Molecular and functional characterization of neutrophils from calves with bovine leukocyte adhesion deficiency : comparison of normal, heterozygous, and homozygous animals
by Karen May Lambrecht Sipes

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Montana State University

Abstract:
Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive disease of Holstein dairy cattle that is characterized by recurrent bacterial infections. The molecular basis for BLAD involves a mutation in CDl8 resulting in the absence of β2 integrin expression on leukocytes. The β2 integrins play key roles in neutrophil adhesion, chemotaxis, phagocytosis, and receptor signaling. The absence of these adhesion molecules severely impairs leukocyte adherence-dependent functions and results in compromised immunity. Thus, BLAD calves provide a model for investigating the role of β integrins in these neutrophil functions. In the studies described here, surface molecule expression, microbicidal activity, cell maturation, and molecular signaling were studied in neutrophils isolated from heterozygous and homozygous BLAD calves and compared with clinically normal calves. With the exception of the β integrins, surface molecule expression on leukocytes from the three bovine genotypes was similar when evaluated with a panel of monoclonal antibodies. Using three different measurements, neutrophils isolated from homozygous BLAD calves expressed increased NADPH oxidase activity after stimulation when compared to normal and heterozygous BLAD neutrophils. Visual comparison of whole blood smears revealed that homozygous BLAD calves had higher levels of immature neutrophils. Antibody cross-linking of the β subunit (CD18) of the β integrins on normal and heterozygous BLAD neutrophils produced a transient rise in intracellular free calcium, exocytosis of specific granules, shedding of L-selectin, up-regulation of CD18, and redistribution of the protein kinase p58fgr. These responses were absent in homozygous BLAD neutrophils. Immunoprecipitation of homozygous BLAD neutrophil membrane lysates with antibodies against β integrins revealed three protein bands with molecular weights corresponding to two of the three a subunits and the β subunit of the β2 integrins. Western blots confirmed that the CD11b α subunit was not present in these lysates, but the lack of antibodies that cross-react with the bovine β2 integrins hindered the identification of the three bands which may represent defective β2 integrin subunits in animals homozygous for BLAD. Overall, these studies contribute to our understanding of the role of β integrins in neutrophil function.

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A thesis submitted in partial fulfillment
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of

Doctor of Philosophy

in

Veterinary Molecular Biology

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

December 1999

APPROVAL

of a thesis submitted by

Karen May Lambrecht Sipes

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date 12/17/99

This thesis is dedicated to my children Courtney and Lindsey who have filled my life with joy and unconditional love.

ACKNOWLEDGMENTS

I would like to thank the people in my life who were fundamental in my quest for my doctoral degree. I would like to express my appreciation to my committee, Dr. Mark Quinn, Dr. Mark Jutila, Dr. Jim Cutler, Dr. Seth Pincus, and Dr. David Pascual. Their insight into the world of science is always inspiring. A special thanks to my advisor Dr. Mark Quinn for his guidance, encouragement, and understanding.

I will cherish the friendships of my fellow graduate students who shared this unique time of my life. I could not have had a better classmate than Jeff Leid (my biggest vice). My best wishes to all.

I am proud to have had the opportunity to work with many talented and interesting people. People in my research group who have been a great source of help and humor: Dr. Steve Swain, Peggy Bunger, Andrew Matala, Dan Seimsen, Laura Nelson, Gayle Watts, Dr. Angela Davis, Kathy Jutila, Angela Hanson, Dr. Jindrich Soltys, Patrice Mascolo, Dr. Katherine Gauss, Dr. Mark Clements, and all others who have come and gone. It is difficult to express how much I have gained both personally and professionally from our time together. Thank you.

Finally, I would like to acknowledge the NIH/NIAID medical mycology predoctoral training grant and MSU for the financial support of my graduate research.

