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Dale E. Shuster, *United States Department of Agriculture*

Marcus E. Kehrl, Jr., *United States Department of Agriculture*

Mark R. Ackermann, *United States Department of Agriculture*

R. O. Gilbert, *United States Department of Agriculture*



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Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle

(animal model/CD18/ β_2 integrins)

DALE E. SHUSTER*, MARCUS E. KEHRLI, JR.*[†], MARK R. ACKERMANN[‡], AND ROBERT O. GILBERT[§]

*U.S. Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Metabolic Diseases and Immunology Research Unit, and [†]Avian Diseases Research Unit, Ames, IA 50010-0070; and [‡]Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

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ABSTRACT Two point mutations were identified within the gene encoding bovine CD18 in a Holstein calf afflicted with leukocyte adhesion deficiency (LAD). One mutation causes an aspartic acid to glycine substitution at amino acid 128 (D128G) in the highly conserved extracellular region of this adhesion glycoprotein, a region where several mutations have been found to cause human LAD. The other mutation is silent. Twenty calves with clinical symptoms of LAD were tested, and all were homozygous for the D128G allele. In addition, two calves homozygous for the D128G allele were identified during widespread DNA testing, and both were subsequently found to exhibit symptoms of LAD. The carrier frequency for the D128G allele among Holstein cattle in the United States is approximately 15% among bulls and 6% among cows. This mutation is also prevalent among Holstein cattle throughout the world, placing this disorder among the most common genetic diseases known in animal agriculture. All cattle with the mutant allele are related to one bull, who through the use of artificial insemination sired many calves in the 1950s and 1960s. The organization of the dairy industry and the diagnostic test described herein will enable nearly complete eradication of bovine LAD within 1 year. These results also demonstrate that bovine LAD is genetically homologous and phenotypically similar to human LAD, thus providing a useful animal model for studies of LAD and β_2 integrin function.

Leukocyte adhesion deficiency (LAD) is an autosomal recessive disease characterized by greatly reduced expression of the heterodimeric β_2 integrin adhesion molecules on leukocytes, resulting in multiple defects in leukocyte function (1–3). Neutrophil extravasation requires β_2 integrin interaction with endothelial intercellular adhesion molecules (2, 4, 5). Without β_2 integrins, neutrophils are unable to enter the tissues to destroy invading pathogens. Consequently, LAD patients suffer frequent and recurrent bacterial infections (1–3). The β_2 integrins include LFA-1, Mac-1, and p150,95, which consist of unique α subunits, CD11a, CD11b, and CD11c, respectively, and a common β subunit, CD18. Because β_2 integrin expression requires intracellular association of the CD11 and CD18 subunits, defects in CD18 prevent expression of all β_2 integrins (2, 4, 6). All human cases of LAD have been traced to defects in the gene encoding CD18 (2, 4, 7–11).

A granulocytopeny syndrome had been described in Holstein cattle (12–14), and Kehrlí *et al.* (15) showed that this disease results from a lack of leukocyte β_2 integrin expression, indicating that this disease is equivalent to LAD. The etiologic mutation for bovine LAD had not been determined, and efforts to study and control this disease were seriously limited by the lack of a diagnostic test to identify carrier

cattle. We now show that LAD in Holstein cattle is caused by a point mutation in the gene encoding CD18[¶] and that this mutation is prevalent among Holstein cattle throughout the world.

MATERIALS AND METHODS

Cattle. Two calves with LAD were purchased from separate commercial dairy farms. The calves were brought to the attention of the local veterinarian for poor growth performance in one case and for chronic, soft tissue swelling under the jaw in the other case. Subsequent examination revealed a pronounced mature neutrophilia in both cases, which caused the calves to be brought to our attention. The absence of neutrophil β_2 integrin expression by flow cytometry indicated that the calves had LAD. Other LAD cattle were submitted during the last 15 years to either the Iowa State or Cornell University veterinary clinics with LAD symptoms, primarily recurrent infection and persistent mature neutrophilia exceeding 40,000 cells per μ l, now known to be characteristic of bovine LAD. Blood or formalin-fixed, paraffin-embedded tissues from these cattle were supplied for DNA testing.

