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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APPROVAL

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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.

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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

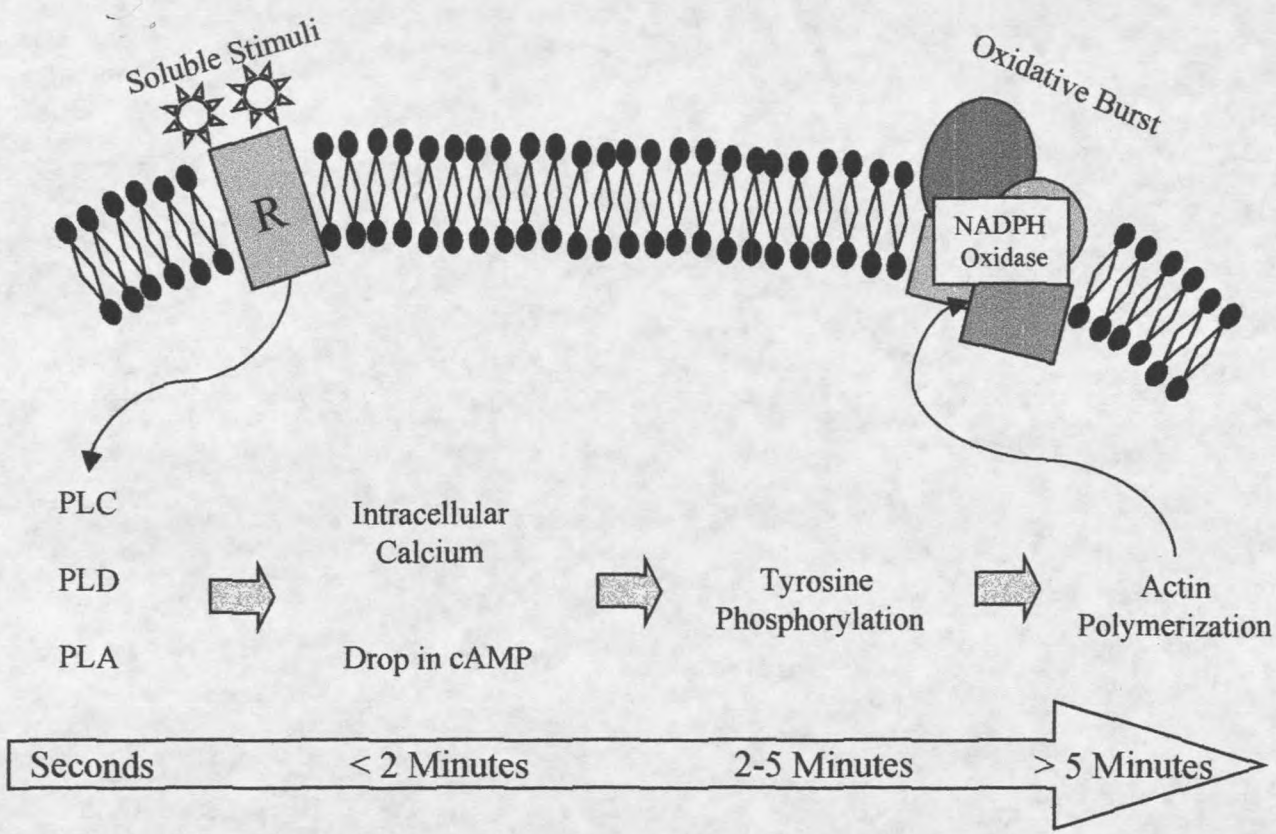

Figure 1.1 Neutrophil Activation. Soluble stimuli can quickly activate neutrophils. Signaling through membrane receptors on adhered neutrophils can result in an oxidative burst within 10 minutes. Different stimuli and various adherent or non-adherent assays can slightly decrease or greatly delay the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

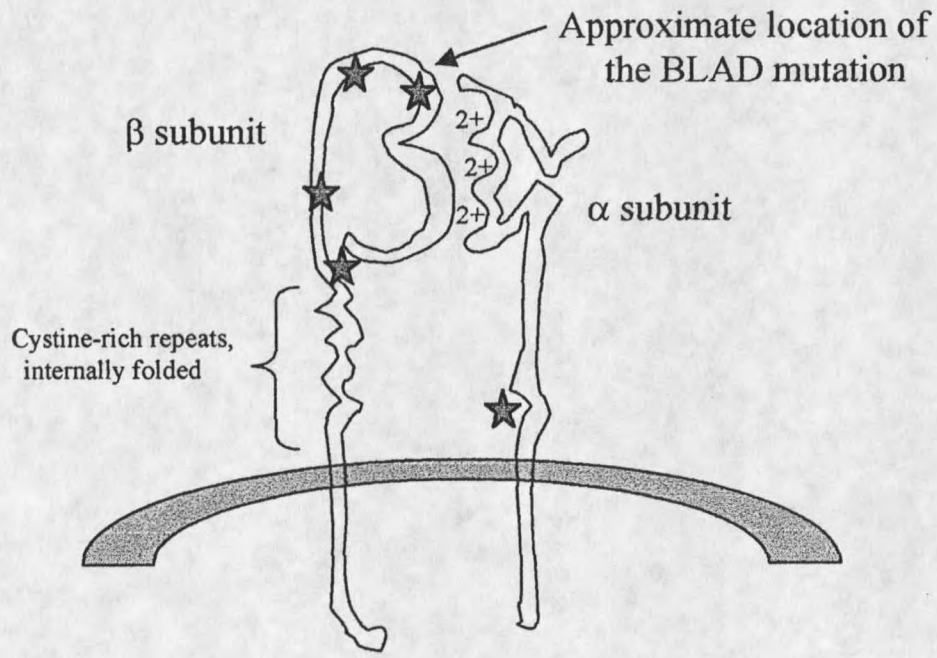

Figure 1.2 β_2 Integrin α and β Subunits. Structural features of the α and β subunits of the β_2 integrins. ★ Indicates disulfide bonds. 2+ indicates divalent cation-binding sites. The four cystine-rich repeat region in the β subunit also contains many disulfide bonds thought to give rigidity to this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

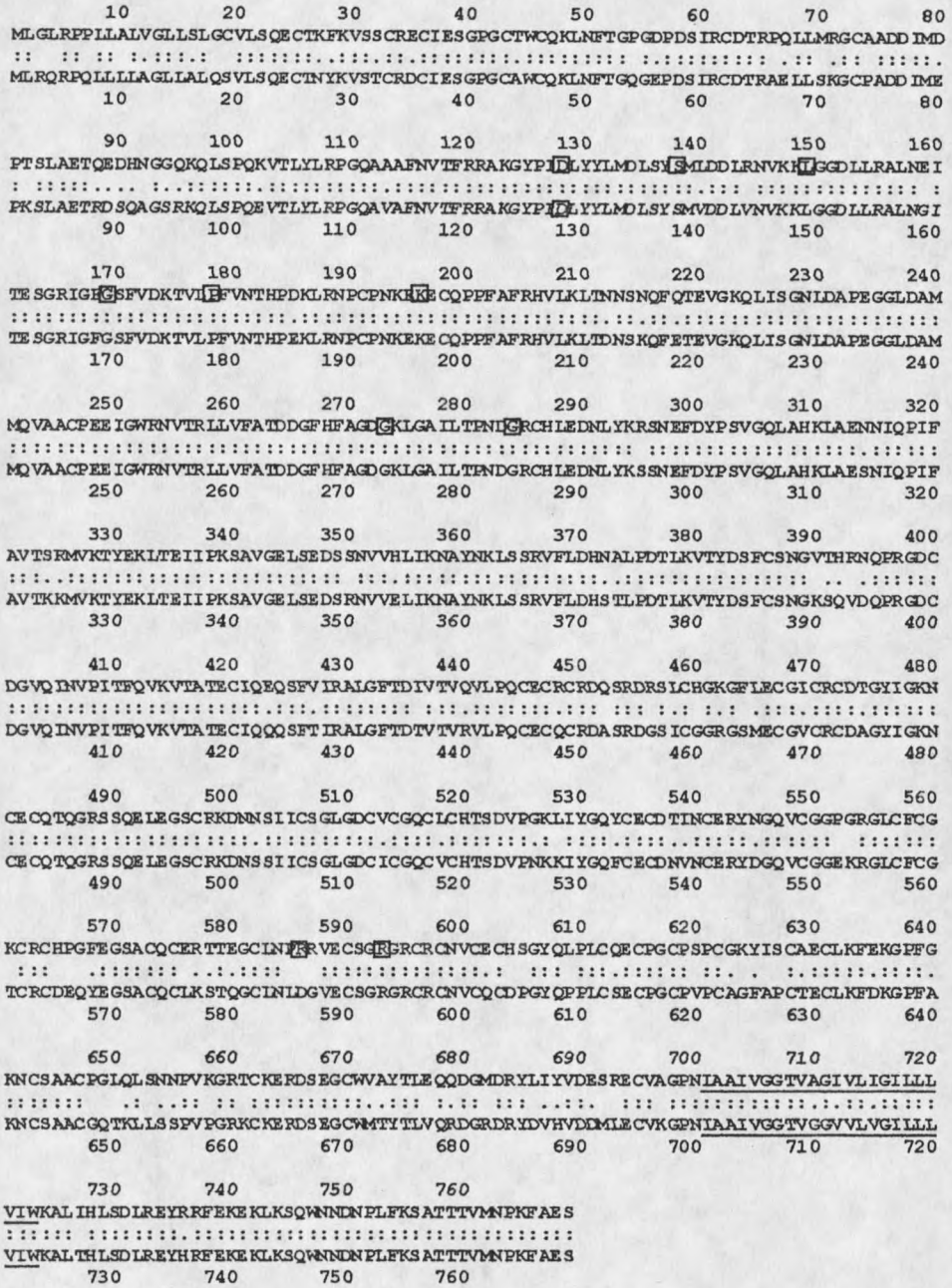

Figure 1.3 Amino Acid Sequence of Human and Bovine CD18. Alignment of human (top) and bovine (bottom) CD18 amino acid sequences. Aspartic acid #128 in bovine (boxed D) is the site of mutation in BLAD. Boxed amino acids in the human sequence depict known mutations in LAD. Underlined amino acids #701-723 depict the transmembrane region. There is 83.4% identity in the complete protein between human and bovine with 97.9% identity in the cytoplasmic tails.

integrins permit the migration of eosinophils into tissues (56). Also, homozygous BLAD cattle that are supported by long term antibacterial therapy are able to respond to virus infections. These observations and mononuclear cell functional assays seem to indicate that, in general, affected animals have no obvious defects of lymphocyte (T-cell or B-cell) function including their ability to transmigrate out of the peripheral blood (18,56).

During the same time that the human LAD and the bovine BLAD diseases were being defined, the comparison of adherent verses non-adherent human neutrophil functional assays was just beginning. At first, researchers reported that the oxidative burst in response to PMA or *f*MLP stimulation occurred but was "quantitatively variable and without a consistent pattern of response" in adhered human LAD neutrophils (57). Attempted stimulation of human LAD neutrophils with Mac-1 dependent protocols produced decreased oxidase production. These results with human neutrophils can be attributed to the various forms of the genetic defects in the human disease, as well as the inconsistencies in the various stimuli and assay methods used in the different studies (58). Similarly, researchers reported diminished hydrogen peroxide generation by BLAD neutrophils stimulated by opsonized zymosan (opZ), yeast (16,17), and PMA (17,59). The BLAD neutrophil should not be expected to produce an oxidative burst when stimulated with opZ or phagocytosed yeast because the receptors for these stimuli are Mac-1-dependent (22). The earlier studies on the BLAD neutrophil oxidative burst using PMA as a stimulus employed non-adherent assays and external superoxide measurements (17). Since then, researchers report that there appears to be no general defect in PKC-dependent responses in human LAD neutrophils (57). Other functions of the human

LAD neutrophils that are now listed as normal include much of the FcR signaling, superoxide production, and initial increases in calcium and plastin phosphorylation (12). Based on these observations, it appears that the PKC-dependent portion of the oxidative burst pathway may also be intact in the BLAD neutrophil (60).

Neutrophils can be stimulated with receptor-specific antagonists, and subsequent activation steps can be measured to determine if the pathway is altered by the absence of β_2 integrins. Not only can ligation of the β_2 integrins (CD11a,c and CD18) directly activate neutrophils, but also each neutrophil surface receptor/receptor class can be divided into groups based on whether the signal transduction appears to require the β_2 integrin molecules. The neutrophil surface receptors included in this distinction are: complement receptors, immune complex receptors, GPI-linked receptors, G-protein receptors, PAF receptors, TNF receptors, CSF receptors, LTB_4 receptors, and apoptosis receptors.

Mac-1 as a Direct Receptor

Complement Receptor

There are four known complement fragment receptors on human neutrophils. Complement receptor 1 (CR1, CD35) binds C3b and C4b with low affinity (2,61), complement receptor 3 (CR3, Mac-1) binds iC3b (inactivated C3b) and C1q (38), complement 3a receptor (C3aR) binds C3a and C4a with low affinity, and complement 5a receptor (C5aR) binds C5a (2). Research now suggests that CR3 might act as an element of the ingestion process rather than a traditional receptor for complement (38,50,62).

CR3 mediates the direct binding of iC3b-coated particles without triggering the oxidative burst (50). CR3 also directly binds to C1q, but it has been reported that C1q is associated with a collagen like region (CLR) and the complete protein C1q-CLR is large enough (180 kD) to bind two receptors to produce a superoxide response without the need for another stimulus (38).

Neutrophils from LAD patients do not adhere to or produce superoxide in response to C1q-CLR (38), indicating that this process may be dependent on the Mac-1 molecule. Anti-CR3 mAb blocks the phagocytosis of C4b-opsonized erythrocytes but doesn't stop the binding of these erythrocytes to the cells (38). Neutrophil initial adhesion and spreading on C3b-coated surfaces are the same in normal, anti-CD18 treated, and LAD neutrophils, but both the β_2 integrin-deficient neutrophils and neutrophils treated with anti-CD18 subsequently detach. It was reasoned that these cells were unable to progress through the signaling pathway of CR1 to actin reorganization without co-stimulation of Mac-1 (47). This again suggests that CR1 primarily promotes the adhesion of particles and that CR3 mediates the subsequent engulfment (62).

Cross-linking surface-bound mAb to CD18, and CD35 (CR1) activates the neutrophil through the PLD pathway and appears to follow the same pathway as when neutrophils phagocytose complement-opsonized particles (39,62). Neutrophils incubated with opZ in suspension exhibited superoxide production only where the plasma membrane contacted particles as well as in the phagosomes. For example, neutrophils adherent to glass coverslips showed diformazan production around the cell bodies. Attached cells that ingested opZ also showed diformazan production in the phagosomes.

The peripheral reaction must be independent of phagocytosis, and neutrophils in suspension may reflect superoxide production relating specifically to phagocytosis (50,63), yet both reactions may be Mac-1 dependent.

In the bovine system, complement receptor 1 (CR1) can recognize and bind to C3b, and un-opsonized zymosan is bound to CR3. Zymosan particles are capable of binding to bovine neutrophils at FcR, CR1, and CR3 receptor sites, depending on the opsonization procedure used, but research in this area is hindered by the lack of blocking mAb that work in the bovine system (64).

Mac-1 Dependent Receptors

FcR, uPAR and LPS Receptor

Interactions of IgG with neutrophils induce a wide variety of responses, including phagocytosis, generation of the oxidative burst, and the release of lysosomal enzymes. All of the responses are initiated through the binding of the Fc domain of IgG to its specific receptor, IgG Fc receptor (FcγR) (65). Freshly isolated human neutrophils express mainly FcγRII (CD32) and FcγRIII (CD16). Exposure to interferon-γ, G-CSF, or GM-CSF results in the additional expression of FcγRI (CD64). FcγRI has a high affinity for IgG and is the only Fc receptor to bind monomeric IgG (53). FcγRI and FcγRII are believed to promote phagocytosis whereas FcγRIII promotes binding (61). FcγRI and FcγRII are transmembrane receptors with cytoplasmic domains of 44 to 76 amino acids, respectively, and both have been shown to mediate intracellular signal transduction efficiently. FcγRIII is coupled to the plasma membrane via a

glycosylphosphatidylinositol moiety (GPI-linked) in human neutrophils but is a transmembrane molecule in murine neutrophils (53) and also appears to be a transmembrane molecule in bovine neutrophils (sequence personally compared from data retrieved from Genebank). The GPI-linked form of FcγRIII in human neutrophils may lack signal transduction ability on its own (66).