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CHAPTER 1

INTRODUCTION

The Neutrophil

Neutrophils are one of the primary cells involved in the host response to infectious disease and inflammation. Not only are they essential to host defense against bacteria and some fungi (1), they have also been implicated in destructive inflammatory responses such as ischemia-reperfusion tissue injury and inappropriate inflammation responses like rheumatoid arthritis (2). Neutrophils have various membrane receptors that recognize and bind pathogens, chemotactic cytokines, growth factors and inflammatory proteins. Additionally, neutrophils cooperate with the other leukocytes in various immune responses (3). As with all leukocytes, neutrophils originate in the bone marrow, developing from pluripotent stem cells. Cytokines, such as the colony stimulating factors (CSF), act on stem cells to differentiate them into the myeloid cell lineage and eventually commit them to become neutrophils, which mature within 8-10 days (4,5). Upon maturation, neutrophils become functional where they can be found in the blood and tissues. Neutrophils only reside in the blood for a short time (6-24 hours) before migrating to the tissue (2,5-7). It has been reported that neutrophils are only functional for an average of 2.5 days in the tissues (1,6) before they routinely undergo apoptosis, and unlike lymphocytes, they cannot recirculate (5,6). Considering this short life span and the fact that neutrophils are the most abundant leukocyte found in normal

leukocyte found in normal healthy adult human blood, the turnover rate for the bone marrow production of neutrophils is impressive (4×10^8 neutrophils/pound of body weight/day) (2).

Neutrophil Response to Inflammation

The immediate host response to infection is a transient neutropenia resulting from increased margination and accelerated delivery of neutrophils to the infected site. Within an hour, neutrophils are released from the bone marrow reserve into the bloodstream. In the early phases of infection, the circulating half-life of neutrophils is shortened, and cell turnover is accelerated. The circulating half-life returns to normal as the infection is resolved. In prolonged inflammation or stress, there are increased numbers of immature neutrophils (band neutrophils) in the circulating blood representing a depletion of the neutrophil storage pools. This is referred to as a "left shift" (8,9). In addition to changes in the neutrophil blood count, neutrophil morphology can be altered by infection. Cytoplasmic granules become prominent, and large bluish, Döhle bodies may be seen. Döhle bodies are remnants of free ribosomes or rough surfaced endoplasmic reticulum left from an immature stage (9). Infection and inflammation can also affect the function of circulating neutrophils: both enhanced and impaired responses have been reported when compared to normal values (5).

To participate in an inflammatory response in the tissue, neutrophils follow three molecular steps known as rolling, tight adhesion and transmigration. Currently, the molecular aspects of these systems are being defined. It is known that L-selectin-

expressing neutrophils survey the vascular endothelium for inflammatory signals (7). Once the neutrophil encounters activated endothelium, L-selectin is shed and the neutrophils engage the endothelium with the P- and E-selectin ligands (10). The sequence of rolling interactions has been defined using monoclonal antibodies (mAb) or blocking peptides to the various selectins and their corresponding ligands at sequential times after neutrophil/endothelial activation (12).

Cellular tight adhesion involves a family of molecules known as the integrins, with various cell types expressing different integrins on their cell surfaces. There are specific integrin subfamilies grouped by the association of one β subunit molecule with one molecule from a particular group of α subunits (10). Neutrophils are dependent on the interaction of their β_2 integrins with the intracellular adhesion molecules (ICAM-1 & ICAM-2) on activated endothelium for tight adhesion and subsequent transmigration. The function of the β_2 integrins was determined by research with mAbs against the integrins and their ligands, transfected cell lines (11), knockout mice (12), human neutrophils from patients with various forms and degrees of genetic β_2 integrin deficiencies (13-15), and Holstein cattle with a genetic mutation in the β_2 subunit (16-23).

