conserved including an AT-rich region believed to regulate mRNA stability and translational

efficiency. Overall, the deduced amino acid sequences were > 80% identical among the three species. Amino acids 96-389 and those in the cytoplasmic domain were very highly conserved. All cysteine residues and potential N-glycosylation sites present in the bovine sequence were also present in the human and murine sequences. Amino acid identity was found in those regions where mutations were found to cause the genetic disease, leukocyte adhesion deficiency. These interspecies conserved regions identify portions of the CD18 molecule which presumably are functionally important.

Within 2 and 7 weeks after completing sequencing of the apparently normal bovine CD18 allele, two Holstein calves (10 and 14 mo of age) with symptoms of LAD were brought to our attention and then purchased from commercial dairy farms. Both calves had mild diarrhea and periodontal gingivitis with gingival recession and tooth loss, and were only 60% of normal weight. One calf had ulcers in and around the mouth that eventually healed after continued topical application of antibiotics. Both calves presented with a persistent and pronounced mature neutrophilia of >47,000 neutrophils/μl, compared with a normal level of <4,000 neutrophils/μl. Neutrophils from both calves expressed <2% of normal level of β_2 integrins by flow cytometry, demonstrating that both calves had LAD. Pedigree information was provided by the owners and the Holstein-Friesian Association of America. Leukocyte RNA was isolated from one of these LAD calves and a Holstein cow that had normal β_2 integrin expression. Northern blot analysis revealed that CD18 transcript was present at normal levels and size in the LAD calf, ruling out genetic defects that block transcription or cause large deletions. Messenger RNA was isolated from one of these LAD calves and used to generate cDNA for sequencing of the bovine CD18 alleles present in BLAD cattle (Shuster, et al., 1992b). The resulting cDNA sequence for CD18 in the BLAD calf was compared to the normal sequence. A point mutation (adenine→guanine) resulting in a substitution of glycine for an aspartic acid at position 128 (referred to as the D128G allele) of the protein sequence in the gene for CD18 was identified in the first heifer and confirmed in the second heifer, and all subsequent cases we have found to date (>100 cases world-wide).

Testing for the mutation at nucleotide 383 of the CD18 locus among additional cases of the bovine granulocytopathy syndrome, the Holstein A.I. bulls and sire dams was performed with genomic DNA. DNA was amplified for 35 cycles (94°C 15 sec, 69°C 20 sec) in a 20 μl reaction containing 1X polymerase chain reaction buffer, 0.2 mM dNTPs, 0.5 U Amplitaq polymerase, and 4 pmol of sense primer (5'-TCCGGAGGGCCAAGGGCTA) and antisense primer (5'-GAGTAGGAGAGGTCCATCAGGTAGTACAGG). Reaction tubes and contents were kept on ice until placed directly into the hot thermal cycler block. Ten-microliter aliquots of amplification product were subjected to restriction endonuclease digestion separately by direct addition of 4 U of Taq I or Hae III followed by incubation for 1.5 hr at 65°C or 37°C, respectively. Digested product was separated by 4% agarose gel electrophoresis and visualized ethidium bromide staining and ultraviolet light illumination.

RESULTS

Because of recurrent bacterial infections in spite of a striking neutrophilia, defective neutrophil function in bovine granulocytopathy syndrome cattle had been suspected (Hagemoser, et al., 1983;Kehrli, et al., 1990;Nagahata, et al., 1987;Takahashi, et al., 1987). Neutrophils from BLAD patients have several functional deficits consistent with deficient expression of CD11/CD18 glycoproteins. Diminished phagocytosis-associated oxidative and secretory functions during ingestion of C3bi opsonized zymosan by neutrophils of BLAD patients' are consistent with a deficiency of the Mac-1 α subunit that contains the complement receptor type 3 (CR-3) epitope reactive with C3bi deposited on zymosan particles (Anderson, et al., 1985;Anderson, et al., 1984). Adherence and surface CD18 levels on neutrophils from normal cattle increased following stimulation with platelet activating factor (PAF) (Kehrli, et al., 1993). These responses were not observed with neutrophils from BLAD patients. PAF-enhanced neutrophil adherence from normal cattle can be inhibited by treating control neutrophils with anti-CD18 mAb but this mAb was without effect when incubated with

BLAD patient neutrophils. Surface expression of CD18, CD11a, and CD11b was initially evaluated using the following mAb: R15.7 (anti-CD18), R3.1 (anti-CD11a) and Leu 15 (anti-CD11b) with neutrophils of one of our BLAD patients. CD11a and CD11b were detected at 6% and 10% of normal values. Repeated analyses of several BLAD patients that we have cared for, demonstrate CD18 to be expressed at ≤ 2% of normal levels for resting and PAF-stimulated neutrophils. Resting neutrophil L-selectin levels in BLAD patients are markedly reduced compared to controls (Kehrli, et al., 1993). The low levels of L-selectin in BLAD patients may be a reflection of chronic subclinical infections.