A FcγR co-localizes with Mac-1 in the plasma membrane upon human neutrophil activation (12). Several research groups conclude that it is specifically the GPI-linked FcγRIII and the GPI-linked urokinase plasminogen activator receptor (uPAR) that co-localizes with the Mac-1 molecule upon activation (12,27,38,59). Disruption of the Mac-1 co-localization with FcγRIII inhibits the ability of human neutrophils to mobilize intracellular calcium and produce superoxide in response to immune complexes (38). LAD neutrophils, BLAD neutrophils, or neutrophils treated with anti-β_2 integrin mAb show decreased IgG Fc receptor mediated phagocytosis, decreased immune complex-stimulated LTB_4 production, and decreased adhesion to immune complex-coated surfaces with decreased spreading (12,21,23). Although the phagocytosis of IgG-opsonized particles by activated neutrophils requires β_2 integrins, binding of the IgG-opsonized particles occurs normally in the absence of these integrins. This again suggests a role for Mac-1 in ingestion of IgG-opsonized particles at some step subsequent to particle binding (23,38,47).

Human LAD neutrophils do not show a calcium response when treated with urokinase plasminogen activator (uPA). Neutrophils incubated with anti-Mac-1 $F(ab')_2$ fragments do not respond to uPA. When placed individually or together in the U937 cell

line (myelomonocytic), only transfected cells expressing both Mac-1 and the uPA receptor (uPAR) exhibit a calcium response to uPA. Because it has been shown that uPAR and FcγRIII can be physically associated with Mac-1, it is reasoned that Mac-1 might act as a transmembrane signaling conduit for uPAR as well as for FcγRIII (27). In the U937 transfected cells, uPA triggers tyrosine phosphorylation of a 38kD protein (27). Cross-linking Mac-1 with either FcγRII or FcγRIII leads to the phosphorylation of tyrosines in the FcγRII cytoplasmic tail. This, in turn, leads to the formation of an immune tyrosine activation motif (ITAM), which can serve as a docking site for other kinases involved in signal transduction (12). It has been suggested that engaging FcγRII can also result in the phosphorylation of PLC by the protein tyrosine kinase $p55^{fgr}$ (12,39,65). Other researchers claim that FcR, like the complement receptors, do not use PLC/IP_3 and instead use the same PLD pathway as complement induced phagocytosis (62). This latter pathway may be the one signaled with the FcγRIII/Mac-1 dependent signaling.

Another GPI-linked receptor on neutrophils is the bacterial lipopolysaccharide (LPS) receptor (CD14) (38). The Mac-1 molecule binds to certain regions of LPS (2). One study suggests that Mac-1 appears to be involved in the redistribution of the LPS receptor, although Mac-1 does not co-cap with CD14 on the surface of LPS activated neutrophils (67). Much of the LPS receptor signaling appears to be independent of Mac-1 as shown by the LPS activation of CHO cells and 70Z/3 cells (pre B-cells) transfected with only the LPS receptor. The absence of the Mac-1 molecule on these cells did not interfere with the activation pathway, as measured by adherence in CHO cells and IgM

production in 70Z/3 cells (68).

Chemoattractant Receptors

Chemoattractants bind to leukocyte receptors that span the membrane seven times (serpentine receptors). These receptors couple to G-proteins, which signal a variety of messenger molecules. In turn, these messenger molecules induce a conformational change in the integrins, which increases their binding affinity. The serpentine receptors play a dual role, directing cellular migration and regulating integrin binding (12,69). The leukocyte chemoattractants can be divided into three basic types (12):

- Classical - act broadly on several cell types including neutrophils and monocytes. They include N-formyl peptides, C5a, LTB_4 and PAF (a product of phosphatidylcholine metabolism).
- C-X-C chemokines (chemoattractant cytokines) - act on leukocytes and fibroblasts involved in wound healing. They include α chemokines like IL-8.
- C-C (β) chemokines - act on monocytes, lymphocyte subpopulations and eosinophils. These include monocyte chemoattractant protein (MCP-1), monocyte inflammatory protein-1 (MIP-1) α and β, and RANTES.

When human neutrophils are attached to BSA-coated nylon fibers, adherence itself does not elicit an oxidative burst. When stimulated with *f*MLP, C5a, PAF, TNF-α and GM-CSF, these adherent neutrophils do produce superoxide (70,71). This adherence is a β_2 integrin-dependent priming effect and is cytochalasin-independent (11,49,72). The

same β_2 integrin-dependent priming and subsequent enhanced oxidative burst to chemoattractants can be induced by cross-linking surface bound anti-β_2 integrin mAb (73). Stimulation of neutrophils by *f*MLP induces the hydrolysis of phosphatidylinositol biphosphate (PIP_2), leading to the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG), which is a PLC signaling event (70). Once chemoattractants signal neutrophil activation through G-proteins, the up-regulation of CD18 is not reversible (12), further implicating the Mac-1 molecule in G-protein signaling.

TNF, CSF and Fas

There are two human neutrophil TNF receptors, p55 and p75. The extracellular portions of these receptors are homologous to p75 belonging to the nerve growth factor (NGF) receptor family and the Fas antigen. These proteins are not homologous in their intracellular domains (28). TNF stimulation of human neutrophils is Mac-1 dependent, as reported with both plate-adherent (28,30) and cross-linking studies (73). Adhered human neutrophils respond to TNF by releasing their granules, reorganizing their cytoskeleton, and producing a delayed oxidative burst. Like TNF, G-CSF and GM-CSF can induce an oxidative burst in adhered neutrophils after at least 60 minutes (74). Neutrophils adhered to serum coated plates and activated with TNF-α produce an oxidative burst that is insensitive to PTX (G protein inhibitor) and staurosporine (PKC inhibitor). This oxidative burst takes place without the formation of inositol phosphates, phosphatidic acid, or arachidonic acid, suggesting that it is independent of G-protein activation and the subsequent pathways through PLC, PLA, and PLD (29,43).

activation and the subsequent pathways through PLC, PLA, and PLD (29,43). Superoxide production in neutrophils that are adhered to fibrinogen coated plates and activated with TNF-α, and neutrophils that are adhered to anti-β_2 integrin-coated plates can be inhibited by Wortmannin (myosin light chain inhibitor) and three different inhibitors of protein tyrosine kinases (43). In response to TNF, neutrophils adhered to protein-coated surfaces, but not suspended neutrophils, induce tyrosine phosphorylation of several proteins (28,73). Tyrosine phosphorylation is evident 5 minutes after the addition of TNF and lasts at least two hours. The tyrosine kinase inhibitors K252a, genistein, and ST638 suppress tyrosine phosphorylation and block TNF-induced hydrogen peroxide production in a reversible manner at low concentrations (28). Additionally, the monocyte receptor for CSF-1 is actually a transmembrane tyrosine kinase capable of auto-phosphorylation (75).

The Mac-1-dependent activation of neutrophils by TNF promotes a fall in cAMP, which is closely related to cell spreading and actin reorganization (76). Pretreatment of neutrophils with cytochalasin-B severely decreases the cell's response to TNF-α without affecting the priming for the *f*MLP response by TNF-α (77). Thus, hydrogen peroxide secretion from TNF-α treated neutrophils appears to be a direct, but delayed response, that requires assembly of microfilaments (78). TNF-α-pretreated neutrophils show depressed locomotion that may be related to TNF-induced increased adhesiveness of the neutrophils to the substrate via the Mac-1 molecule (77). Cellular focal adhesions are the predominant location for tyrosine phosphoproteins in TNF-treated neutrophils, and they may also be the sites for the accumulation of the β_2 integrins, the TNF receptors, and/or

the cytoskeletal-associated components of the oxidative burst. Inhibiting spreading by blocking actin polymerization with cytochalasin-B leads to rapid de-phosphorylation of previously tyrosine-phosphorylated proteins. This suggests that tyrosine phosphoproteins may be protected from de-phosphorylation when associated with the cytoskeleton (28).

Fas (APO-1, CD95) is a gene whose product is a membrane spanning protein homologous to TNF and NGF receptors. Cross-linking by antibody to Fas can induce apoptosis. This requires a cytoplasmic death domain that activates a family of caspases (cysteine proteases), which signal apoptosis (79). Apoptosis is a biochemical sequence of events evident in various cell types when cell death is physiologically determined. Apoptosis has been described in the death of cells with short half lives like neutrophils; the elimination of self-reactive T-cells; involution of cells deprived of necessary growth factors; morphogenetic death of cells during embryonic development; and killing of cells which serve as targets for T-cells, natural killer cells, or antibody-dependent cell mediated cytotoxic mechanisms (80). Adhesive interactions between cells and ECM proteins play a vital role in embryonic morphogenesis. Many of the interactions between cells and the ECM are mediated by the integrin family; however, the precise mechanism of signals from ECM proteins via integrins to the intracellular machinery that controls cell growth, behavior, and differentiation remains to be defined (81). The pathway of apoptosis is different in various cell types, but the mechanism of death itself may always be the same in a final common pathway (80).

An important feature of regulated apoptosis is removal of apoptotic cells and factors involved in the resolution of inflammation. One component of inflammation

resolution is the clearance of neutrophils and their potentially cytotoxic contents. The large daily production of neutrophils needs to be balanced by a systematic elimination process whether there is an inflammatory-induced neutrophilia or not. In neutrophil turnover, it seems as if the cell has an autonomous internal clock controlling its death program (40). Several activation-dependent neutrophil functions lead to changes in second messenger systems that are associated with induction of apoptosis. These include transient elevations in cytosolic free calcium, superoxide generation, and protein tyrosine phosphorylation (40). Various cytokines and chemotactic and priming agents present during inflammation prolong neutrophil survival. This is observed with GM-CSF, but, in contrast, TNF-α accelerates apoptosis in human neutrophils (82). The engagement of Mac-1 by transmigration through activated endothelium induces apoptosis in human neutrophils. Likewise, the cross-linking of the Mac-1 molecule with mAb enhances neutrophil apoptosis in response to TNF-α (82). The link of CD11b/CD18-dependent phagocytosis with induced apoptosis provides a counter-regulatory mechanism to accelerate apoptosis in the inflamed tissue under external conditions that nurture neutrophil survival. Such a mechanism eliminates phagocytic cells that have reached the end of their useful life span in inflamed tissues, thus limiting the effects from the toxic contents of neutrophils (40). These Mac-1-dependent enhanced apoptotic processes can be blocked with tyrosine kinase inhibitors (82).

β_2 Integrin Cell Signaling

Quantitative β_2 Integrin Up-regulation

Even though they are constitutively expressed, β_2 integrins on the neutrophil surface require cellular activation for function (10,26,83). In resting neutrophils, integrins are thought to be in a conformational-inactive state. This inactive state can be recognized by certain mAbs, but the integrins are unable to bind their ligands (26). Upon cell stimulation, the integrins are primed and they exhibit increased ligand avidity. This increased avidity can be measured with specific mAb that only recognize a site on the activated integrin (26,84). Integrins change configuration in preparation for and in response to binding to activated endothelial ligands, thereby increasing affinity for the endothelium (12). Quantitative up-regulation can also come from intracellular release of granules. CD11a/CD18 molecules are not up-regulated, whereas both CD11b/CD18 and CD11c/CD18 can be up-regulated several fold from intracellular granules, causing a marked increase in adhesion (44,85). The precise series of intracellular events responsible for the changes in integrin affinity and the intracellular mediators of this process are unknown.

Outside-In Signaling (Direct)

Direct binding of the β_2 integrins with their ligands induces outside-in signaling (see Figure 1.4), and different ligands can induce different signals. For example, Mac-1 binding to ICAM signals diapedesis, yet Mac-1 binding to opsonized particles signals phagocytosis (26). Specific peptide sequences are thought to regulate this process

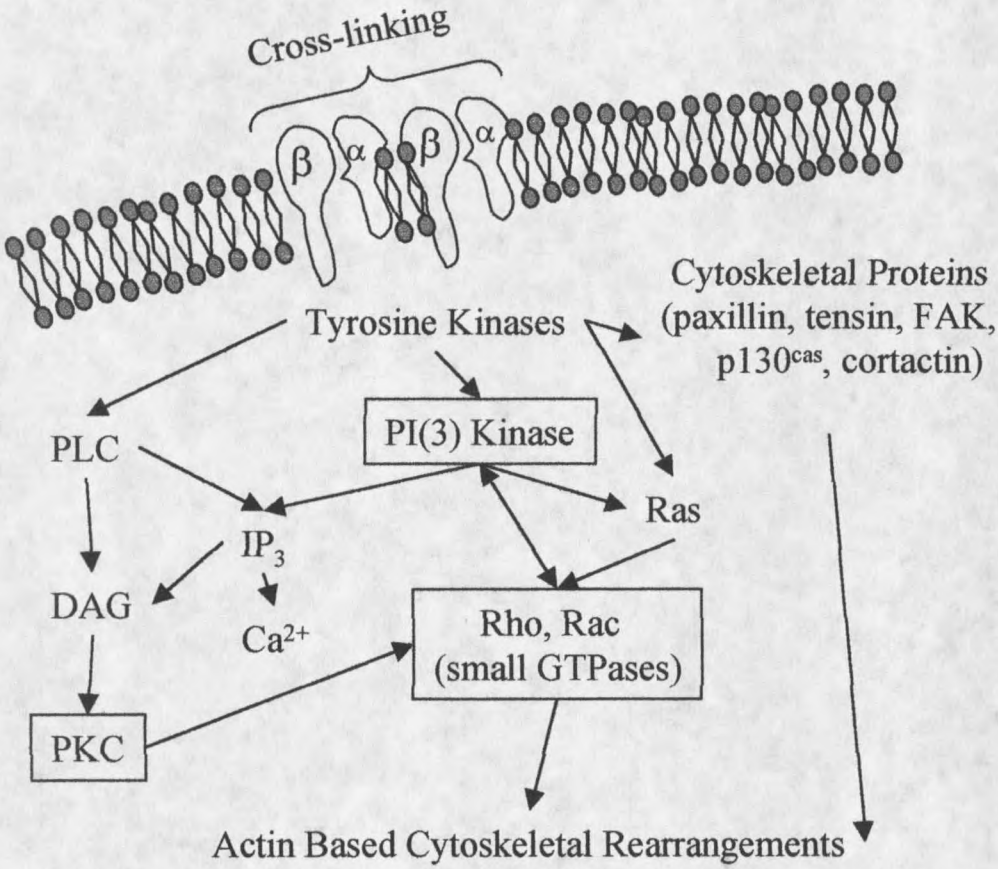

Figure 1.4 Outside-in Signaling through the β_2 Integrins. Putative signal-transduction pathways initiated by β_2 integrin cross-linking in neutrophils. (Figure compiled from references 26, 48, 83, 89)

through various binding sites in the integrins' I-domain (26). Large ligand molecules can possess more than one binding site. Collagen has a RGD sequence that is important for β_2 integrin adhesion as well as a DGGRYY sequence that initiates neutrophil degranulation and the oxidative burst (51). Stimulation of the oxidative burst through ligation of CD11/CD18 is independent on pertussis toxin-sensitive G-proteins but, is inhibited by tyrosine kinase inhibitors (26,84).