The third step in the neutrophil's exit from the blood stream to the tissue is transmigration through the endothelium. Neutrophil transmigration does not permanently alter the binding between the endothelial cells at the intercellular junctions and, therefore, does not create a detectable hole (12). In fact, once the endothelial crossing is complete, the endothelial cells and the neutrophils remain intact (12). The molecules involved in

neutrophil transmigration include the platelet-endothelial cellular adhesion molecule (PECAM-1, CD31) and at least one of the β_3 integrins ($X_v\beta_3$, CD51/CD61). PECAM-1 is present at the intercellular junction of endothelial cells and is expressed on both neutrophils and lymphocytes (12). $X_v\beta_3$ is also expressed on lymphocytes and neutrophils (12). PECAM-1 can interact with itself and with $X_v\beta_3$. The β_3/PECAM-1 and the homophilic PECAM-1 interactions are believed to account for the close apposition between the neutrophil and the endothelial layer that allows the endothelium to remain intact (12).

Once the endothelial crossing is complete, the neutrophil encounters the subendothelial basal lamina, which consists of a dense meshwork of extracellular matrix proteins (ECM) (12). The leukocyte response integrin (LRI) is a β_3 integrin involved in neutrophil activation at this level in the tissue. LRI requires the presence of another molecule known as the integrin associated protein (IAP) or CD47 (12). Ligands for the LRI can induce an oxidative burst in non-β_2 integrin dependent adhered neutrophils, and this response can be blocked by anti-IAP mAb (24).

After neutrophils have entered the tissue, their main function is to search, find and destroy pathogens (phagocytosis and killing) and to interact with the other cells of the immune system to clean up and repair sites of infection and inflammation. Neutrophils express many surface receptors that participate in these various functions. These include chemokine receptors, cytokine receptors, immune complex receptors, complement component receptors, and specific peptide receptors. The signal transduction cascades induced by these receptors can lead to a repertoire of various responses, including

aggregation, phagocytosis, degranulation, and the oxidative burst (25).

Neutrophil Activation Pathways

Neutrophils can be primed and/or activated by a number of different compounds through a number of different pathways. Primed neutrophils are functionally up-regulated but do not complete cellular signaling through the oxidative burst. They do, however, respond to subsequent activating stimuli in an exaggerated fashion compared to unprimed cells (25,26). Priming stimuli include: monomeric IgG, IFN-γ, TNF-α, LPS, IL-6, IL-8, G-CSF, GM-CSF, PMA, LTB_4, calcium ionophores, insulin-like growth factor, PAF, and human growth hormone (27).

Neutrophils can be fully activated by a number of soluble and particulate stimuli, including chemotactic factors, cytokines, immune complexes, phorbol esters, and serum treated zymosan (β-glucan). Soluble stimuli can activate neutrophils within seconds to minutes, whereas some particulate agonists tend to activate neutrophils more slowly (2). The earliest events in the sequence leading to neutrophil activation is the phosphorylation of PLA, PLD, and/or PLC (seconds) (2). This leads to the intracellular release of stored calcium and the drop in cAMP levels (< 2 min) (2,28). Increases in tyrosine kinase activity leads to an accumulation of phosphorylated proteins (2 - 5 min). Both the drop in cAMP and the phosphorylation of certain protein seem to be essential for the subsequent actin polymerization (> 5 min), which in turn is necessary for activation of the respiratory burst (> 10 min) (28). Note that these times are general and are based on published research with soluble activators and adherent neutrophils (see Figure 1.1). By using