Blood or extended semen from bulls in commercial artificial insemination programs was submitted by the owners. These bulls included >90% of all United States bulls commonly used for artificial insemination. Blood or milk samples from cows in several commercial or university dairy herds were provided by collaborating university personnel. These bulls and cows are representative of Holsteins in the United States and were sampled and tested without regard to their possible genetic status for the LAD mutation. Pedigree information, which was available for all bulls and all but one of the LAD calves, was provided by the owners and the Holstein–Friesian Association of America.

Flow Cytometry. Indirect immunofluorescence analysis of peripheral blood neutrophils was performed using murine R15.7 monoclonal antibody to CD18 and fluorescein-conjugated antibody to mouse IgG (1, 15). Labeled cells were analyzed in a flow cytometer, and neutrophils were identified by forward and side scatter characteristics. Fluorescence after labeling with IgG1 control antibody was low for all cattle. Relative expression of CD18 was calculated as mean channel fluorescence with R15.7 antibody minus fluorescence with control antibody. With this procedure, neutrophils from LAD carriers expressed \approx 70% as much CD18 as neutrophils from homozygous normal cows (15).

Abbreviation: LAD, leukocyte adhesion deficiency.

[†]To whom reprint requests should be addressed.

[¶]The normal bovine CD18 cDNA sequence and the mutations reported herein have been deposited in the GenBank data base (accession no. M81233).

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Northern Blot and Sequence Analyses. Bovine peripheral blood leukocytes were isolated by centrifugation and hypotonic lysis of erythrocytes, and leukocyte RNA was isolated by using guanidinium isothiocyanate (16). For Northern blot analysis, total RNA (10 μ g) was electrophoresed in a 1% agarose gel containing formaldehyde, transferred to a nylon membrane, and then hybridized with bovine *CD18* cDNA labeled with [α - 32 P]ATP (17). The hybridization solution was 50% formamide/5 \times Denhardt's solution/5 \times SSPE/0.5% SDS containing salmon sperm DNA at 0.1 mg/ml, and hybridization was performed at 42°C overnight (1 \times SSPE is 150 mM NaCl/10 mM NaH₂PO₄/1 mM EDTA, pH 7.4). The membrane was washed twice in 6 \times SSPE/0.5% SDS at 25°C and twice in 1 \times SSPE/0.5% SDS at 37°C. To sequence bovine *CD18*, poly(A)⁺ RNA was fractionated by oligo(dT) chromatography (18), and 1 μ g was reverse transcribed with an oligo(dT) primer. The cDNA was amplified with bovine *CD18*-specific sense (5'-CCCTGCCAGTCCAGCTGGACACC-3') and anti-sense (5'-CCACGCCCATCATTC-TGGGGCAG-3') primers in the polymerase chain reaction (PCR). To sequence the amplified products, single-stranded DNA was generated from purified PCR product by linear PCR with a single primer and sequenced directly (19) by using bovine *CD18*-specific primers. Overlapping segments of bovine *CD18* were independently amplified and sequenced to enable complete bidirectional sequencing of the entire *CD18* coding region.