Inside-Out Signaling (Indirect)

When receptors other than the β_2 integrins are engaged, intracellular signals can prime the integrins. This is referred to as inside-out signaling (see Figure 1.5). It has been suggested that the small molecular weight G-protein, Rho, is the common protein in a number of pathways leading to this type of integrin priming (86-89). Upon stimulation, chemoattractant receptors (G-protein-coupled, seven-transmembrane) inhibit adenyl cyclase activity, thus lowering cAMP (26). Protein kinase A (PKA) activity is dependent on cAMP levels. Lowering the cellular cAMP level releases PKA from blocking guanine nucleotide exchange on Rho (86). Engagement of the TNF receptor also lowers intracellular cAMP levels. The rapid adhesion caused by the cAMP/PKA pathway may be a transient adhesion mechanism for chemotaxis (26). Chemoattractant receptors can also activate PKC (diacylglycerol-independent form) through phosphatidylinositol 3-kinase (PI-3 kinase). PKC then activates Rho (26). A slower, more sustained activation of the β_2 integrins is maintained as Rho completes the cycle through its ability to activate PI-3 kinase (26). Wortmannin, an inhibitor of PI-3 kinase, can block the integrin-mediated responses to chemoattractants and TNF (26,43).

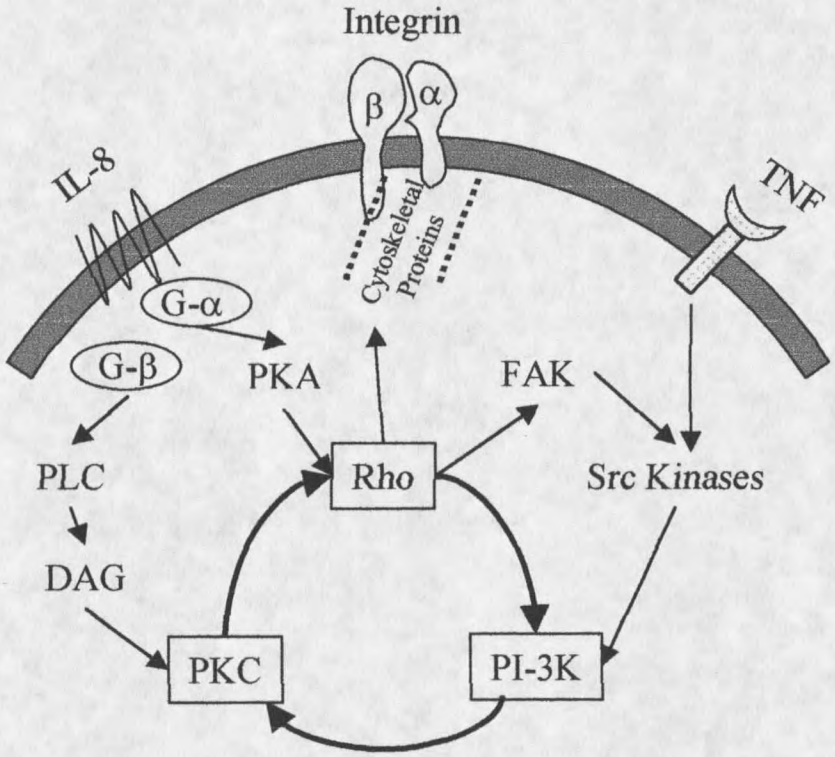

Figure 1.5 Inside-out Signaling to the β_2 Integrins. Putative signal-transduction pathways initiated by chemokines (example IL-8) or Mac-1 associated receptors (example TNF) in neutrophils. Final common pathway is through Rho GTPase which culminates in cytoskeletal rearrangement. (Figure compiled from references 26, 83, 89)

Indirect signaling through the β_2 integrins involves tyrosine kinase activities and the polymerization of actin and other cytoskeletal proteins, which eventually leads to the assembly of NADPH oxidase components in an active form (26,43,90). PI-3 kinase is activated by association with tyrosine phosphorylated proteins (26,91), and in turn PI-3 kinase activation leads to the phosphorylation of L-plastin. L-plastin is involved in the bundling of actin filaments and in the up-regulation of integrin avidity (26,88,89). The β_2 integrin cytoplasmic tail associates with α-actinin in stimulated human neutrophils (88,92), providing an area of focal contact with the cell membrane and an anchor for actin filaments to allow cell spreading and migration (26). Actin-binding proteins, such as vinculin, talin and paxillin, join the actin cytoskeleton and the integrin/receptor cytoplasmic tail (26,88) (Figure 1.6). Since protein tyrosine kinase inhibitors block both the spreading and respiratory burst in human neutrophils (39) and LAD neutrophils are deficient in their ability to spread (14,17), it appears that polymerization of the cytoskeleton is downstream to the activation of tyrosine kinases (39). It has been suggested that the α-actinin/CD18 complex stimulates a serine/threonine protein kinase, and this acts on tyrosine kinases later in the integrin-signaling cascade (26,93).

Focal Adhesion Kinase (FAK)

Transmembrane proteins, including cytokine receptors, integrins, and GPI-linked proteins, associate with the focal adhesion kinase (FAK) and with one or more of the Src family kinases (26,54,88). Clustering of these receptors, triggered by their ligands, stimulates tyrosine phosphorylation by their linked kinases (54). Src-related tyrosine

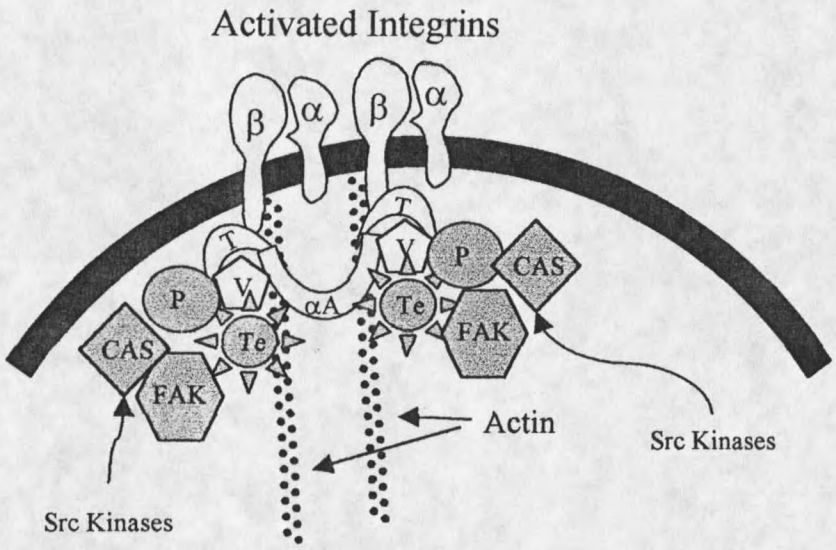

Figure 1.6 β_2 Integrin Association with Cytoskeletal Proteins. A model showing signaling molecules involved in anchoring the actin filaments with activated β_2 integrins. T = talin, αA = α-actinin, V = vinculin, P = paxillin, Te = tensin, CAS = $p130^{cas}$, FAK = focal adhesion kinase. Shading indicates molecules that exhibit tyrosine phosphorylation upon activation. (Figure compiled from references 26, 48, 88)

kinases do not possess extra-cellular or transmembrane sequences, and they are believed to play roles in signal transduction by making complexes with other molecules localizing on the cell surface at the focal contacts (26,81,94). Kinase activity of members of the Src family can be regulated by de-phosphorylation of critical phosphotyrosine residues at the COOH-terminus or by tyrosine phosphorylation within the kinase domain. Either activation of a tyrosine phosphatase acting on the COOH-terminal phosphotyrosine or of a tyrosine kinase causing hyper-phosphorylation of the tyrosine within the kinase domain can occur (39). PI-3 kinase appears to be involved downstream from the Src family kinases, because wortmannin will block neutrophil adhesion and oxidative burst in the

presence of the phosphoproteins (26).

Clustering of integrins causes enhanced tyrosine phosphorylation of the tyrosine kinase $p125^{fak}$ in at least three different cell types (26,88,95). This cytoplasmic kinase lacks a transmembrane domain and an obvious site for lipid anchors and appears to be a substrate for the Src-kinases. This kinase also lacks the SH2 and SH3 domains that are usually found in cytoplasmic tyrosine kinases (81,95). After FAK-dependent phosphorylation, activated paxillin binds several signaling molecules in addition to the scaffold molecules of the cytoskeleton. This anchoring/signaling mechanism is diagramed in Figure 1.6. Immune complex-induced paxillin tyrosine phosphorylation is absent in LAD PMN and, therefore, points to a role for Mac-1 in this signaling process (12).

Fgr Proteins (Src-family Kinases)

Protein-tyrosine kinases associate with surface receptors that lack an intracellular catalytic domain. Fgr, which is specifically expressed in granulocytes, monocytes, and natural killer cells (28) is associated with FcγRII and is involved in FcγRII-mediated signal transduction pathways in human neutrophils (39,65). Both $p58^{fgr}$ and $p55c^{fgr}$ are associated with the plasma membrane, and during release of secondary granules, are translocated from a granule location to the plasma membrane (39,96,97). During direct β_2 integrin signaling, $p58^{fgr}$ is activated in the absence of kinase activity directly associated with the β_2 integrin molecules in immunoprecipitates from Triton-X 100 or NP-40 human neutrophil lysates (39). The mechanism of $p58^{fgr}$ activation by β_2 integrins

in neutrophils appears to be more complex than that identified with members of the Src family, which are associated with the cytoplasmic tail of FcγRII (39). Other members of the Src-kinase family that have been linked to Mac-1-dependent TNF stimulation of human neutrophils include $p53/56^{lyn}$ and $p59/61^{hck}$ (82).

Degranulation

Activated neutrophils release intracellular granules in a sequential order. Upon human neutrophil activation, secretory and specific granules, followed shortly by azurophil granules, exocytose to the membrane surface or empty their contents into the phagocytic vacuole (9). Secretory granules, as seen in human neutrophils, have not been described in bovine neutrophils. Yet, the accepted marker for this granule, alkaline phosphatase, exists in relatively high amounts in bovine neutrophil plasma membrane preparations (98,99). The total alkaline phosphatase content of bovine neutrophils is 50-55% higher than unstimulated cell surface alkaline phosphatase activity, indicating latent, potentially granular alkaline phosphatase. This latent alkaline phosphatase and its recruitment during stimulation by degranulating agents is commonly used as a marker for secretory granules in human neutrophils (100,101).

Lactoferrin release is a common marker for specific granule degranulation in human and bovine neutrophils (31,102-104). The amount of membrane-associated lactoferrin increases with the activation by PMA, fMLP, and TNF in human neutrophils (105,106) and by PMA and PAF in bovine neutrophils (104). In bovine neutrophils activated with various doses of PAF, secretory granules degranulate at low

concentrations, specific granules degranulate at moderate concentrations, and high concentrations cause azurophil degranulation (104). This supports the idea that azurophil granules are specialized lysosomal structures, which do not participate in secretion but are involved with digestion of phagocytosed material (104). In stimulated neutrophils, different signaling pathways may cause release of different granules depending on the desired neutrophil response

L-selectin Shedding

L-selectin is a surface molecule that is constitutively expressed on leukocytes. Cell-specific priming signals transiently increase the expression of L-selectin on the cell surface (104,107). L-selectin is rapidly removed from the cell surface upon neutrophil activation (108), and L-selectin shedding is regarded as an important measurable consequence of leukocyte activation and adhesion. Emigrated neutrophils reportedly express little if any L-selectin (109,110). It has been shown that low doses of PAF or β_2 integrin mAb cross-linking results in up-regulation of Mac-1 without any detection of L-selectin shedding in bovine neutrophils (84,111). This suggests a more complex regulation of L-selectin shedding as compared to degranulation (84).

Summary

The β_2 integrins are essential for the neutrophils' transmigration from the blood stream into the tissues, phagocytosis, and associated receptor functions. A single genetic mutation in the β subunit of the β_2 integrins can result in an absence of the β_2 integrins on

leukocyte cell surfaces. Additionally, because of the evolutionary conserved sequences in the integrin family as a whole, a better understanding of β_2 integrins may be relevant to the understanding of many integrin processes.

Hypothesis

In this dissertation, I have investigated the hypothesis that priming and activation of bovine neutrophils can be modulated by β_2 integrins. More specifically, the following questions were addressed in my research:

1. What is the level of expression of the β_2 integrins on the surface of bovine leukocytes and how does it compare to other cell surface markers?
2. Can homozygous BLAD neutrophils be activated with a non-β_2 integrin-dependent stimulus?
3. Can cross-linking the surface β_2 integrins on bovine neutrophils cause β_2 integrin-dependent cell surface priming effects?
4. Can cross-linking the surface β_2 integrins on bovine neutrophils cause association of cytoskeletal proteins in a β_2 integrin-dependent fashion?
5. Do homozygous BLAD neutrophils express cytoskeletal β_2 integrin proteins that may not be expressed on the cell surface?

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CHAPTER 2

ANALYSIS OF SURFACE ANTIGEN EXPRESSION AND HOST DEFENSE FUNCTION IN LEUKOCYTES FROM CALVES WITH BOVINE LEUKOCYTE ADHESION DEFICIENCY: COMPARISON OF HETEROZYGOUS AND HOMOZYGOUS ANIMALS

Abstract

Bovine leukocyte adhesion deficiency (BLAD) is a genetic disease of cattle that results in impaired immune function due to the absence of leukocyte β_2 integrin expression. In the present studies, we have compared surface antigen expression and functional responses of leukocytes from normal, heterozygous, and homozygous BLAD calves. Our results demonstrate that, while there were some small differences in leukocyte populations in the blood of normal and heterozygous calves, the leukocytes from heterozygous animals were essentially identical to those of normal calves with respect to surface antigen expression, *Candida albicans* phagocytosis and killing, and NADPH oxidase activity. By comparison, neutrophils from homozygous BLAD calves exhibited a dramatically reduced phagocytic capacity (<5% of normal or heterozygous neutrophils), yet they were still able to kill ~30% of the yeast as compared to normal and heterozygous neutrophils. One explanation is the higher respiratory burst activity, which was ~2 times higher than the activity from normal or heterozygous calves. Thus, these results support the conclusion that animals deficient in β_2 integrins may up-regulate other host defense capabilities in order to compensate for the absence of normal adherence-dependent host defense functions.

Introduction

Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive disease of Holstein dairy cattle that is characterized by recurrent bacterial infections (1-3). The molecular basis for BLAD involves a single mutation in CD18 (D128G), resulting in lack of β_2 integrin expression on leukocytes, which severely impairs adherence-dependent functions, such as adhesion, chemotaxis, and integrin-dependent phagocytosis (1,3-6). These defects in leukocyte adherence result in compromised mucosal immunity, leading to numerous alimentary and respiratory tract infections (2). As a result, calves with BLAD die when they are < 1 year old unless they are maintained on antibiotic treatment (1,7).

Functional characteristics of neutrophils and monocytes from homozygous BLAD calves have been described (3,5-8); however, less is known about the phenotypic characteristics of cells from heterozygous (carrier) animals, or the effect of the lack of β_2 integrins on the expression of other leukocyte antigens (myeloid or lymphoid). Nagahata *et al.* (9) reported enhanced expression of Fc receptors on neutrophils from calves with BLAD, but Fc receptor-mediated responses were lower than or equivalent to those of clinically normal animals (10), supporting the hypothesis that CD11b/CD18 is needed for correct Fc receptor-mediated neutrophil function (9,10).

Several groups have analyzed the respiratory burst of neutrophils from calves with BLAD; however, the results have been inconsistent (1,3,9-11). Depending on the stimulus and measurement protocol used, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity of neutrophils of calves with BLAD was enhanced (12), equivalent (11), partially decreased (3,10), and severely decreased (3,13) as compared

with that of neutrophils from clinically normal cattle. When NADPH oxidase activity was severely decreased, stimuli were CR3 (CD11b/CD18) specific or dependent (3,13). In studies using phorbol myristate acetate (PMA) as a stimulus, NADPH oxidase activity was similar to that of healthy bovine neutrophils, suggesting that the NADPH oxidase in neutrophils from calves with BLAD was at least functional (3). In contrast, neutrophils from patients with human leukocyte adhesion deficiency (LAD) had substantially increased NADPH oxidase activity when stimulated with opsonized sheep red blood cells (RBC) (12). Clearly, further studies on the NADPH oxidase response of neutrophils from calves with BLAD are necessary in order to determine the host defense capabilities that remain in these animals. One possibility is that the observed variability in neutrophil function is because of heterogeneity in leukocyte populations. Blood from calves with BLAD is characterized by a substantial peripheral neutrophilia (3,14); but maturity of the circulating cells has not been characterized sufficiently. Several workers have reported that mature segmented neutrophils were predominant (15,16), whereas others have reported increased concentrations of immature band neutrophils (3,17). Because neutrophil heterogeneity has been shown to play a role in functional capacity (18,19), a more comprehensive analysis of neutrophil populations in calves with BLAD is important.