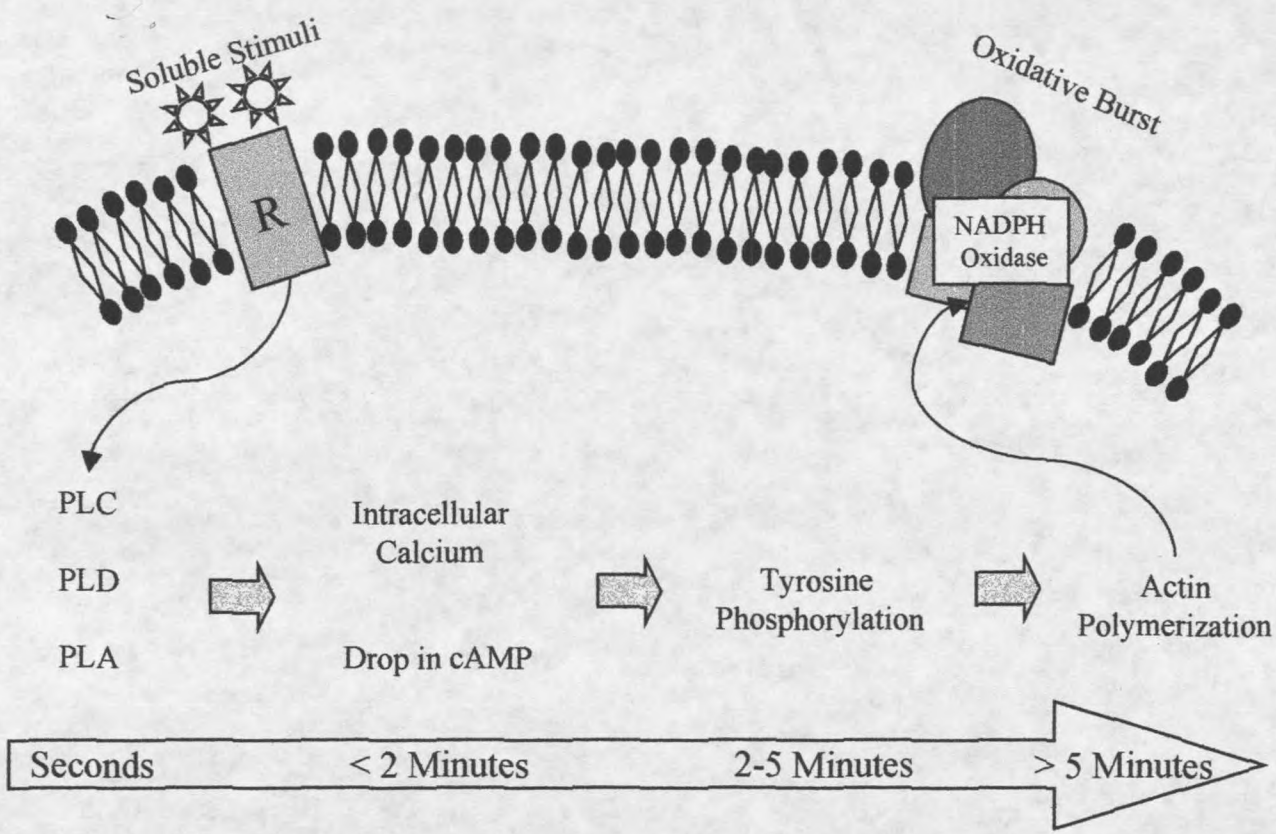

Figure 1.1 Neutrophil Activation. Soluble stimuli can quickly activate neutrophils. Signaling through membrane receptors on adhered neutrophils can result in an oxidative burst within 10 minutes. Different stimuli and various adherent or non-adherent assays can slightly decrease or greatly delay the oxidative burst.

different stimuli and various adherent or non-adherent assays, the time frame can be slightly decreased (29) or delayed for up to 120 minutes (30,31). Other variables can also change the activation parameters. For example, some neutrophil isolation procedures have been shown to prime or activate the neutrophils (25,32,33). Priming can then influence the time to onset of a number of neutrophil functions, including oxidative burst activity, phagocytosis, chemotaxis, and the expression of cell surface markers (34-36).

During the late 1980's, some stimuli were found to induce superoxide production in adherent, but not in suspended neutrophils (37). Neutrophils adherent to surfaces coated with serum or ECM proteins and induced by soluble stimuli produced oxygen metabolites over a prolonged period, while these same stimuli did not activate non-adherent neutrophils (31,38). Triggering of the oxidative burst in adherent conditions is in turn dependent on the spreading and reorganization of the cytoskeleton. The NADPH oxidase system is assembled at the sites of the polymerized cytoskeleton (39) and this assembly is required for the subsequent oxidative burst. Neutrophil adherence occurs independently from degranulation and membrane surface molecule up-regulation; however, inhibiting neutrophil adherence can prevent the other two processes from occurring (25,31). Since a number of receptor-mediated signal transduction pathways in the neutrophil appear to be dependent on β_2 integrins, especially Mac-1 (CD11b/CD18) (40,41), *in vitro* assays with adherent neutrophils may present a more physiological model when studying neutrophil function (31,37).