Two point mutations were detected in the sequence from the LAD calf. One mutation replaced adenine at nucleotide 383 with guanine, and the other mutation replaced the cytosine at nucleotide 775 with thymine. Both parents, obligate heterozygotes, of the other LAD calf were heterozygous for both mutations. The mutation at nucleotide 775 was silent as it did not alter the deduced amino acid sequence. The mutation at nucleotide 383 caused an aspartic acid to glycine substitution at amino acid 128 (D128G). This mutation occurs near the center of 26 consecutive amino acids that are identical in normal bovine, human, and murine CD18 and lies within a large extracellular region that is highly conserved across integrin β subunits (Kishimoto, et al., 1989b;Shuster, et al., 1992a;Shuster, et al., 1992b;Wilson, et al., 1989). Retrospective case studies indicated the presence of only one allele in the Holstein cattle population which causes LAD (Gilbert, et al., 1993;Stöber, et al., 1991;Takahashi, et al., 1987).

DISCUSSION

Granulocytopathies in dogs (Renshaw, et al., 1975), humans (Crowley, et al., 1980) and cattle (Hagemoser, et al., 1983;Nagahata, et al., 1987) characterized by persistent progressive neutrophilia in patients affected with severe recurrent bacterial infections and failure to form pus were reported between 1975 and 1987. In all three species, these conditions were eventually determined to be heritable deficiencies of leukocyte surface glycoproteins associated with diminished cell adherence between 1984 and 1990 (Anderson, et al., 1984;Dana, et al., 1984;Giger, et al., 1987;Kehrli, et al., 1990;Takahashi, et al., 1987). Although published reports of its diagnosis are few, the bovine granulocytopathy syndrome has been diagnosed at Veterinary Schools throughout the world during the past 8 years. In vitro assessments have identified abnormalities of motile, phagocytic, and oxidative functions of neutrophils which appear to mediate inflammatory deficits in vivo (Hagemoser, et al., 1983;Kehrli, et al., 1990;Nagahata, et al., 1987;Takahashi, et al., 1987). To date, over 100 Holstein bovine LAD cases have been diagnosed and confirmed to be homozygous for the D128G mutation (Gilbert, et al., 1993;Shuster, et al., 1992b;Stöber, et al., 1991). Many of these cases were found using formalin-fixed tissues from cattle suspected to have bovine granulocytopathy syndrome in Iowa, New York, Wisconsin, and Germany, dating back to 1977. Thus, it is probable that all BLAD patients among Holsteins represent a homozygous genotype for a single mutant allele of CD18 (D128G).

The severe clinicopathologic consequences of CD11/CD18 deficiency in people as well as dogs and apparently cattle reflect the diverse contributions of the β_2 integrins to leukocyte adherence reactions of importance in inflammation and host defense. The severity of neutrophil function abnormalities and clinical complications among recognized human beings with LAD is directly related to the degree of glycoprotein deficiency. Human patients with the severe phenotype (expressing < 1% of normal αβ complexes on cell surfaces) are susceptible to life-threatening infectious complications in infancy, whereas patients with moderate deficiency (expressing 3 to 10% of normal amounts) develop less severe complications and generally survive into adulthood (Anderson, et al., 1985). Studies of their neutrophils in vitro indicate less-severe functional impairment than has been observed among subjects with the severe phenotype (Anderson, et al., 1985).

It is our opinion, that BLAD patients have a moderately severe phenotype since affected cattle express ~2% of normal amounts of CD18 protein on neutrophil surfaces. It is clear that BLAD is an eventually lethal genetic condition for homozygous animals. It is also likely that BLAD calves would

be the first die if a disease outbreak would occur on a dairy farm. It is also possible that BLAD calves may die as a result of disease caused by normal flora. Based on our limited clinical and research experience with eight BLAD patients, the majority of these calves would die before one year of age. It is possible, however, for some of the animals to live past two years of age but they would be severely stunted (~50% of expected body weight) in growth and would suffer from the various infectious conditions of the skin, gastrointestinal and respiratory tracts. Of the older survivors, a striking periodontal gingivitis with marked recession of the gingiva may be the most obvious clinical sign.