The objective of the study reported here was to analyze surface antigen expression and functional responses of leukocytes from calves heterozygous and homozygous for BLAD, and compare those features with cells from clinically normal calves.

Materials and Methods

Materials

Dulbecco's phosphate-buffered saline (DPBS) was purchased from GIBCO-BRL (Gaithersburg, MD), phorbol 12-myristate 13-acetate (PMA) was from Calbiochem (San Diego, CA), phycoerythrin (PE-) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody were purchased from Jackson Immunoresearch, (West Grove, PA), dihydrorhodamine-123 and fluorescein isothiocyanate were from Molecular Probes (Eugene, OR), Thermonex cover slips were purchased from Nunc Corporation (Naperville, IL). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Blood Samples

Blood from clinically normal Holstein calves (n=4) and from Holstein calves heterozygous (n=4) and homozygous (n=4) for BLAD was collected in 50 ml polypropylene tubes containing acid citrate dextrose (ACD) at the National Animal Disease Center in Ames, Iowa, shipped to Montana State University, and analyzed within 24 hours of collection. A total of 200 blood samples were analyzed for this study. We received weekly shipments (approximately 50 shipments) of blood from 4 different sets of animals; each set consisted of a clinically normal calf, a BLAD-heterozygous calf and a BLAD-homozygous calf. The calves were age- and sex-matched to the homozygous BLAD calf in each set. To ensure that the time required for shipping did not substantially affect test results, fresh blood from 4 clinically normal Holstein calves was collected at our

facility and analyzed in parallel with test samples. Results obtained from fresh blood samples were used as a reference only and were not combined together with data obtained from shipped blood. Although studies on shipped bovine blood have not been previously described, Prince and Lapé-Nixon (20) found that human blood collected in ACD can be used to reliably assess granulocyte NADPH oxidase activity even as long as 24 hours after collection.

Leukocyte Isolation

For analysis of leukocyte surface antigens, RBC were removed from the blood samples by H_2O lysis, and the leukocytes were resuspended in cold Dulbecco's phosphate-buffered saline solution, (DPBS; pH 7.4). Neutrophils were isolated as previously described (21). Briefly, whole leukocyte suspensions were layered onto a 2-step density gradient consisting of 15 ml Histopaque 1077 over 15 ml of a 1:1 mixture of Histopaque 1077 and 1119. After centrifuging at 2,500 x g for 25 minutes at 20^0C, the mononuclear cell and neutrophil bands were collected and washed 2 times with 50 ml cold DPBS. Cell purity was assessed by microscopic visualization and flow cytometric analysis. Because of neutrophilia in the homozygous calves, neutrophil isolation consistently produced preparations containing >98% neutrophils, whereas preparations from clinically normal and heterozygous calves contained 93 to 98% neutrophils. To account for contribution of leukocytes other than neutrophils, leukocytes taken from the non-neutrophil layer of the density gradient were added back to the assay preparation to adjust the neutrophil

population so that the percentage of neutrophils was the same for each genotype (> 93%). Based on exclusion of trypan blue, cell viability was > 98%.

Measurement of Cell Surface Antigen Expression

Antigen expression was measured by flow cytometric analysis as described previously (22). For labeling, $1x10^6$ cells were incubated with either 50 μg primary antibody/ml or 100 μl hybridoma culture supernatant for 30 minutes on ice, washed with DPBS, and incubated with PE- or FITC-conjugated goat anti-mouse IgG secondary antibody at a dilution of 1:1000 for 30 minutes. The cells were washed again, resuspended in DPBS, and analyzed by flow cytometry using a FACScan or FACSCalibur (Becton Dickinson, Palo Alto, CA). Ten thousand events were collected for each sample. For analysis of leukocyte surface antigen expression, distinct leukocyte populations were electronically gated on the basis of their distinct forward and side scatter characteristics. Unstained leukocyte samples and samples stained with isotype-matched control mouse antibodies were analyzed as controls. Seventeen monoclonal antibodies were tested against each leukocyte population obtained from clinically normal, heterozygous, and homozygous calves, including antibodies recognizing bovine neutrophils and monocytes (BN1-15.6) (23), most γδ T cells (GD3.5) (24), all γδ T cell receptors (TCR; GD3.8) (24), γδ TCR-defined subsets (GD3.1, GD197) (24,25), CD2 (CC42) (26), CD4 (CC30) (26), CD5 (CC17) (26), CD8 (CC58) (26), CD45RO (GD6-10) (M.A. Jutila, unpublished antibody), CD44 (Hermes 3) (27), CD62L (Dreg-56) (27), B cell antigen CD21 (CC21)

(26), CD18 (MHM23, R15.7, IB4) (28-30), and CD11b (MM12A) (VMRD, Inc., Pullman, WA).

Production of FITC-labeled Yeast

Candida albicans, strain CA-1, was grown in 2% glucose-yeast extract peptone broth at 37°C with shaking until mid-stationary phase. Yeast cells were washed 3 times in DPBS, fixed with 10% formaldehyde in cold DPBS for 30 minutes to 1 hour, washed 3 more times with cold DPBS, and stored at 4^0C until use. Heat killed yeast were produced by boiling yeast suspended in DPBS for 30 minutes; cells were washed three times with cold DPBS and stored at 4^0C until use. All yeast were labeled as described by Lyman and Walsh (31). Briefly, live, formaldehyde-fixed or heat-killed yeast were washed in 0.2 M DPBS (pH 8.0) and incubated in the dark (10^7 cells/ml) with 250 μM FITC for 30 minutes at 25^0C. Cells were then washed 3 times with 0.2 M DPBS (pH 7.4) and stored at 4^0C until use.

Yeast Phagocytosis Assay

To evaluate phagocytosis, 500 μl of neutrophils (5.4 x 10^6 cells/ml in RPMI) were added to cover slips inside the wells of a 6-well tissue culture plate. Cells were allowed to adhere for 30 minutes to 1 hour at 37°C, the media was removed, and 500μl of FITC-labeled yeast suspended in pre-warmed (37^0C) RPMI containing either 10% normal bovine serum, 10% complement-inactivated serum (56°C for 30 minutes), or 10% yeast-absorbed normal bovine serum were added. The yeast-absorbed serum was produced by mixing 10

parts serum with 1 part packed, formalin-killed yeast cells for 10 minutes on ice. The yeast cells were centrifuged at 1,000 x g for 10 minutes, and the supernatant was absorbed with yeast twice more.

Neutrophils and yeast were allowed to interact for 1 hr at 37°C. To stop the interaction, cold 1% paraformaldehyde solution was added to each well and samples were incubated overnight at 4^0C or for 1 to 2 hours at 25^0C. Ingested yeast were differentiated from attached yeast by quenching any external FITC with 2 mg trypan blue /ml in 0.02 M citrate buffer containing 0.15 M NaCl (pH 4.4). Because trypan blue does not penetrate the neutrophil plasma membrane, only ingested yeast were visualized by fluorescent microscopy. Samples were run in triplicate.

Yeast Killing Assay

To analyze yeast killing, 2.7×10^6 neutrophils and 5.3×10^6 live yeast in 1 ml RPMI with 10% normal bovine serum were incubated for 1 hour at 37°C with rotation. Samples were serially diluted into DPBS, and aliquots were plated onto Sabouraud dextrose agar plates. After incubation for 48 hours at 37°C, yeast colonies were counted. Samples were run in triplicate.

Analysis of NADPH Oxidase Activity

NADPH oxidase activity of control and PMA-stimulated neutrophils was determined by use of 3 protocols. In the first protocol, the rate of O_2^- production was

measured as the rate of superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction as described (32).

In the second protocol, respiratory burst activity was determined by use of a flow cytometric assay with the fluorescent indicator dihydrorhodamine 123 (DHR). Because DHR oxidation can be influenced by the absolute and relative number of neutrophils in the leukocyte suspension (33), the relative neutrophil to mononuclear cell percentages were adjusted by adding back mixed mononuclear cells from the mononuclear cell layer so that all samples had comparable leukocyte compositions. Neutrophil preparations (10^7 cells/ml in DPBS) were incubated with 100 ng DHR/ml for 5 minutes at 37°C. The oxidative burst was then activated by the addition of 100 nM PMA, and cells were kept at 37°C. At 0, 5, 10 and 15 minutes, 1 x 10^6 cells were removed from the reaction and placed in cold flow cytometry tubes kept on ice. Fluorescence of DHR was measured using flow cytometry as described (34).

In the third protocol, a DHR-based microtiter plate fluorescence assay was used to detect reactive oxidants. Neutrophils loaded with DHR were placed in 96-well microtiter plates (2.5 x 10^5 cells/well in DPBS) and activated with 100 ng PMA /ml at 25°C. Fluorescent readings were recorded every 2 minutes at 530 nm using a FL-500 fluorescence microtiter plate reader (BioTek Instruments, Winooski, VT).

Differential Cell Counts

Well-mixed whole blood samples were used to make standard blood smears. After air drying, the smears were stained using a Wright's staining kit (LeukoStat Stain Kit,

Fisher Scientific, Pittsburgh, PA). Leukocyte differential counts (100 leukocytes/smear) were performed using light microscopy (100X magnification under oil). Three smears from each calf were analyzed each week for 4 weeks (1200 cells for each genotype) by a certified medical technologist (KMS). Results for each leukocyte population were expressed as percentage (mean ± SEM) of total leukocytes.

Blood smears were also analyzed for neutrophil maturation. One hundred neutrophils were counted on each slide and classified as bands, as having 2 segments, or as having ≥ 3 segments. The nuclei of band neutrophils had parallel sides but twisted to conform to the space within the cytoplasm, and the nuclear membranes were smooth. Indentations or irregularities of the nuclear membrane were considered features of mature neutrophils (35). A nuclear membrane with 1 indentation was classified as having 2 segments; a membrane with >1 indentation was classified as having ≥ 3 segments. Three smears from each calf were analyzed each week for 4 weeks (1200 cells for each genotype) by a certified medical technologist (KMS). Results for each leukocyte population were expressed as percentage (mean ± SEM) of total leukocytes.

Statistical Analysis

One way analysis of variance (ANOVA) was performed on all data, followed by Tukey's pairwise comparisons. Differences were considered significant at $P<0.05$.

Results

Leukocyte Surface Antigen Expression

Significant differences in percentages of lymphocytes stained by monoclonal antibodies directed against surface antigens were not detected between heterozygous and homozygous calves, except for the expected lack of CD18 expression on cells from calves homozygous for BLAD (Table 2.1). Monocytes (Table 2.2) and neutrophils (Table 2.3) from heterozygous and homozygous calves also had similar expression of surface antigens, again with the exception of CD11b and CD18, which were not expressed on homozygous cells.

The effect of shipping on neutrophil surface antigen expression was evaluated by measurement of CD11b, CD18 and L-selectin expression, since changes in the level of expression of these antigens provides a sensitive measure of neutrophil priming or activation (36-39). Expression of these antigens on neutrophils from shipped blood of clinically normal or heterozygous calves was not significantly different from that of fresh bovine blood from clinically normal calves (Table 2.3), demonstrating the effects of shipping on surface antigen expression were minimal.

Yeast Phagocytosis

Neutrophils from heterozygous calves phagocytosed yeast as efficiently as neutrophils from clinically normal calves (Figure 2.1). Furthermore, phagocytosis by neutrophils from clinically normal and heterozygous calves was decreased by serum

Table 2.1 Surface Antigen Expression on Lymphocytes from Calves Heterozygous or Homozygous for BLAD.

Monoclonal antibody	Antigen/cell recognized	Heterozygous		Homozygous	
		Calf 1	Calf 2	Calf 1	Calf 2
CC42	CD2	41.3	35.4	42.3	54.5
CC30	CD4	19.6	NT	26.0	NT
CC17	CD5	77.8	NT	81.1	NT
CC58	CD8	23.4	NT	28.2	NT
GD3.1	γδ T-cell subset	8.0	13.2	20.0	24.6
GD197	γδ T-cell subset	28.0	25.0	20.7	18.0
GD3.5	Most γδ T-cells	52.9	41.7	38.8	41.1
GD3.8	All γδ T-cells	40.4	33.0	28.7	38.3
CC21	All B-cells	19.5	16.9	21.8	12.6
GD6-10	CD45RO	62.9	61.8	63.6	71.3
Hermes	CD44	100	100	100	100
R15.7	CD18	100	100	<1	<1
Lymphocyte count (%)		48.8	41.6	18.6	25.1

Results are expressed as percentage of lymphocytes staining above background (secondary antibody only). NT = not tested.

Table 2.2 Surface Antigen Expression on Monocytes from Calves Heterozygous or Homozygous for BLAD.

Monoclonal antibody	Antigen/cell recognized	Heterozygous	Homozygous
Control*	--	3.6±0.2	3.7±0.2
BN1-15.6	Bovine neutrophils & monocytes	42.2±2.5	57.9±7.9
BN1-80	Bovine monocytes	63.9±1.4	81.4±10.2
R15.7	CD18	95.8±2.9	11.9±0.2
MHM23	CD18	28.2±0.3	3.0±0.1
IB4	CD18	29.4±0.5	2.1±0.2
MM12A	CD11b	30.3±5.5	4.0±0.4
Dreg56	L-selectin	826.6±63.1	736.1±66.8
GD6-10	CD45RO	205.9±8.3	146.4±28.7
Hermes	CD44	12.4±0.5	18.6±1.7

Results are expressed as mean ± SEM fluorescence intensity (arbitrary units). Four calves from each group were analyzed. *Cells were stained with secondary antibody only.

Table 2.3 Surface Antigen Expression on Neutrophils from Clinically Normal Calves and Calve Heterozygous or Homozygous for BLAD.

Monoclonal Antibody	Antigen/cell recognized	Clinically normal (fresh†)	Clinically normal (shipped††)	Heterozygous	Homozygous
Control*	--	1.4±0.1	2.6±1.3	3.6±0.1	3.6±0.3
BN1-15.6	Bovine neutrophils & monocytes	334.3±18.0	416.3±13.1	400.9±13.4	366.5±20.6
R15.7	CD18	77.8±1.4	80.8±5.1	70.7±8.5	5.5±0.1
MHM23	CD18	12.0±0.8	11.2±1.0	25.9±1.4	1.9±0.6
IB4	CD18	35.6±3.4	32.4±0.7	28.6±1.4	4.7±0.4
MM12A	CD11b	17.5±0.5	19.9±0.8	22.3±1.1	2.4±0.9
Dreg56	L-Selectin	696.7±53.2	579.4±70.0	581.3±34.0	721.8±48.6
GD6-10	CD45RO	NT	NT	273.0±28.6	183.2±59.2
Hermes	CD44	NT	NT	12.8±0.5	12.0±0.8

Results are expressed as mean ± SEM fluorescence intensity (arbitrary units). Four calves from each group were analyzed. *Cells were stained with the secondary antibody only. †Fresh blood samples were analyzed. ††Blood samples were shipped to the laboratory and analyzed within 24 hours. NT = not tested.

complement inactivation. Pre-absorbing the serum with yeast also resulted in decreased phagocytosis, although to a slightly lesser extent than with complement inactivation. In comparison, neutrophils from homozygous calves were unable to phagocytose yeast (Figure 2.1), as is consistent with the importance of β_2 integrins in phagocytosis.