Specific neutrophil activation steps can be blocked with inhibitors, which gives insight into the pathways activated by different stimuli. For example, pertussis toxin

inhibits neutrophil activation resulting from the stimulation of G-protein linked receptors. Cytochalasin B blocks both the polymerization of actin filaments and up-regulation of the β_2 integrins without impairing adherence to a substrate. This agent also inhibits spreading and calcium fluxes, suggesting that cell movement may initiate increases in intracellular calcium (42). Wortmannin acts on myosin light chain kinase (MLCK) which, by phosphorylating the myosin light chain, initiates the interaction of myosin with actin. Wortmannin acts downstream from G-proteins, as this drug does not influence the calcium fluxes caused by G-protein linked receptor activation (43). Because cytochalasins, elevation of cytosolic cAMP, and wortmannin all inhibit both spreading and the respiratory burst, physiological stimulation of the neutrophil respiratory burst is thought to be adhesion-dependent (39).

β_2 Integrins

The three β_2 integrins consist of a common β subunit, CD18, and one of three α subunits: CD11a, CD11b, or CD11c (see Table 1.1). Molecular weights of the α subunits

Table 1.1 β_2 Integrins

Integrin	Integrin Names	CD Number	Ligands	Functions
$\alpha_L\beta_2$	LFA-1	CD11a/ CD18	ICAM-1; ICAM-2; ICAM-3	Initial rolling/tethering; Homotypic adhesion
$\alpha_M\beta_2$	Mac-1; CR3	CD11b/ CD18	ICAM-1;ICAM-2; Factor X; Fibrinogen; C3bi; LPS; β-glucan; elastase	Chemotaxis; Degranulation; Diapedesis; Oxidative burst; Phagocytosis;
$\alpha_X\beta_2$	P150/95; CR4	CD11c/ CD18	Fibrinogen; C3bi; LPS	Adherence

Table adapted from references (26, 83)

are 177 kDa (CD11a), 165 kDa (CD11b), and 150 kDa (CD11c) in human leukocytes. Linking of the α subunit with the β_2 subunit occurs in the Golgi apparatus, and the assembled receptors are then transported to the cell surface or to intracellular stores. The α subunit has a short cytoplasmic domain, while the β subunit has a highly conserved cysteine rich region, which gives it a rigid tertiary structure (44). The external portion also contains 5-6 N-glycosylation sites, which are linked with complex-type oligosaccharides (45) (see Figure 1.2). The primary sequences of the α and β subunit cytoplasmic domains of the β_2 integrins have been well conserved through evolution (46).