The dairy industry relies on the use of artificial insemination. Testing of bulls and cows mated for the purpose of producing future sires used in artificial insemination throughout the world has been in progress since 1991. Results from this testing has provided the necessary information to phase out the use of carrier bulls designated (*BL) within 5 years without any significant loss of the existing gene pool necessary for high quality milk production. BLAD is an especially serious condition within the Holstein breed because some of the most prominent sires of the breed are heterozygous for the D128G allele. Osborndale Ivanhoe, Penstate Ivanhoe Star and Carlin-M Ivanhoe Bell are some of the elite sires of the breed diagnosed as carriers of this allele on the basis of DNA testing. It is certain that a small proportion of calfhood deaths in this breed are, in fact, attributed to infectious complications of BLAD. If we assume random mating and a carrier rate for the D128G allele of 13.5% among bulls and 8% among cows, not more than 1.1% of all random matings would be between carrier animals. Of these matings only 25% would result in a homozygous affected BLAD progeny, therefore, only 0.27% of all random matings would result in an affected BLAD calf. With 9.7 million dairy cows in the United States, this predicts about 26,000 BLAD calves born each year.

The frequency of the mutant allele and the lack of pathognomonic clinical symptoms suggest that most LAD calves die undiagnosed. Our experience indicates that most affected calves will die at an early age (<1 year) and those that survive will exhibit poor growth performance. Some calves may live past two years of age, but their lactational and reproductive potential is likely very low because of their small size and poor health. The organization of the dairy industry such that relatively few bulls sire most of the progeny allows rapid elimination of autosomal recessive diseases. Virtually all of the Holstein bulls used for A.I. have been tested, and carriers are being culled or used cautiously. Within one year, few calves will be sired by carrier bulls, so the incidence of LAD will decline dramatically. This research on bovine LAD supports what is becoming the first worldwide attempt to select animals for a specific DNA sequence.

Due to the existence in dizygotic bovine twins of the leukochimeric condition, it is important to identify even slight aberrations in DNA polymorphism assays. The potential risk of false genotyping of cattle, and the expense to breeders, emphasizes the importance of this issue. Ours and previous reports of leukochimerism, warrant against the exclusive use of blood samples for DNA typing when twinning is properly documented.(Ryncarz, et al., 1994) Breed associations have determined it is important to eliminate the presence of genetic lethal conditions from populations of animals. The dairy cattle industry affords the most striking example of how rapidly the use of DNA-PCR technologies can be applied with minimal negative impact on the available finite gene pool. Previous efforts to screen for a genetic defect in Australian Holstein cattle also used DNA-PCR technology (typically DNA extracted from blood) (Dennis, et al., 1989). Testing of animals for citrullinemia using blood leukocyte DNA led to the false genotyping of a few animals which were not originally known to be twins. Apparently, a certain percentage of live births (~1 in 1500 animals) involve a calf with a twin that died in utero and was resorbed during gestation or was never observed in the placental membranes (personal communication, Dr. Peter Healy, 1992). These unidentified twins pose a potential delay to breed associations who desire to quickly eliminate genetic defects through DNA testing methods. A similar lack of a known co-twin has recently been reported in a parentage dispute involving a mare who was excluded as a parent based upon blood-derived DNA (Bowling, et al., 1993).

Limitations of applying DNA-PCR for genotype identification include financial as well as technical errors. If 1499 out of 1500 births are accurately recorded as twin or single births, then a test

performed on blood-derived DNA would be 99.93% accurate. This level of accuracy should be acceptable provided twinning is documented where possible and it offers the cattle industry the ability to test the DNA of calves shortly after birth, thus saving considerable expense associated with performing skin biopsies or waiting until semen is available from bulls which need to be tested. For cows, only an alternative source of DNA (such as skin) is a satisfactory solution to the potential leukochimerism condition causing a false genotype. We recommend testing the most influential Holstein sires and any sires descendent from a known carrier through the use of semen. Matings between known carriers should be avoided unless a deliberate effort to identify homozygotically normal embryos or fetuses is made. This type of mating might result in valuable progeny but would be achieved only through considerable expense and effort. We urge that this condition be removed from the bovine gene pool through the screening of potential A.I. sires as has been taking place since 1991.

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