Testing for the mutation at nucleotide 383 was performed with genomic DNA isolated from blood, semen, milk, or fixed tissues. Whole blood (250 μ l) with EDTA anticoagulant was hypotonically lysed with 1 ml of 0.01 M sodium phosphate (pH 7.2) and centrifuged (10,000 \times g, 1 min) twice. The leukocyte pellet was washed once with 1 ml of 10 mM sodium phosphate, pH 7.2/3 mM KCl/140 mM NaCl (phosphate-buffered saline). The pellet was then resuspended in 10 μ l of 0.1 M NaOH/2 M NaCl, heated at 95°C for 2 min, and centrifuged (10,000 \times g, 10 min). The supernatant containing the DNA was removed and diluted with 25 μ l of sterile distilled water. Approximately 0.5 ml of extended semen was washed with 1 ml of phosphate-buffered saline and centrifuged (10,000 \times g, 1 min) three times. The final pellet was resuspended in 90 μ l of 10 mM Tris, pH 7.5/1 mM EDTA/100 mM NaCl and 10 μ l of 1 M NaOH and then heated at 95°C for 5 min. Milk samples were centrifuged (10,000 \times g, 1 min), supernatant was poured off, and milk fat was removed from the sides of the tube with a sterile cotton swab. Milk leukocytes were then washed and treated in the same manner as semen. Formalin-fixed, paraffin-embedded tissues were deparaffinized and processed according to the procedure of Greer *et al.* (20). DNA (0.3 μ l) was amplified for 35 cycles (94°C, 15 sec; 69°C, 20 sec) in a 20- μ l reaction containing 1 \times PCR buffer, 0.2 mM dNTPs, 0.5 unit of AmpliTaq polymerase (Perkin-Elmer/Cetus), and 4 pmol of sense primer (5'-TCCGGAGGGCCAAGGGCTA-3') and antisense primer (5'-GAGTAGGAGAGGTCCATCAGGTAGTACAGG-3'). Reaction tubes and contents were kept on ice until placed directly into the hot thermal cycler block. Aliquots (10 μ l) of amplification product were subjected to restriction endonuclease digestion separately by direct addition of 4 units of *Taq* I or *Hae* III followed by incubation for 1.5 hr at 65°C or 37°C, respectively. Digested product was analyzed by 4% agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Identification of Mutations in Bovine CD18. Two Holstein calves (10 and 14 months of age) with symptoms of LAD were 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

calves 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 per μ l, compared with a normal level of <4000 neutrophils per μ l. Neutrophils from both calves expressed <2% of the normal level of β_2 integrins by flow cytometry (Fig. 1), demonstrating that both calves had LAD.

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 (Fig. 2A), ruling out genetic defects that block transcription or cause large deletions. Poly(A)⁺ RNA was reverse transcribed, and the entire *CD18* coding region for the LAD calf and the normal cow was sequenced. Sequences from these animals were compared with each other and with the previously determined bovine *CD18* cDNA sequence (21). 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 (Fig. 2B and C). 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 (2, 21-23).

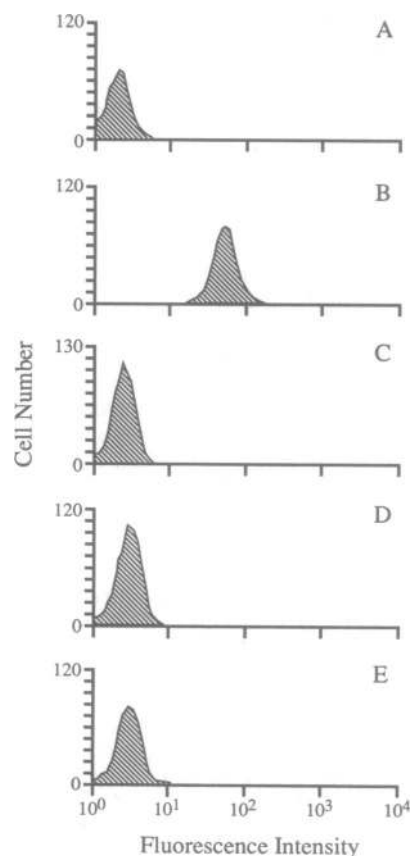


FIG. 1. Indirect immunofluorescence analysis of CD18 expression on bovine neutrophils from a normal cow (B), from the two purchased LAD calves (C and D), and from the first LAD calf produced by selective mating (E). Fluorescence after labeling with IgG1 control antibody was low for all cattle (A).