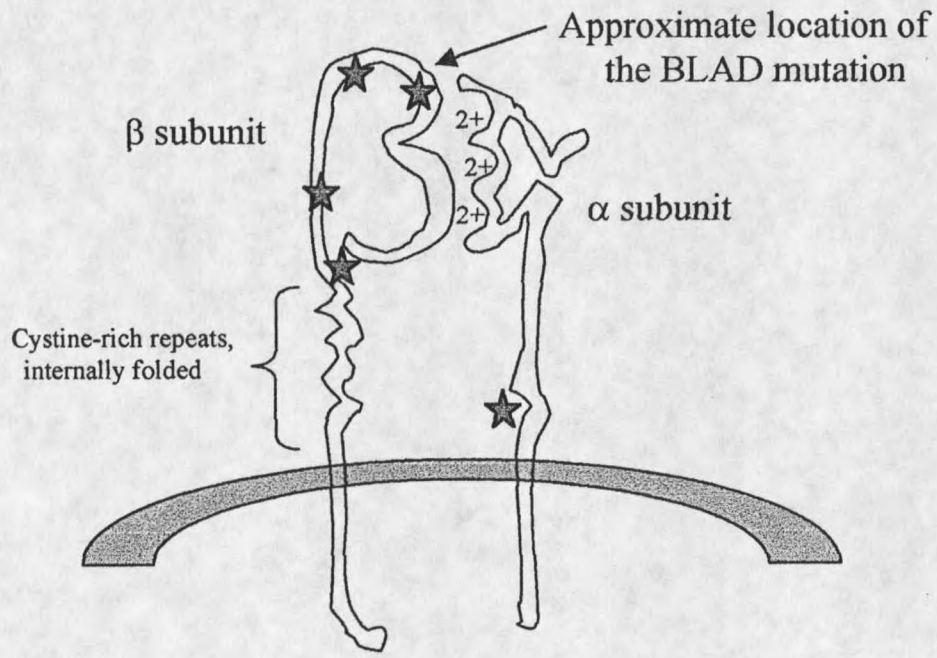

Figure 1.2 β_2 Integrin α and β Subunits. Structural features of the α and β subunits of the β_2 integrins. ★ Indicates disulfide bonds. 2+ indicates divalent cation-binding sites. The four cystine-rich repeat region in the β subunit also contains many disulfide bonds thought to give rigidity to this part of the subunit. (Figure compiled from references 112, 113)

Although the β_2 integrins are expressed exclusively on leukocytes, the various leukocyte subtypes express different combinations and percentages of the three β_2 integrins (2,15). On resting neutrophil surfaces, the relative expression of the β_2 integrin molecules is Mac-1>LFA-1>gp150/95 (2,10). Even though they are constitutively expressed, β_2 integrins on the neutrophil surface require cellular activation for function (10,26). Integrins change configuration in preparation for and in response to binding of activated endothelial ICAM ligands, thereby increasing affinity for the endothelium (12). CD11a/CD18 molecules are not up-regulated on the cell surface, whereas CD11b,c/CD18 can be up-regulated several fold from intracellular granules, causing a marked increase in adhesion (44). The precise series of intracellular events responsible for the changes in integrin affinity and the intracellular pathways mediating this process are unknown.

Mac-1 (CD11b/CD18)

The Mac-1 molecule is involved in neutrophil tight adhesion with ICAM-1 and ICAM-2 as described above, and it functions as a receptor for iC3b (complement fragment), coagulation factor X, fibrinogen (27,30,44), zymosan (β-glucan), *E. coli*, LPS, and *Leishmania* (27,44). Mac-1 is also required for neutrophil interaction/signaling with other molecules that do not directly bind to Mac-1, such as various ECM proteins (30), IgG (47), and urokinase (27). The Mac-1 molecule has been implicated in specific neutrophil functions such as LTB_4 generation (39) and apoptosis regulation (40). Human neutrophil activation directly through Mac-1 occurs concomitantly with an increase in intracellular calcium levels, activation of multiple protein kinases, alterations in lipid

composition of the plasma membrane, and rearrangement of the cytoskeleton (7,44,48).

The identification of specific Mac-1 ligands has led to specialized adherence assays. The β_2 integrins are the only known fibrinogen receptors on human neutrophils (28), yet the β_2 integrins do not directly bind certain plastics, glass, fibronectin, vitronectin, laminin, or immune complexes (12). Functional assays with adherent neutrophils were first performed on serum coated nylon fibers, and then on ECM-protein coated plates (30). When bound to solid-phase fibrinogen, thrombospondin, laminin, fibronectin, and vitronectin, adherent neutrophils secrete hydrogen peroxide in response to cytokines, whereas neutrophils in suspension do not. These interactions require expression of Mac-1 on the neutrophil (30). As mAb against the different integrins were produced, β_2 integrin-specific adherent neutrophil testing was performed by allowing the cells to spread on mAb attached to protein G-coated plates. More recently, these mAb have been used for specific pathway activation in cross-linking studies (49). Cross-linking human neutrophil LFA-1 (CD11a) or gp150/95 (CD11c) molecules with mAb bound to protein G-coated plates signals the activation of the neutrophil respiratory burst. Similar cross-linking using mAb specific to CD18 can also trigger the oxidative burst, but mAb to the specific α subunit of Mac-1 (CD11b) do not directly stimulate neutrophils (28,50-52). These specific signaling properties are believed to be due to the structure of the β_2 integrin cytoplasmic tails. The cytoplasmic tails of CD11a, CD11b and CD11c are 53, 19, and 29 amino acids, respectively (50), while CD18 has a cytoplasmic tail of 46 amino acids (53).