Yeast Killing

Analysis of yeast killing revealed that neutrophils from heterozygous calves were as capable of killing yeast as cells from clinically normal calves (Figure 2.2). As expected, neutrophils obtained from homozygous BLAD calves had significantly lower capacity to kill yeast (Figure 2.2).

Respiratory Burst Activity

Results of the cytochrome c assay indicated that neutrophils from homozygous calves generated approximately twice as much O_2^- as neutrophils from clinically normal or heterozygous calves (Figure 2.3). These results were confirmed by flow cytometric analysis of dihydrorhodamine 123 (DHR) fluorescence in PMA-stimulated cells (Figure 2.4, top panel) and by a microtiter plate DHR assay (Figure 2.4, bottom panel).

Differential Leukocyte Count

Calves homozygous for BLAD had substantial neutrophilia, whereas clinically normal and heterozygous calves had differential counts within normal reference ranges over the 4-week period of testing (Figure 2.5). Neutrophil maturation analysis revealed a

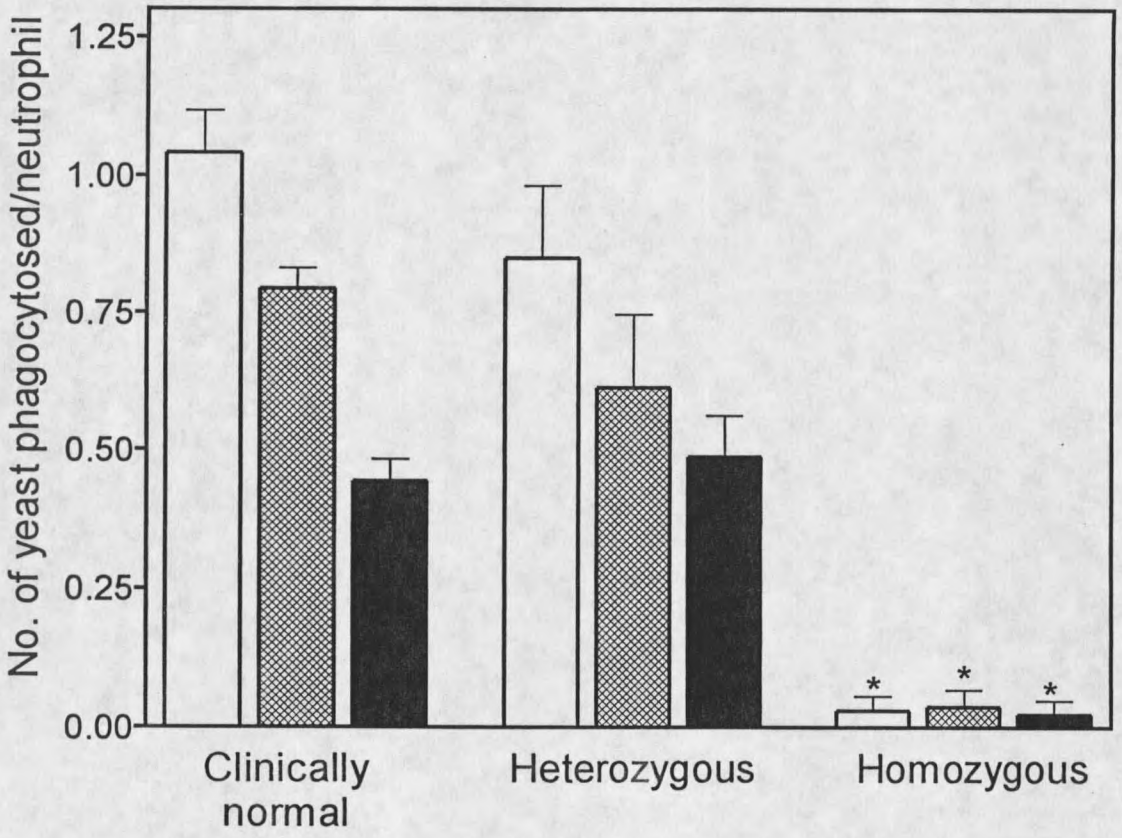

Figure 2.1 Phagocytosis of *Candida albicans* by Neutrophils Isolated from Clinically Normal Calves and Calves Heterozygous or Homozygous for BLAD. Neutrophils were incubated with FITC-labeled *C. albicans* opsonized with normal bovine serum (open bar), yeast absorbed serum (hatched bar), or complement-inactivated serum (solid bar) and the number of yeast phagocytosed per neutrophil was determined as described under Materials and Methods. Results are expressed as mean ± SD of 3 independent experiments. *Indicates significant ($P<0.005$) difference from neutrophils from clinically normal and heterozygous calves.

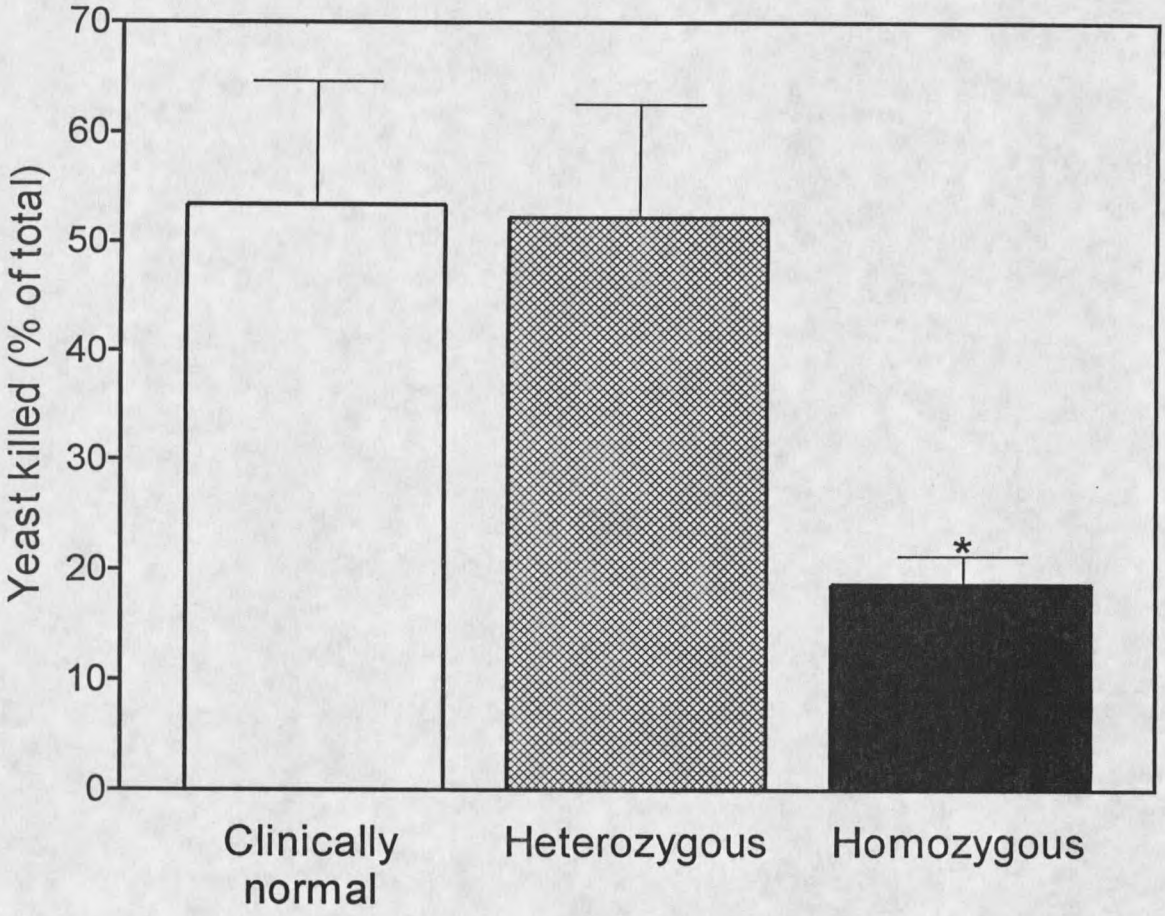

Figure 2.2 Killing of *Candida albicans* by Neutrophils Isolated from Clinically Normal Calves and Calves Heterozygous or Homozygous for BLAD. Neutrophils obtained from clinically normal calves (open bar) and calves heterozygous (hatched bar) or homozygous for BLAD (solid bar) were incubated with *C. albicans* (1:2 ratio) opsonized with normal bovine serum. The samples were plated on agar, and the number of surviving yeast was determined as described under Materials and Methods. Results are expressed as mean ± SD and represent 3 independent experiments. *Indicates significant ($P<0.005$) difference from neutrophils from clinically normal and heterozygous calves.

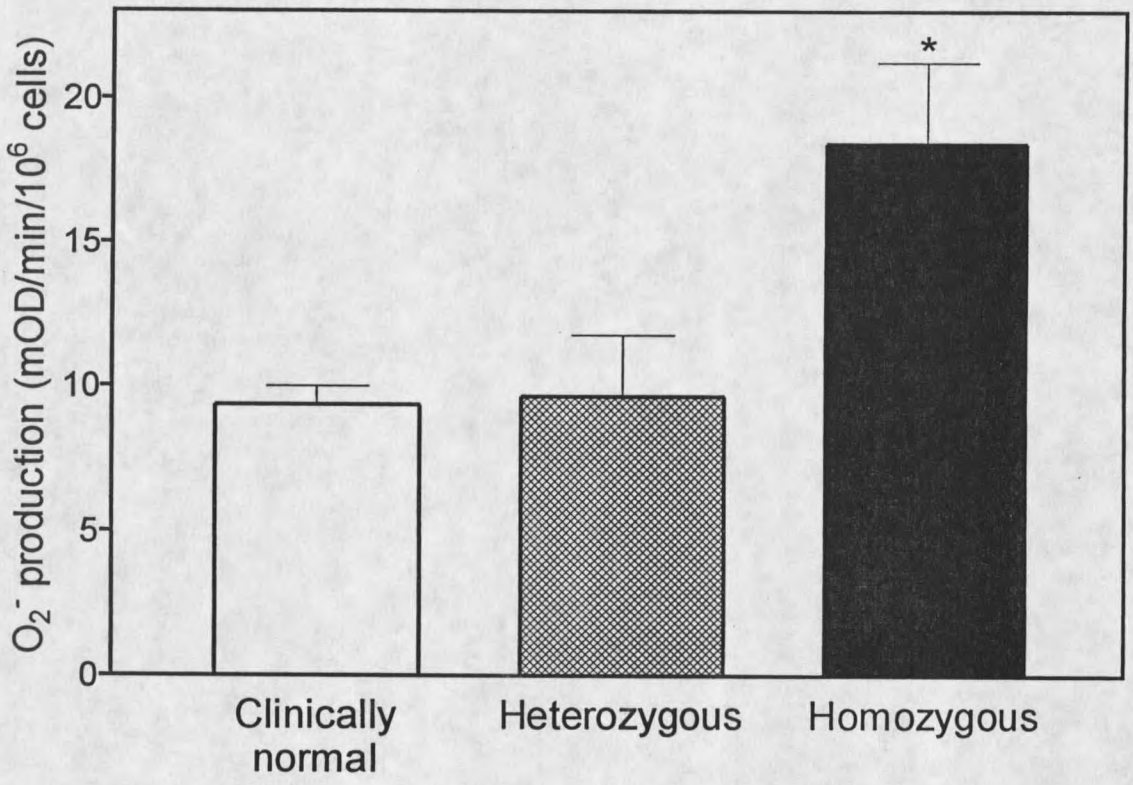

Figure 2.3 Respiratory Burst Activity of Neutrophils Isolated from Clinically Normal Calves and Calves Heterozygous or Homozygous for BLAD. O_2^- production by neutrophils isolated from clinically normal calves (open bar), heterozygous calves (hatched bar), and homozygous calves (solid bar), measured by use of a cytochrome c assay. Results are expressed as mean ± SEM of 8 independent experiments. *Indicates significant ($P<0.001$) difference from neutrophils of clinically normal and heterozygous calves.

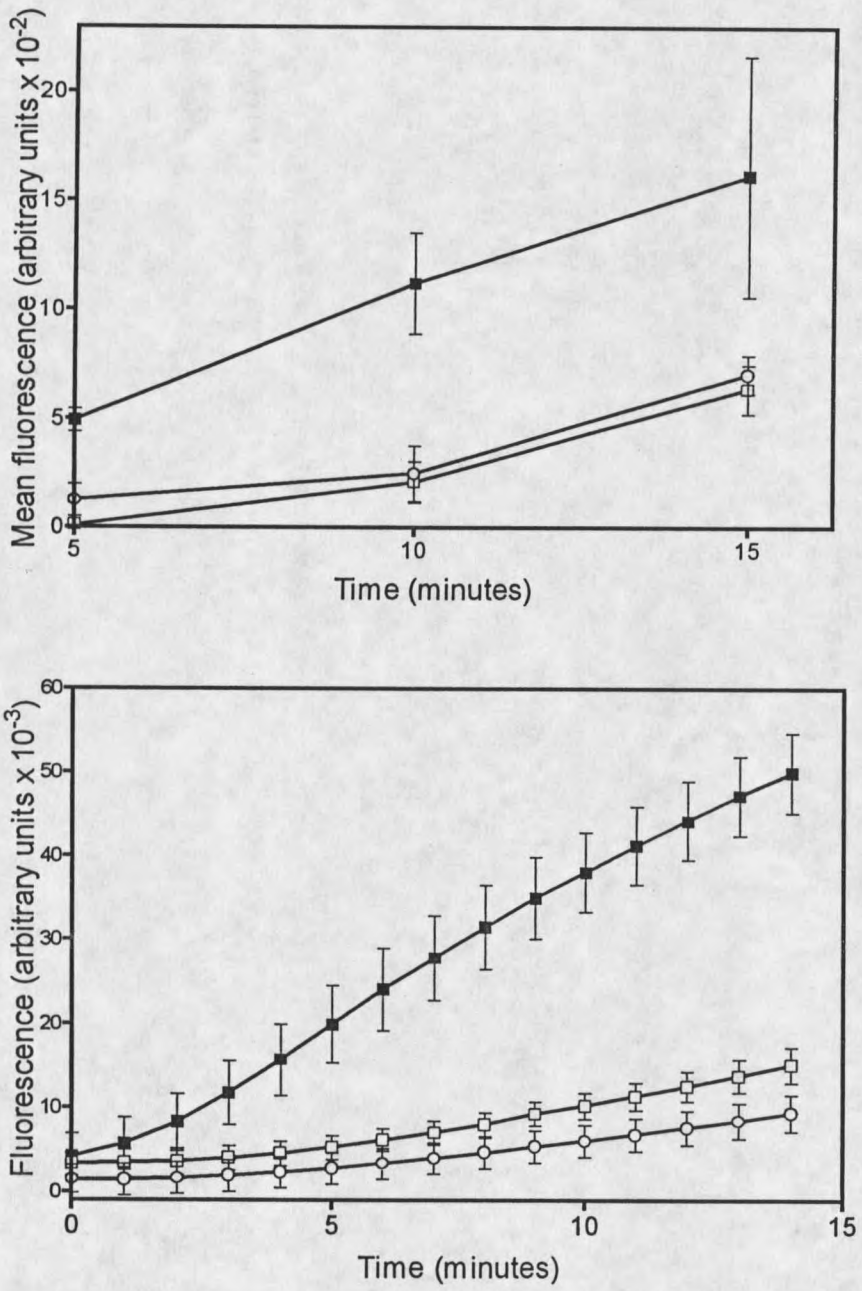

Figure 2.4 Respiratory Burst Activity of Neutrophils Isolated from Clinically Normal Calves and Calves Heterozygous or Homozygous for BLAD. Dihydrorhodamine (DHR) fluorescence of neutrophils from clinically normal calves (open circles), heterozygous calves (open squares), and homozygous calves (solid squares), measured by flow cytometry (top panel) and in a fluorescent microtiter plate assay (bottom panel). Results are expressed as mean ± SEM of 3 (top panel) and 4 (bottom panel) independent experiments.

significant shift toward band neutrophils in the blood from homozygous BLAD calves when compared with blood from clinically normal or heterozygous calves (Figure 2.6). In addition, this shift toward band neutrophils was consistent in the homozygous calves over the entire 4-week period of testing. The percentage of neutrophils with 2 segments was significantly increased in blood from heterozygous calves as compared with clinically normal calves (Figure 2.6), indicating a slight shift toward a more immature neutrophil population. Additionally, in both the heterozygous and homozygous calves there was a slight increase in the percent of eosinophils as compared to the normal calf (Figure 2.5).