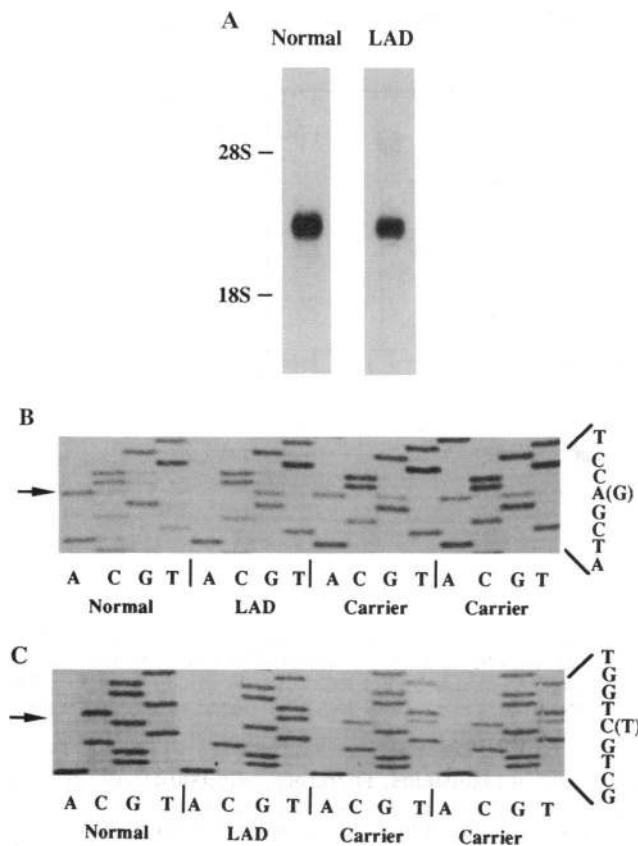


FIG. 2. (A) Northern blot analysis of *CD18* transcripts in total RNA isolated from peripheral blood leukocytes from a LAD calf and a normal cow. (B) Sequence autoradiograph showing the point mutation at nucleotide 383 (arrow). The normal cow is homozygous A, the LAD calf is homozygous G, and both parents of another LAD calf are heterozygous A/G. (C) Sequence autoradiograph showing the point mutation at nucleotide 775 (arrow). The normal cow is homozygous C, the LAD calf is homozygous T, and both parents of another LAD calf are heterozygous C/T at this nucleotide.

Genotype of LAD Calves. A method was developed to rapidly screen cattle for the D128G allele. Genomic DNA was amplified with primers specific to regions near nucleotide 383, and the amplification product was digested with *Taq* I and *Hae* III to specifically cut the normal and mutant alleles, respectively (Fig. 3). With this technique, five Holstein

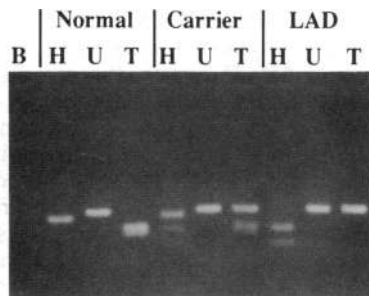


FIG. 3. Agarose gel electrophoresis of amplified DNA from normal, carrier, and LAD cattle following restriction enzyme digestion (B, amplification blank; H, *Hae* III; U, undigested; T, *Taq* I). DNA from normal cattle is digested by *Hae* III (at a position distinct from nucleotide 383) to generate 9- and 49-base-pair (bp) fragments and by *Taq* I to generate 26- and 32-bp fragments. DNA from LAD cattle is digested by *Hae* III to generate 9-, 19-, and 30-bp fragments but is not digested by *Taq* I. DNA from carrier cattle is digested by *Hae* III to generate 9-, 19-, 30-, and 49-bp fragments and by *Taq* I to generate 26-, 32-, and 58-bp fragments.

calves that had been diagnosed with LAD by the lack of β_2 integrin expression were found to be homozygous for the D128G mutation. Both parents of four LAD calves were also screened for this mutation, and each was found to be heterozygous. Postmortem testing was completed with formalin-fixed tissues from 12 calves that had exhibited the LAD phenotype and had been submitted to the veterinary clinic at Cornell University between 1975 and 1991. Chief complaints at admission were respiratory disease, diarrhea, inappetence, unthriftiness, or a combination of these symptoms. Each calf exhibited at least one mature neutrophil count in excess of 40,000 per μ l (range, 41,000–320,000). All of these calves were homozygous for the D128G mutation. Two additional LAD calves were produced by repeated mating of both parents of our proband LAD calf through the use of superovulation and embryo transfer technology. Both calves were homozygous for the D128G mutation, their neutrophils expressed very low levels of CD18 (Fig. 1E), and they suffered symptoms of LAD. During widespread screening of Holstein cattle for the D128G allele, two calves homozygous for the D128G allele were identified, and both calves were subsequently found to exhibit LAD symptoms. Together, these results demonstrate the causative role of the D128G mutation in bovine LAD.