CD11b exhibits at least two functional binding sites, one for iC3b and RGD-

containing proteins, and another for carbohydrates and LPS (54). Blocking the Mac-1 receptor on neutrophils with mAb prevents spreading and/or chemotaxis on a variety of substrates. Adhesion mediated by Mac-1 is transient, allowing for the cycles of adhesion and detachment that are necessary for cell locomotion and transmigration (42). Anti-Mac-1 mAb blocks spreading and locomotion by preventing the adherence of pseudopods to the substrate, but does not block polarization, degranulation, or actin polymerization in response to chemoattractants (12).

Bovine Leukocyte Adhesion Deficiency (BLAD)

Leukocyte adhesion deficiency (LAD) was first described in humans in the early 1980's. This disease includes genetic abnormalities in the molecular structure of the β_2 integrins. A small molecular defect in one of the α subunits can cause little change in leukocyte function whereas certain genetic changes in the β subunit can render this molecule completely nonfunctional, thus blocking the joining of the two subunits. The latter produces the most severe of the human LAD phenotypes, resulting in the absence of all β_2 integrin expression on the leukocyte surface. In humans, various genetic mutations in the different β_2 integrin subunits results in a wide range of pathology and symptoms (13,15,17).

In the late 1980's, it was discovered that the Holstein cattle industry had inbred a β_2 integrin genetic defect into the breeding bull population. Low leukocyte counts in milk production was targeted as a good breeding characteristic without the realization that this was caused by a genetic alteration limiting leukocyte transmigration from the

blood stream into the tissues. In the Holstein cattle disease, there is a single point mutation, a substitution of guanine to adenine at position 383 in the cDNA of the CD18 gene, which results in the substitution of aspartic acid to glycine at amino acid #128 in CD18 (D128G) (3,17) (see Figure 1.3). Although the mutated mRNA of the CD18 gene is expressed in the bovine neutrophil, no form of the protein has been found in the cells (20). Unlike the human disease where different mutations result in various levels of surface β_2 integrin expression, the inbred mutation in BLAD results in less than 1% of β_2 integrin surface expression. Since this mutation matched the genetic description of human LAD, the disease was named Bovine Leukocyte Adhesion Deficiency or BLAD. There is also a similar disease reported in dogs as a result of inbreeding (22).

Neutrophils constitute about 60 to 75% of the blood leukocytes in most carnivores (55) and 50 to 75% in adult humans (5). Neutrophils constitute about 20 to 30% of the total circulating leukocytes in ruminants, such as cattle, and in laboratory rodents (55). The general pathology of BLAD cattle mimics the severe human disease with the most striking characteristic being pronounced neutrophilia ($>3 \times 10^4$ /μl, Normal = 1.3-2.9 x 10^3/μl) (16). The high number of circulating neutrophils may result from a higher production in the bone marrow due to inflammation, from prevention of tissue migration, or from a longer life span. Anemia is a feature that is also reported in dogs and humans with LAD and is thought to be a consequence of reduced erythropoiesis due to hyperplastic granulopoiesis (19,56). In skin biopsies of one BLAD calf, the eosinophils, in contrast to neutrophils, remained capable of leaving the blood stream. This is consistent with findings in human LAD, indicating that adhesion molecules other than β_2