Discussion

Currently, little is known about how bovine leukocyte adhesion deficiency (BLAD) affects the expression of cell surface antigens, other than the β_2 integrins, on leukocytes of affected animals. In the present study, we found that the expression of a number of surface antigens on myeloid and lymphoid cells from calves heterozygous and homozygous for BLAD was similar to that reported for clinically normal calves, (24,25,40). Thus, even though animals with BLAD have severely compromised host defense capacity, there is very little effect on the expression of leukocyte cell-surface antigens, except for the β_2 integrins.

In the study reported here, we performed experiments using BLAD blood that was shipped overnight. To control for any artifacts caused by the shipping process, we also analyzed fresh blood collected from clinically normal Holstein calves at our facility.

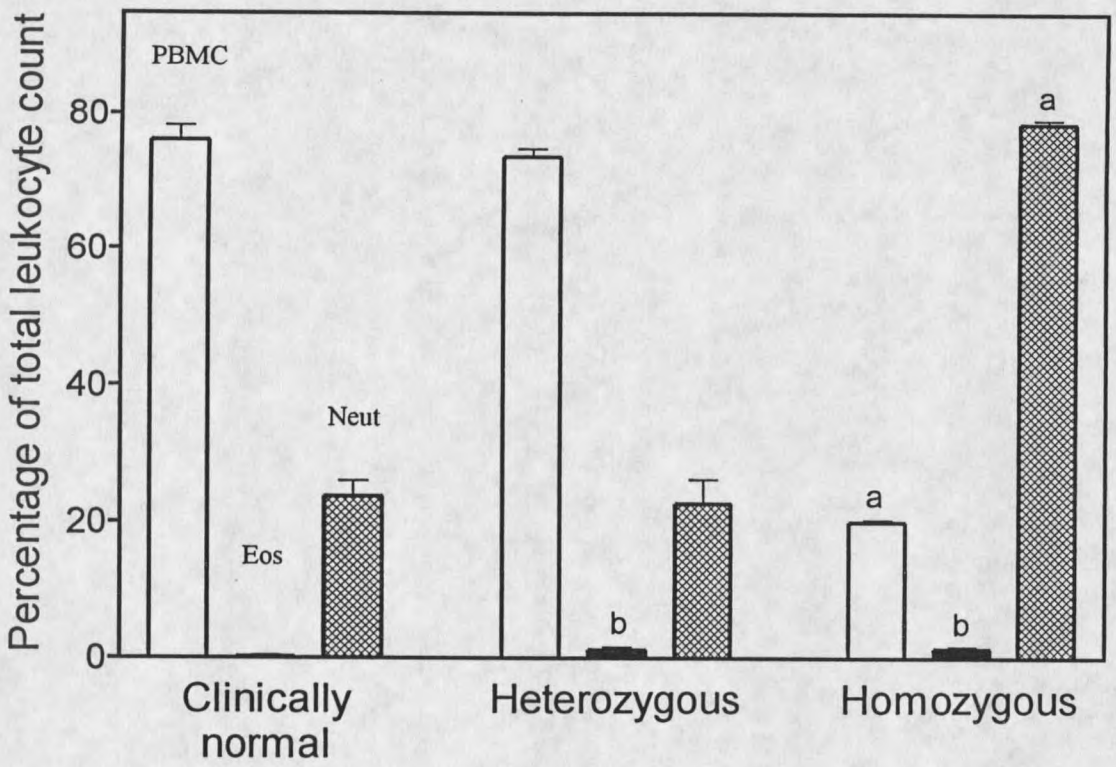

Figure 2.5 Differential Leukocyte Counts in Peripheral Blood Obtained from Clinically Normal Calves and Calves Heterozygous or Homozygous for BLAD. Percentages (mean ± SD of triplicate samples analyzed each week for 4 weeks) of peripheral blood mononuclear cells (PBMC/open bars), eosinophils (Eos/solid bars), and neutrophils (Neut/hatched bars). a = significant ($P<0.001$) difference from cells of clinically normal and heterozygous calves. b = significant ($P<0.05$) difference from cells of clinically normal calves.

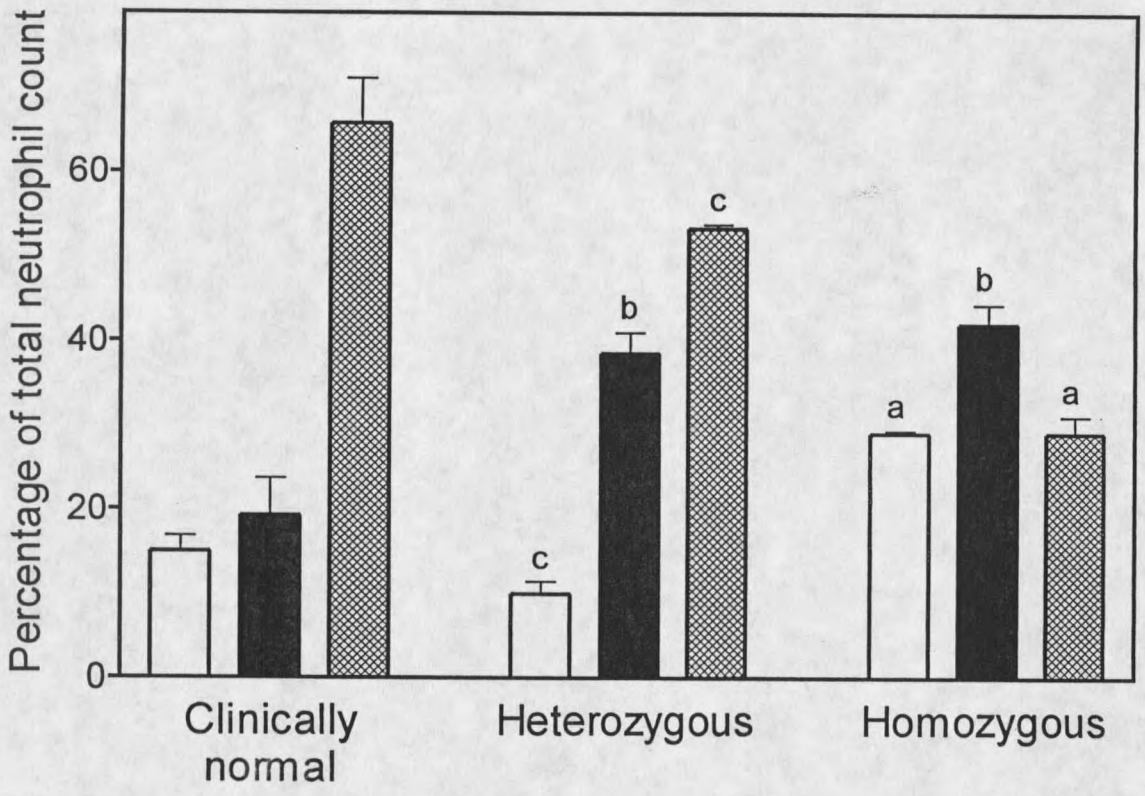

Figure 2.6 Differential Neutrophil Counts in Peripheral Blood Obtained from Clinically Normal Calves and Calves Heterozygous or Homozygous for BLAD. Neutrophils were classified as bands (open bars), having 2 segments (solid bars), or having ≥ 3 segments (hatched bars). Results are expressed as percentage of total neutrophils counted (mean ± SD of triplicate samples analyzed each week for 4 weeks). a = significant ($P<0.001$) difference from cells of clinically normal and heterozygous calves. b = significant ($P<0.001$) difference from cells of clinically normal calves. c = significant ($P<0.01$) difference from cells of clinically normal calves.

Expression of leukocyte surface markers on cells from shipped blood that had been collected in ACD and analyzed within 24 hours was similar to that of cells from fresh blood, indicating that the effects of shipping the blood were minimal. Similarly, Prince and Lapé-Nixon (20) found that neutrophils in human blood collected with ACD as the anticoagulant were stable for up to 24 hours, as determined by analysis of NADPH oxidase activity.

Neutrophils from heterozygous calves phagocytosed and killed *C. albicans* as well as those from clinically normal calves, demonstrating that the heterozygous genotype likely does not have an effect on adhesion-dependent functions of leukocytes. In further support of this conclusion, phagocytosis by neutrophils from heterozygous calves was dependent on complement and immunoglobulin opsonization, indicating that phagocytosis was mediated in part via β_2 integrins. Defective complement- and immunoglobulin-mediated phagocytosis by neutrophils from homozygous calves has been attributed to impaired cooperation between immunoglobulin Fc receptors and CR1 with CR3 (CD11b/CD18) (11). In contrast to cells from clinically normal and heterozygous calves, neutrophils from homozygous calves were not able to efficiently phagocytose yeast. This result confirms previous reports that neutrophils from homozygous calves had severely decreased phagocytic capacity compared with cells from clinically normal calves (3,41). Yeast killing was not evaluated in these previous studies.

Even though neutrophils from homozygous calves were unable to phagocytose yeast, they still maintained approximately 30% of the capacity of clinically normal or heterozygous cells to kill *C. albicans*. Diamond and coworkers (42,43) showed that

neutrophils could damage or kill hyphae and pseudohyphae of *C. albicans* via oxygen-dependent, nonphagocytic mechanisms, as these yeast forms were too large to phagocytose. Thus one explanation for the results of the study reported here is that neutrophils from calves homozygous for BLAD, which cannot phagocytose, might use similar nonphagocytic mechanisms to attack even small organisms such as yeast blastoconidia. Indeed, we found, using 3 different assay methods, that neutrophils from homozygous animals generated approximately twice the amount of O_2^- as cells from clinically normal and heterozygous calves. These results are consistent with a previous report showing that murine IgG2a-opsonized sheep RBC stimulated enhanced chemiluminescence by neutrophils isolated from a patient with LAD, as compared with healthy human neutrophils (12). Thus, the ability of neutrophils from calves homozygous for BLAD to kill yeast without phagocytosing them indicates that other host defense capabilities are functional and may even be up-regulated in thee cells.

Although a number of studies measuring respiratory burst activity in neutrophils from calves homozygous for BLAD have been reported, the results of these studies have been inconsistent (3,9-11). In the study reported here, PMA stimulated a greater respiratory burst in neutrophils from homozygous calves than in neutrophils isolated from clinically normal or heterozygous calves. Whether this increase in oxidase activity is attributable to an increase in the amount of NADPH oxidase proteins or to priming effects is not known. Another possibility is that the greater NADPH oxidase activity of neutrophils from homozygous calves might result from enrichment of a subpopulation of cells that has a higher respiratory burst capacity. Differential counts did reveal a

significant increase in the number of band neutrophils in blood from homozygous animals; however, Krause *et al.* (44) reported that immature peripheral blood neutrophils do not respond as well as mature cells in functional assays, such as the production of reactive oxidants. Thus, the presence of greater numbers of band neutrophils may not be sufficient to explain the enhanced oxidative burst found in PMA-stimulated neutrophils from homozygous calves. In addition, neutrophils from the heterozygous calves, which also had elevated levels of less mature neutrophils, still generated similar amounts of O_2^- as cells from clinically normal calves.

The reason for the highly variable NADPH oxidase response of cells from homozygous calves is not clear. A similar variability in oxidase response was observed by Gresham et al. (45), who analyzed neutrophils from humans with LAD and suggested that this variability reflected differences in prior in vivo exposure of the cells to inflammatory stimuli. Previous exposure of neutrophils to a priming agent can substantially enhance respiratory burst activity when the cells are activated by a subsequent stimulus (46). Thus, it is also possible that the variability in bovine neutrophil oxidase response may reflect differences in neutrophil priming prior to cell isolation. Another possible explanation for the variability in NADPH oxidase activity of cells from calves with BLAD is the variety of methods that have been used to analyze respiratory burst activity. Most previous studies utilized luminol-dependent chemiluminescence (LDCL) to measure reactive oxidant production (3,8,9,11,12). Although highly sensitive, luminol is not specific for O_2^- (47). In addition, LDCL is not a useful assay for continuous monitoring of the respiratory burst because luminol reaction products can interfere with the light reaction (48). In the study

reported here, we utilized 3 methods for evaluating respiratory burst activity: a standard cytochrome c-based assay, which is specific for measurement of external O_2^- production (32,49) and two DHR-based assays, which both measured internal H_2O_2 production (34). Although assays with DHR are more sensitive than those with other fluorescent dyes for flow cytometric measurements of reactive oxygen species (34), there are some limitations in using DHR to analyze respiratory burst activity. For example, DHR oxidation is influenced by the absolute and relative number of neutrophils in the leukocyte suspension (50). Thus, care was taken to ensure that the cell populations were alike in the different samples analyzed.

Previously, studies on neutrophils from calves homozygous for BLAD (51) and neutrophils from CD18-deficient mice (52) revealed that these cells can utilize alternative, β_2 integrin-independent pathways to perform various adherence-dependent functions. Accordingly, the results of the study reported here further support the hypothesis that animals deficient in β_2 integrins utilize or up-regulate alternate host defense capabilities to partially compensate for the lack of normal adherence-dependent host defense functions. Unfortunately, even up-regulation of such compensatory mechanisms cannot overcome the incapacitating effects of this disease on the innate immune system, and untreated animals still die shortly after birth.

Note: The studies described in Chapter 2 were published as follows:

Sipes, K.M., Edens, H., Kehrli, M.E., Jr., Cutler, J.E., Miettinen, H.M., Jutila, M.A., and Quinn, M.T. 1999. Analysis of surface antigen expression and host defense function in leukocytes from calves with bovine leukocyte adhesion deficiency: Comparison of heterozygous and homozygous animals. *Am. J. Vet. Res.* **60**:1255-1261.

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CHAPTER 3

ANTIBODY-INDUCED ENGAGEMENT OF β_2 INTEGRINS ON BOVINE NEUTROPHILS: A COMPARISON OF CLINICALLY NORMAL, HETEROZYGOUS, AND HOMOZYGOUS BLAD ANIMALS

Abstract

β_2 integrins mediate selective cell signaling functions in neutrophils. Engagement and clustering of the β_2 integrins on the neutrophil membrane leads to a number of signaling events resulting in cytoskeletal rearrangement and functional responses such as chemotaxis, degranulation, and activation of the respiratory burst. Bovine neutrophils from cattle afflicted with bovine leukocyte adhesion deficiency (BLAD) do not express the β_2 integrin molecules on their cell surface, therefore they represent a unique model to study β_2 integrin-dependent cell functions. To investigate β_2 integrin-dependent cell-signaling processes, we analyzed intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), cell surface L-selectin and CD18 expression, cell surface lactoferrin, and redistribution of cytoskeletal and signaling proteins as a consequence of antibody-induced cross-linking on bovine neutrophils. In cross-linked normal bovine neutrophils, we found a decreased and delayed increase in ($[Ca^{2+}]_i$) compared to responses observed after treatment with platelet activating factor (PAF). As expected, similar cross-linking experiments failed to induce a comparable increase in $[Ca^{2+}]_i$ in neutrophils isolated from a homozygous BLAD calf. Cross-linking induced an increased staining of cell surface L-selectin, CD18, and lactoferrin in neutrophils isolated from normal or heterozygous BLAD cattle. A comparable change in the surface expression of these molecules was not observed on similarly treated neutrophils from a homozygous BLAD animal, implicating specific

granule degranulation as a response to cross-linking. Cross-linking surface-bound β_2 integrin monoclonal antibodies (mAb) induced a redistribution of the tyrosine kinase $p58^{fgr}$ to the Triton X-100 insoluble cell fraction, while similarly treated neutrophils from cattle homozygous for BLAD did not show a redistribution of $p58^{fgr}$. To identify β_2 integrin-associated proteins in bovine neutrophils, cell lysates were immunoprecipitated with anti-β_2 integrin mAbs. Assuming that neutrophils isolated from cattle homozygous for BLAD lacked all four of the β_2 integrin subunits, neutrophil cell lysates from these animals were used as controls in these experiments. Surprisingly, in the neutrophil lysates from the homozygous animal, anti-CD18 mAb 68-5A5 immunoprecipitated two protein bands with molecular weights in the ~150-180 kD range and one protein band with the molecular weight of ~97 kD, and anti-Cd11c mAb 3.9 immunoprecipitated one protein band with the molecular weight of ~180 kD. These findings suggest that some of the β_2 integrin proteins may be present in neutrophils from cattle homozygous for BLAD, yet these proteins do not appear to be expressed on the surface of the cell and may not be functional.