Prevalence of the D128G Allele among Holstein Cattle. To determine the prevalence of the D128G allele, 2025 United States Holstein bulls that are used for artificial insemination were screened for the presence of the mutation. The carrier frequency was 14.1% overall, and 17.1% among the top 100 bulls for genetic merit for milk production, before selection against this disease began. These superior bulls sire thousands of calves in countries throughout the world. We also screened 1559 Holstein cows from several farms throughout the United States and detected a 5.8% carrier frequency. From these data, a conservative estimate of the incidence of LAD in United States Holstein calves at birth is 0.2%.

All LAD calves that have been studied are distantly related to one another, and their pedigrees were traced over five generations to a common male ancestor in both parental lines (Fig. 4). In addition, all carriers of the D128G allele that we identified and for which we were able to obtain pedigree information are descendants of this bull or, in a few cases, his immediate relatives. This bull, Osborndale Ivanhoe, was born in 1952 and was shown to be a carrier of the D128G allele by testing of frozen semen. Because of his high genetic merit for milk production, he and several of his sons and grandsons have each sired thousands of progeny so that he now is the animal with the greatest genetic relationship (relationship coefficient = 0.102) to the United States Holstein breed (24).

DISCUSSION

These results define a mutation that causes LAD in Holstein cattle. To date, all bovine LAD cases that have been diagnosed and genetically tested are homozygous for the D128G mutation. Furthermore, genetic screening of >3000 cattle revealed only 2 D128G homozygotes, and both of these individuals were afflicted with clinical abnormalities typical of LAD. Therefore, at least in Holstein cattle, the D128G mutation is the only known cause of bovine LAD. It is possible that other LAD alleles are present among cattle, but their prevalence is apparently low and economically insignificant. If a case of bovine LAD is identified that is not homozygous for the D128G allele, the possibility of another mutant allele will need to be investigated.

The D128G mutation occurs in a highly conserved region of the extracellular portion of the CD18 glycoprotein. Several mutations that cause human LAD have also been found in this region (3, 7–11), indicating that the structure of this region is functionally important. Intracellular processing and

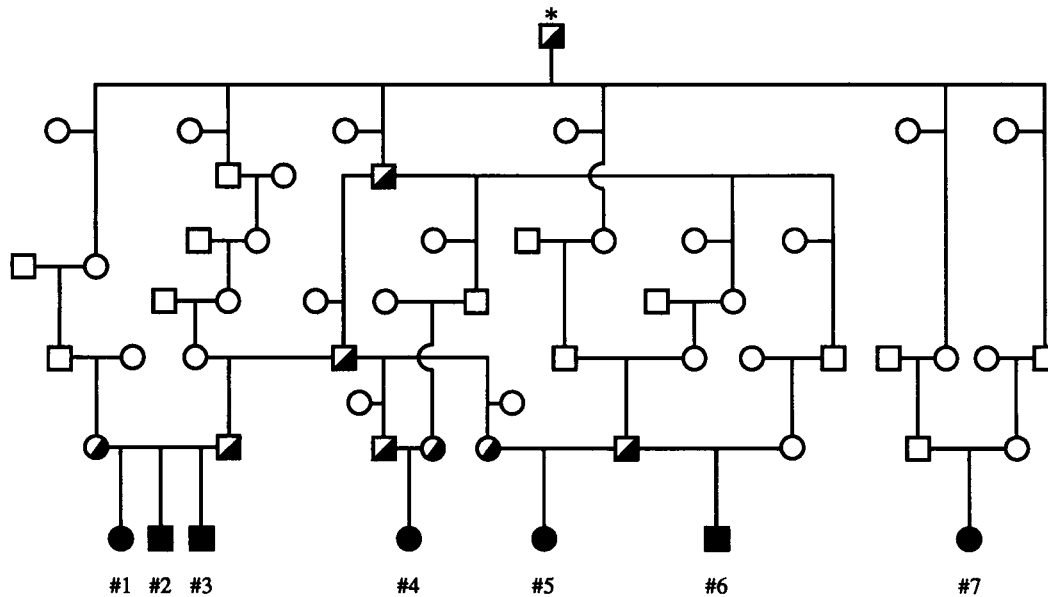


FIG. 4. Pedigrees of LAD calves (filled symbols) showing their relationship to a common carrier sire, Osborndale Ivanhoe, who has been labeled as a "Father of the Holstein Breed" with thousands of registered sons and daughters (24) and is indicated here with an asterisk. All LAD calves are homozygous for the D128G mutation. Open symbols represent animals that were not tested for the D128G mutation, and half-filled symbols denote carriers. Calves 1 (15) and 7 (12) are described in the literature. Calves 2 and 3 were produced by repeated mating of the parents of calf 1. Calves 4 and 5 are the two purchased calves.

surface expression of the β_2 integrins require association of the α and β subunits (2, 4, 6). Mutations in the highly conserved extracellular region of CD18 may disrupt α/β subunit association so that processing and expression fail to occur for any of the β_2 integrin subunits. The lack of β_2 integrin expression by leukocytes from LAD calves is nearly complete, and the clinical presentations of human and bovine LAD patients—e.g., gingivitis, tooth loss, and high susceptibility to recurrent respiratory and gastrointestinal tract infections (1, 25)—are strikingly similar. Thus, bovine LAD provides a good animal model for studies of human LAD and its possible treatment with genetic therapy. This model may also be useful for investigations of the role of β_2 integrins in various physiologic and pathologic conditions.

The results demonstrate that the D128G allele and, therefore, LAD is common among United States Holsteins. A carrier frequency of 14.1% among bulls and 5.8% among cows predicts an incidence of 0.2% among United States Holstein calves at birth. In addition, many calves with the LAD phenotype have been observed in Japan and Europe (13, 14, 26). Their pedigrees include Osborndale Ivanhoe, and all of these calves that were tested were D128G homozygotes (M.E.K., Joachim F. L. Pohlenz, Matthaeus Stöber, and Wolfgang Leibold, unpublished data). Thus, this mutation is prevalent among Holstein cattle throughout the world. Other genetic diseases have been identified in Holstein cattle in recent years. For example, deficiency of uridine monophosphate synthase was identified in United States Holsteins (27). The frequency of carriers for this disease was approximately 2% among cows and 7% among bulls (27). Citrullinemia was found to be common among Australian dairy cattle (28) but is rare among U.S. cattle (29). Thus, LAD is more prevalent and widespread than other bovine genetic diseases.

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 2 years of age, but their lactational and reproductive potential is likely to be very low because of their small size and poor health. In the United States, 80% of the 10 million

dairy cows are Holsteins. Therefore, $\approx 16,000$ calves are born with LAD each year. The average economic loss per calf would be roughly \$300. Thus, bovine LAD costs dairy consumers and producers about \$5 million annually in the United States alone, placing bovine LAD among the most economically significant genetic diseases known in animal agriculture. Among remaining concerns to be addressed are the possibilities of beneficial effects of the carrier state or valuable genetic traits closely linked to the *CD18* locus. Similar linkage has complicated selection against Weaver syndrome, a heritable neurologic disorder in Brown Swiss cattle (30).

The development of artificial insemination has enabled the advent of modern dairy cattle breeding practices. These practices involve intense selection of bulls based upon lactational performance of their daughters and the widespread use of these few genetically superior bulls. During the last three decades, these practices have increased the milk production potential of Holstein cows by >900 kg per lactation (31, 32) but have also increased the genetic relatedness of individuals within the breed (24). This increased relatedness is conducive to phenotypic expression of recessive genetic diseases such as LAD. However, the organization of the dairy industry so that relatively few bulls sire most of the progeny allows rapid elimination of autosomal recessive diseases. Most of the Holstein bulls are already being screened, and carriers are being culled or used cautiously. Within 1 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.

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