Introduction

The β_2 integrins are constitutively expressed on the cell surface of leukocytes. These heterodimeric, transmembrane glycoproteins consist of one of three α subunits (CD11a, CD11b, or CD11c) noncovalently associated with a common β subunit (CD18). The β_2 integrins mediate several important functions of the neutrophil. They are required for cellular tight adhesion to the vascular endothelium (1-12), cell spreading and

cytoskeletal formation (13,14), binding of complement C3bi (15-17), and the proper function of other neutrophil receptors (1,2,12,15,18-25).

Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive disease of Holstein dairy cattle that is characterized by recurrent bacterial infections (26-28). The molecular basis for BLAD involves a single mutation in CD18 (D128G), resulting in the lack of β_2 integrin expression on leukocytes, which severely impairs adherence-dependent functions such as adhesion, chemotaxis, and integrin-dependent phagocytosis (11,26,28-30).

There is evidence that the β_2 integrins participate in intracellular signal transduction and regulation of neutrophil function (16,25,31-33). Adherence of neutrophils to immobilized anti-β_2 integrin monoclonal antibodies induces β_2 integrin capping, reorganization of the actin-based cytoskeleton and activates the NADPH oxidative burst (21,34-36). It has been suggested that this outside-in signal transduction pathway activated by the β_2 integrins is different from that induced by the soluble chemoattractants via pertussis toxin-sensitive G-proteins (14,35,37). In adherent neutrophils, the β_2 integrin-mediated oxidative burst is inhibited by tyrosine kinase inhibitors (37,38) and has been shown to involve phospholipase C (PLC) (35,39) and/or phospholipase D (PLD) (17,40). Attempts to identify tyrosine kinases involved in integrin signaling in human neutrophils have established that Src-family kinases, like $p58^{fgr}$, are activated in integrin-dependent adherent neutrophils (36). In contrast to adherent neutrophils, CD18 cross-linking on suspended human neutrophils does not induce the oxidative burst (35).

Cross-linking the β_2 integrins on suspended human neutrophils induces a rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), degranulation of azurophilic granules, up-regulation of surface CD18 expression, actin polymerization, and shedding of L-selectin (40). These findings prompted us to investigate whether cross-linking the surface β_2 integrins on bovine neutrophils causes cell surface priming effects and association of cytoskeletal proteins in a β_2 integrin-dependent fashion. We were able to utilize neutrophils from calves heterozygous or homozygous for BLAD to investigate bovine neutrophil responses to β_2 integrin cross-linking.

Materials and Methods

Materials

Dulbecco's phosphate-buffered saline (DPBS) was purchased from GIBCO-BRL (Gaithersburg, MD). Fura-2/AM was purchased from Molecular Probes, Inc. (Eugene, OR). Stock 10mM PAF solutions were made up in DPBS containing 0.2% bovine serum albumin, and aliquots were stored at -80^0C; dilutions were made in the same buffer. Monoclonal antibody MHM23, which recognizes CD18 (42), was from Dako Corp. (Carpinteria, CA); mAb DREG-56, which recognizes L-selectin (43), was a kind gift of Dr. Mark Jutila (Veterinary Molecular Biology, Montana State University); mAb BL97, which recognizes bovine lactoferrin, was generated in our lab (44); mAb IB4 was obtained from ATCC #10164 (Manassas, VA) then purified and FITC-conjugated in our lab (45). The cross-linking mAb, goat anti-mouse IgG Fc specific, and the secondary mAb FITC-conjugated, goat anti-mouse IgG heavy and light chain, were purchased from

Jackson ImmunoResearch (West Grove, PA). UVT acrylic fluorimeter cuvettes were from Evergreen Scientific (Los Angeles, CA). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Blood Samples

Blood from clinically normal Holstein calves and Holstein calves heterozygous and homozygous for BLAD was collected into 50 ml polypropylene tubes containing acid citrate dextrose (ACD) at the National Animal Disease Center in Ames, Iowa, shipped to Montana State University, and analyzed for this study. Blood from clinically normal Holstein cattle and human blood was collected at Veterinary Molecular Biology, Montana State University, into 20 ml vacutainer tubes (Becton Dickinson, Palo Alto, CA) containing 0.2 ml 5mM EDTA.

Leukocyte Isolation

To isolates neutrophils, red blood cells were removed from blood samples by H_2O lysis, and the leukocytes were resuspended in cold DPBS (pH 7.4). The leukocyte suspensions were layered onto a 2-step density gradient consisting of 15 ml Histopaque 1077 over 15 ml of a 1:1 mixture of Histopaque 1077 and Histopaque 1119. After centrifugation at 2,500 x g for 25 minutes at 20°C, peripheral blood mononuclear cell and neutrophil bands were collected and washed 2 times with cold DPBS. Cell purity was assessed by microscopic visualization and flow cytometric analysis. Because of neutrophilia in the homozygous BLAD calf, neutrophil isolation procedures consistently

produced preparations containing >98% neutrophils, whereas preparations from clinically normal and heterozygous calves contained 93 to 98% neutrophils. To account for contribution of leukocytes other than neutrophils, leukocytes taken from the non-neutrophil layer of the density gradient were added back to the assay preparation to adjust the neutrophil population so that the percentage of neutrophils was the same for each genotype (>93%). Based on exclusion of trypan blue, cell viability was >98%.

Flow Cytometry

All flow cytometry measurements were performed on a FACSCalibur (Becton Dickinson, Palo Alto, CA) and 10,000 gated events were collected for each sample. When whole leukocyte populations were tested, leukocyte subpopulations were gated based on their distinct forward and side light scatter characteristics. For staining, 1×10^6 cells were incubated with 50 μg/ml primary antibody for 30 minutes on ice, washed with cold DPBS plus 2% goat serum (DPBSgs), and incubated with FITC-conjugated goat anti-mouse IgG secondary antibody at a dilution of 1:500 for 30 minutes on ice. The cells were washed again, resuspended in DPBSgs, and analyzed by flow cytometry. To analyze PMA-stimulated cell surface expression, isolated neutrophils were incubated with 30 ng/ml PMA at 37°C for 10 minutes, washed, and resuspended with cold DPBSgs. The anti-β_2 mAb tested are shown in Table 3.1.

Cross-linking of Surface β_2 Integrins

Isolated bovine neutrophils (1×10^7/ml DPBS) from normal, heterozygous and

Table 3.1 Monoclonal Antibodies for Cell Surface Staining

mAb	β_2 Integrin Subunit	Isotype	Source	Reference
IB4	CD18	Mouse anti-human IgG2a	ATCC #10164, Manassas, VA	(45)
MHM23	CD18	Mouse anti-human IgG1	Dako Corporation, Carpinteria, CA	(42)
44a	CD11b	Mouse anti-human IgG2a	ATCC #HB-249, Manassas, VA	(46)
OKM1	CD11b	Mouse anti-human IgG2b	ATCC #CRL-8026 Manassas, VA	(47)
MM12a	CD11b	Mouse anti-bovine IgG1	VMRD, Inc., Pullman, WA	(48)
2LPM19c	CD11b	Mouse anti-human IgG1	Dako Corporation, Carpinteria, CA	(42)
B-Ly6	CD11c	Mouse anti-human IgG1	Zymed Laboratories, Inc., So. San Francisco, CA	(49)
RDI 3.9	CD11c	Mouse anti-human IgG1	Research Diagnostics, Inc., Flanders, NJ	(50)
SHCL3	CD11c	Mouse anti-human IgG2b	Kind gift of Dr. C.A. Parkos, Emory University, Atlanta, GA	(51)
BAQ153	CD11c	Mouse anti-bovine IgM	VMRD, Inc., Pullman, WA	(52)
DF1524	CD11a	Mouse anti-human IgG2b	Serotec Ltd., Oxford, England	N/A
TS 2/4	CD11a	Mouse anti-human IgG1	ATCC #HB-244, Manassas, VA	(53)

homozygous BLAD animals were incubated with 50 μg/ml mAb MHM23 (CD18) (42) for 30 minutes on ice. Following one wash with cold DPBS, the neutrophils were resuspended in cold DPBS plus calcium ($DPBS^{+Ca}$) to the original concentration. After incubation with the primary mAb, a sample from each bovine phenotype was incubated with FITC-conjugated secondary antibody to ensure adequate binding of the primary mAb and to check for background staining on the homozygous BLAD neutrophils, as measured on a FACSCalibur (Data not shown). Antibody-pretreated or untreated neutrophils were cross-linked with 75 μl/10^6 cells of goat anti-mouse IgG Fc specific antibody (Jackson ImmunoResearch, West Grove, PA) at 37°C for the times indicated

then immediately washed with cold DPBS and centrifuged at 1000 x g for 10 minutes. Cells were resuspended in Relax buffer plus 2mM $MgCl_2$, 1:200 Protease Inhibitor Cocktail, 2 mM Na_3VO_4, 5 mM EDTA, and 0.2% Triton X-100, then placed on ice for 15 minutes with stirring. The cell lysates were ultracentrifuged at 8,000 x g for 15 minutes at 4°C. Supernatant fluid (Triton X-100 soluble fraction) was mixed with equal volumes of 2x sample buffer, boiled for 5 minutes and kept on ice until used. Triton X-100 insoluble pellets were resuspended in 2x sample buffer, boiled for 5 minutes and kept on ice until use.

Measurement of Calcium Flux

Antibody-treated or untreated control neutrophils were suspended in DPBS at a concentration of 1 x 10^7 cells/ml and the cells were incubated at room temperature in the dark for 20 minutes with 1 μM Fura-2/AM. For each assay, 10^6 Fura-2-loaded cells were added to 1.5 ml of $DPBS^{+Ca}$ and calcium flux was measured at 37°C with a Perkin-Elmer LS50B scanning fluorimeter using excitation wavelengths of 380 and 340 nm and an emission wavelength of 510 nm. Baseline fluorescence was recorded for a minimum of 2 minutes, cross-linking mAb or control buffer was added to the desired concentration, and recording was continued for 5-10 minutes. To quantify $[Ca^{2+}]_i$, 0.2% Triton X-100 was added to the cuvette to lyse the cells, and a maximum fluorescence was measured. After a brief recording, 20 mM EGTA was added to determine minimum fluorescence (54). Previous reports have indicated that CD11b surface expression and adhesion is dependent upon $[Ca^{2+}]_i$, with maximal CD11b/CD18 expression on neutrophils additionally

dependent on extracellular Ca^{2+} (55). In human neutrophils suspended in Ca^{2+}-free media, containing 1mM EDTA, cross-linking of CD18 still induced increased $[Ca^{2+}]_i$, although it was decreased in size. Therefore, for maximal $[Ca^{2+}]_i$, neutrophils were suspended in Ca^{2+}-supplemented buffer for the cross-linking studies.

Cell Surface Lactoferrin, β_2 Integrin, and L-selectin Measurements

The relative amount of lactoferrin on the surface of neutrophils was determined by flow-cytometric analysis using a FITC-conjugated anti-bovine lactoferrin mAb generated in our lab (BL97) (44). Relative amounts of neutrophil cell surface β_2 integrin molecules were determined by using a FITC-conjugated anti-CD18 mAb (IB4) (45) purified and conjugated in our lab. Cell surface L-selectin was determined using FITC-conjugated anti-L-selectin mAb DREG-56 (43). Antibody-pretreated or untreated neutrophils (1×10^6) were cross-linked with 75 µl of the cross-linking antibody at 37°C for the times indicated then immediately mixed with equal volumes of 1% cold paraformaldehyde and placed on ice for 15 minutes. Cells were washed with cold DPBS and resuspended to the original volume. 50 µg/ml FITC-conjugated mAb was added, and the samples were incubated on ice for 15 minutes before flow cytometric analysis on the FACSCalibur as described above.

There is some discrepancy in studies reporting the release of neutrophil granules at different doses of stimuli (44,56,57). We believe that this is due to the presence or omission of cytochalasin B in the assays. We chose not to use cytochalasin B in our

experiments so that we would be able to compare the relative mobilization of different granules under similar and physiologically relevant conditions (44).

Neutrophil Membrane and Cytosol Preparations

Isolated bovine neutrophils (3×10^8/ml) were suspended in homogenization buffer (10 mM Hepes, 0.34 M Sucrose, 0.1 mM $MgCl_2$, 1.0 mM EDTA; pH 7.4) plus 1:200 Protease Inhibitor Cocktail (Sigma-Aldrich, Inc., St. Louis, MO) and 1 μg/ml DFP and kept at 4°C throughout the procedure. Cells were sonicated (Sonic Desmembrator, Fisher Scientific, Pittsburgh, PA) to disrupt cell membranes, and the lysates were centrifuged at 1000 x g for 10 minutes to remove large cellular debris. The supernatant fluid was then centrifuged for 45 minutes at 175,000 x g in an Ultima-XL 100K ultracentrifuge (Beckman Instruments, Inc., San Jose, CA). The supernatant fluid (cytosol) was aliquoted and frozen at –80°C. The membrane pellet was resuspended in the homogenization buffer plus inhibitor and sonicated gently to disrupt the pellet. Membrane suspensions were aliquoted and kept at –80°C until further use.

To prepare detergent extracts of neutrophil membranes, membrane were diluted 1:4 with Relax buffer (10mM Hepes, 10 mM NaCl, 100 mM KCl, 2 mM $MgCl_2$) plus 1mM PMSF, and 1:200 Protease Inhibitor Cocktail, sonicated, and ultracentrifuged at 35K for 25 minutes at 4°C. The pellets were resuspended in Relax buffer containing 1M NaCl plus inhibitors, sonicated, and ultracentrifuged at 105,000 x g for 25 minutes. Membrane pellets were resuspended in 1% Octyl glucoside (OG) in Relax buffer plus inhibitors, sonicated, and stirred at 4°C for 1 hour. The detergent extracts were

ultracentrifuged at 100,000 x g for 25 minutes, and the supernatant fluid (OG extract) was stored on ice until use.

Immunoprecipitation and Immunoblotting

For immunoprecipitation, 5 μg of OG-extract were incubated with 100 μl 68-5A5 (RDI-CD18) (58) or 3.9 (RDI-CD11c) (50) overnight at 4°C with constant mixing. The membrane-antibody mixture was then added to 100 μl protein G beads (Amersham Pharmacia, Uppsala, Sweden) that were resuspended in Relax buffer plus inhibitors and OG and rotated end over end at room temperature for 3 hours. Samples were centrifuged and the supernatant (unbound fraction) was mixed with equal volumes of 2x reducing sample buffer, boiled, and frozen until use. The bead pellet was washed 6 times with Relax buffer plus inhibitors and detergent then resuspended in 150 μl 2X sample buffer and boiled for five minutes. After flash centrifugation, the supernatant fluid was collected and frozen at 80°C until use.

For immunoblotting, samples were electrophoresed through a 7-18% gradient acrylamide gel and transferred to Protran nitrocellulose (Schleicher and Schuell, Dassel, Germany) at 4°C. Immunoblots were blocked in Blotto/Tween for 2 hours at room temperature and incubated with a 1:1000 dilution of the primary antibody in antibody diluting buffer overnight at 4°C. Blots were washed 5 times and then incubated with a 1:1000 dilution of alkaline phosphatase-conjugated secondary antibody for 1 hour at room temperature. After a final wash, immunoblots were visualized by developing with a BioRad AP conjugate substrate kit (Hercules, CA). Protein band densitometry was

analyzed on an AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA). For silver staining, gels were fixed in 50% methanol and 12% acetic acid for 1 hour, washed in 50% ethanol 3 times for 20 minutes, and soaked in 0.02% sodium thiosulfate for 1 minute. After washing 3 times in distilled water, gels were soaked in 0.2% silver nitrate with 1ml/L formaldehyde for 30 minutes. Gels were washed again in distilled water and developed in 6% sodium carbonate, 1ml/L formaldehyde plus 30 ml/L of the thiosulfate solution. The staining was stopped with 50% methanol plus 12% acetic acid.

In addition to the antibodies listed in table 3.1, the following antibodies were used in the immunoblot and immunoprecipitation studies (Table 3.2);

Table 3.2 Antibodies for Immunoblot and Immunoprecipitation

Antibody	Antigen	Isotype	Source	Reference
Fgr	$p58^{fgr}$	Rabbit anti-peptide Polyclonal	Kind gift of Dr. Giorgio Berton, Verona, Italy	(59)
EA-53	α-actinin	Mouse anti-rabbit IgG1	Sigma-Aldrich, Inc., Saint Louis, MO	N/A
RDI 68-5A5	CD18	Mouse anti-human IgG2a	Research Diagnostics, Inc., Flanders, NJ	(58)
R7928a	CD11b	Rabbit anti-peptide Polyclonal	Kind gift of Dr. C.A. Parkos, Emory University, Atlanta, GA	(60)
25.3.1	CD11a	Mouse anti-human IgG1	Immunotech, Inc., Westbrook, ME	(42)
13.22	CD18/ CD11b	Mouse anti-human	Developed in our lab, MSU, Bozeman, MT	Un-published
TS1/18	CD18	Mouse anti-human IgG1	ATCC #HB-203 Manassas, VA	(53)
R7928c	CD11b	Rabbit anti-peptide Polyclonal	Kind gift of Dr. C.A. Parkos, Emory University, Atlanta, GA	(60)
BN1-15.6	Unknown	Mouse anti-bovine IgG	Kind gift of Dr. M.A. Jutila, MSU, Bozeman, MT	(61)

Statistics

One-way analysis of variance was performed on all data, followed by Dunnett's multiple comparison test. Statistically significant differences from appropriate controls are indicated for each result described.

Results

Cross-reactivity of Anti-β_2 Integrin mAb with Human and Bovine Leukocytes

To identify appropriate mAb for our cross-linking studies, we analyzed a large panel of β_2 integrin mAb for reactivity with human and bovine antigens. Significant differences in percentages of leukocytes stained by anti-β_2 integrin monoclonal antibodies were detected between human and bovine cells (Tables 3.3, 3.4, and 3.5). Two CD18-specific mAb (IB4 and MHM23) stained both human and bovine leukocytes (Table 3.3) and the level of staining was significantly enhanced after activation with PMA. Two anti-CD11b mAb (44a and 2LPM19c) exhibited a staining preference for human leukocytes (Table 3.3 and 3.4); whereas little staining was observed with bovine cells. In contrast, one anti-CD11b mAb (MM12a) preferentially stained bovine leukocytes (Table 3.3 and 3.4). We tested a number of anti-CD11c and anti-CD11a mAb but found relatively lower levels of staining on both human and bovine cells. Of the mAb tested, one anti-CD11c mAb (BAQ153) stained bovine cells (Table 3.3 and 3.4) and one anti-CD11a mAb (DF1524) preferentially stained human cells (Table 3.4). For the subsequent cross-linking studies, mAb MHM23 was chosen for the primary antibody due to its intensity of staining on bovine and human leukocytes.

Table 3.3: Neutrophils

mAb	Subunit	Human	Human/PMA	Bovine	Bovine/PMA
IB4	CD18	18.8 ± 5.5	116.5 ± 35.8	17.1 ± 5.6	138.0 ± 41.8
MHM23	CD18	61.3 ± 12.4	310.7 ± 159.3	65.5 ± 10.9	155.2 ± 13.0
44a	CD11b	18.0 ± 2.8	126.5 ± 23.0	6.5 ± 2.8	35.0 ± 12.7
OKM1	CD11b	14.6 ± 3.7	50.7 ± 4.9	5.6 ± 2.8	32.7 ± 5.9
MM12a	CD11b	5.4 ± 4.2	2.0 ± 1.6	199.4 ± 17.4	621.6 ± 74.7
2LPM19c	CD11b	73.0 ± 16.7	563.2 ± 167.9	1.7 ± 0.8	4.5 ± 5.1
B-Ly6	CD11c	6.8 ± 2.8	5.5 ± 2.7	7.1 ± 3.7	39.4 ± 3.1
RDI 3.9	CD11c	10.4 ± 2.4	8.4 ± 1.4	0.6 ± 0.8	13.0 ± 15.0
SHCL3	CD11c	4.3 ± 2.3	6.8 ± 3.6	5.4 ± 2.0	4.9 ± 4.0
BAQ153	CD11c	4.0 ± 5.0	3.5 ± 5.3	5.8 ± 3.8	79.0 ± 18.3
DF1524	CD11a	27.8 ± 5.2	16.5 ± 4.9	0.1 ± 0.2	0.8 ± 1.2
TS 2/4	CD11a	6.3 ± 2.0	5.8 ± 2.1	9.9 ± 3.7	12.0 ± 7.2

Table 3.3 Cross-reactivity of Anti-β_2 Integrin mAb with Human and Bovine Neutrophils. Untreated and PMA-treated (30 ng/ml) human and bovine neutrophils were incubated with 50 μg/ml of primary mAb, washed, and incubated with appropriate FITC-conjugated secondary mAb. Cells were analyzed by flow cytometry as described under Materials and Methods. Results are expressed as mean fluorescent intensity ± SEM minus background secondary control, N=3.

Table 3.4: Monocytes

mAb	Subunit	Human	Human/PMA	Bovine	Bovine/PMA
IB4	CD18	34.8 ± 19.9	36.7 ± 14.7	34.2 ± 8.6	37.3 ± 2.8
MHM23	CD18	122.3 ± 69.0	96.5 ± 46.1	120.7 ± 10.9	176.0 ± 6.4
44a	CD11b	19.3 ± 8.6	26.1 ± 4.4	21.5 ± 8.7	17.3 ± 3.0
OKM1	CD11b	15.0 ± 7.7	19.4 ± 2.8	8.2 ± 5.7	9.2 ± 7.4
MM12a	CD11b	4.6 ± 1.6	2.8 ± 0.6	147.2 ± 50.4	164.0 ± 19.3
2LPM19c	CD11b	108.0 ± 76.8	122.4 ± 56.3	7.9 ± 1.3	8.5 ± 1.0
B-Ly6	CD11c	12.3 ± 5.5	9.2 ± 3.3	20.2 ± 3.3	22.9 ± 2.4
RDI 3.9	CD11c	22.1 ± 5.7	24.4 ± 3.6	9.6 ± 5.4	10.7 ± 4.7
SHCL3	CD11c	7.6 ± 1.2	7.0 ± 0.1	16.3 ± 0.9	16.4 ± 1.8
BAQ153	CD11c	3.9 ± 3.7	4.1 ± 6.2	25.5 ± 14.0	44.8 ± 14.4
DF1524	CD11a	72.0 ± 26.2	63.4 ± 15.3	1.3 ± 0.4	1.1 ± 0.5
TS 2/4	CD11a	13.0 ± 8.0	13.9 ± 8.8	20.2 ± 9.5	8.6 ± 0.7

Table 3.4 Cross-reactivity of Anti-β_2 Integrin mAb with Human and Bovine Monocytes. Untreated and PMA-treated (30 ng/ml) human and bovine monocytes were incubated with 50 μg/ml of primary mAb, washed, and incubated with appropriate FITC-conjugated secondary mAb. Cells were analyzed by flow cytometry as described under Materials and Methods. Results are expressed as mean fluorescent intensity ± SEM minus background secondary control, N=3.

Table 3.5: Lymphocytes

mAb	Subunit	Human	Human/PMA	Bovine	Bovine/PMA
IB4	CD18	21.7 ± 6.7	14.1 ± 2.0	7.9 ± 1.9	10.4 ± 0.5
MHM23	CD18	82.5 ± 22.2	62.0 ± 18.3	66.4 ± 2.4	103.3 ± 12.9
44a	CD11b	2.6 ± 0.9	3.8 ± 1.3	1.8 ± 0.7	1.5 ± 0.7
OKM1	CD11b	2.1 ± 0.8	2.4 ± 1.1	1.0 ± 0.6	1.1 ± 0.3
MM12a	CD11b	2.1 ± 0.7	1.7 ± 1.1	17.5 ± 6.8	16.9 ± 2.6
2LPM19c	CD11b	13.8 ± 3.0	24.7 ± 4.8	1.3 ± 0.7	0.9 ± 0.3
B-Ly6	CD11c	3.2 ± 1.2	2.2 ± 0.6	3.8 ± 1.3	2.7 ± 0.5
RDI 3.9	CD11c	4.2 ± 4.7	2.0 ± 0.5	1.2 ± 0.9	1.0 ± 0.4
SHCL3	CD11c	1.6 ± 0.2	1.0 ± 0.3	4.3 ± 0.6	2.8 ± 0.5
BAQ153	CD11c	0.7 ± 0.8	0.7 ± 0.8	2.2 ± 0.9	6.2 ± 6.0
DF1524	CD11a	74.9 ± 13.2	63.5 ± 8.0	0.6 ± 0.5	0.3 ± 0.2
TS 2/4	CD11a	10.2 ± 4.0	9.3 ± 4.0	4.3 ± 3.0	1.9 ± 1.1

Table 3.5 Cross-reactivity of Anti-β_2 Integrin mAb with Human and Bovine Lymphocytes. Untreated and PMA-treated (30 ng/ml) human and bovine lymphocytes were incubated with 50 μg/ml of primary mAb, washed, and labeled with FITC-conjugated secondary mAb. Cells were analyzed by flow cytometry as described under Materials and Methods. Results are expressed as mean fluorescent intensity ± SEM minus background secondary control, N=3.

The effects of shipping on neutrophil surface antigen expression was previously evaluated in this lab (62) by measurement of CD11b, CD18, and L-selectin expression, since changes in the level of expression of these antigens provides a sensitive measure of neutrophil priming or activation (63-66). Expression of these antigens on neutrophils from shipped blood of clinically normal calves or heterozygous BLAD calves was not significantly different from that of fresh bovine blood from clinically normal calves (62), suggesting the effects of shipping on cell surface antigen expression were minimal.

Calcium Response in Cross-linked
Bovine Neutrophils

To determine if β_2 integrin cross-linking induced bovine neutrophil cell signaling

events, we analyzed intracellular free calcium concentration $[Ca^{2+}]_i$ in cells treated with cross-linking mAb. Upon CD18 cross-linking, we observed an increased $[Ca^{2+}]_i$ in neutrophils isolated from clinically normal animals, and addition of increasing amounts of cross-linking mAb caused a dose-dependent increase in $[Ca^{2+}]_i$ with the peak response at 75μl of mAb (Figure 3.1). The calcium response observed with cross-linking of the β_2 integrins corresponded with the second, prolonged peak seen after PAF stimulation in human neutrophils. Interestingly, this phase of the $[Ca^{2+}]_i$ response has been reported to be a result of the activation of 5-lipoxigenase and the subsequent generation of leukotriene B_4 (LTB_4) (67), suggesting that β_2 integrin cross-linking may utilize the lipoxygenase signaling pathway. Primary and secondary control mAb elicited no $[Ca^{2+}]_i$ response (Figure 3.1).

The increase in $[Ca^{2+}]_i$ due to cross-linking of the β_2 integrins on bovine neutrophils was significantly ($P<0.001$) lower than that induced by stimulation with PAF (44) (Figure 3.2). However, the peak $[Ca^{2+}]_i$ increase in PAF-stimulated normal bovine neutrophils was comparable to that seen in similarly treated human neutrophils (approximately 220 nM) (67). In addition, the rise of $[Ca^{2+}]_i$ induced by CD18 cross-linking of clinically normal bovine neutrophils was undistinguishable from that induced by β_2 integrin cross-linking in heterozygous BLAD neutrophils (155 ± 6.6 and 157 ± 7.9, respectively). (Figure 3.2). As expected, the increase in $[Ca^{2+}]_i$ in homozygous BLAD neutrophils subjected to the same treatment was significantly lower ($p<0.001$) compared to cross-linked normal and heterozygous BLAD neutrophils (58.9 ± 2.1) (Figure 3.2).

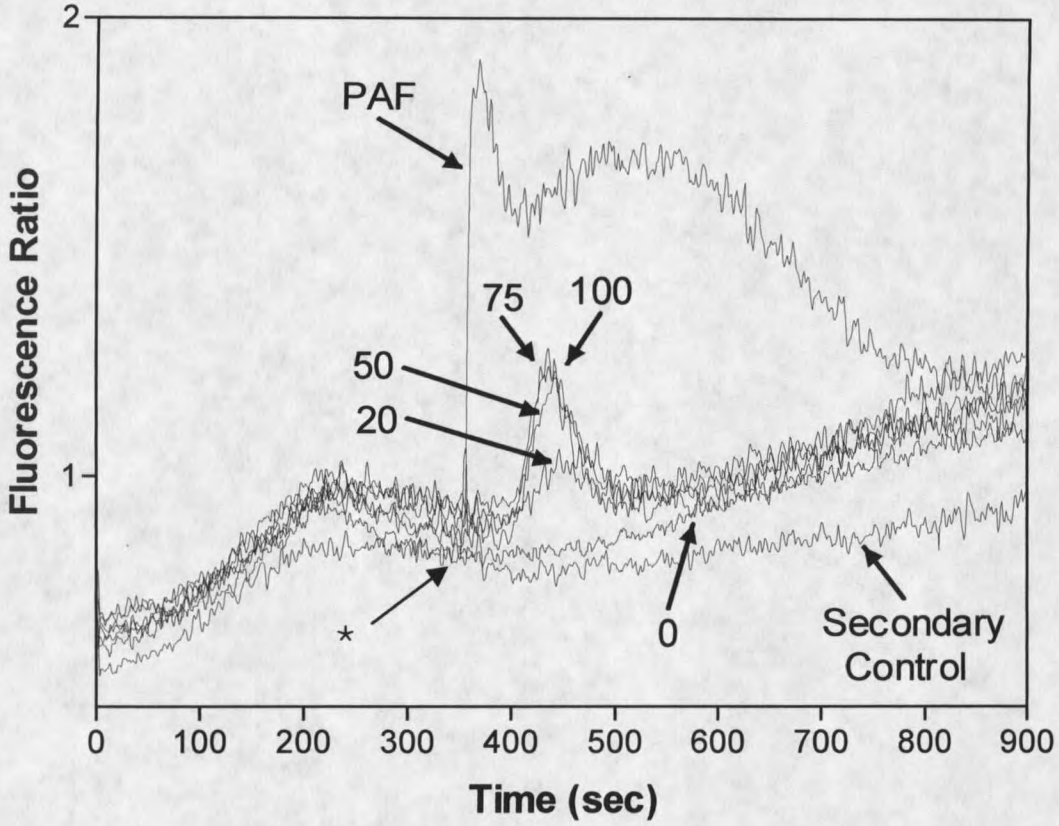

Figure 3.1 Dose-dependent Increase in Intracellular Free Calcium in Response to CD18 Cross-linking. Fura 2-loaded normal bovine neutrophils pretreated with anti-CD18 mAb (MHM23) were stimulated with secondary cross-linking mAb (20µl – 100 µl). Secondary control represents neutrophils without primary antibody and "0" represents primary treated neutrophils without the addition of secondary cross-linking mAb. Calcium response to cross-linking mAb is compared to that induced with 10^{-7} M PAF. * Indicates point at which cross-linking mAb or PAF was introduced. Representative of two separate experiments.

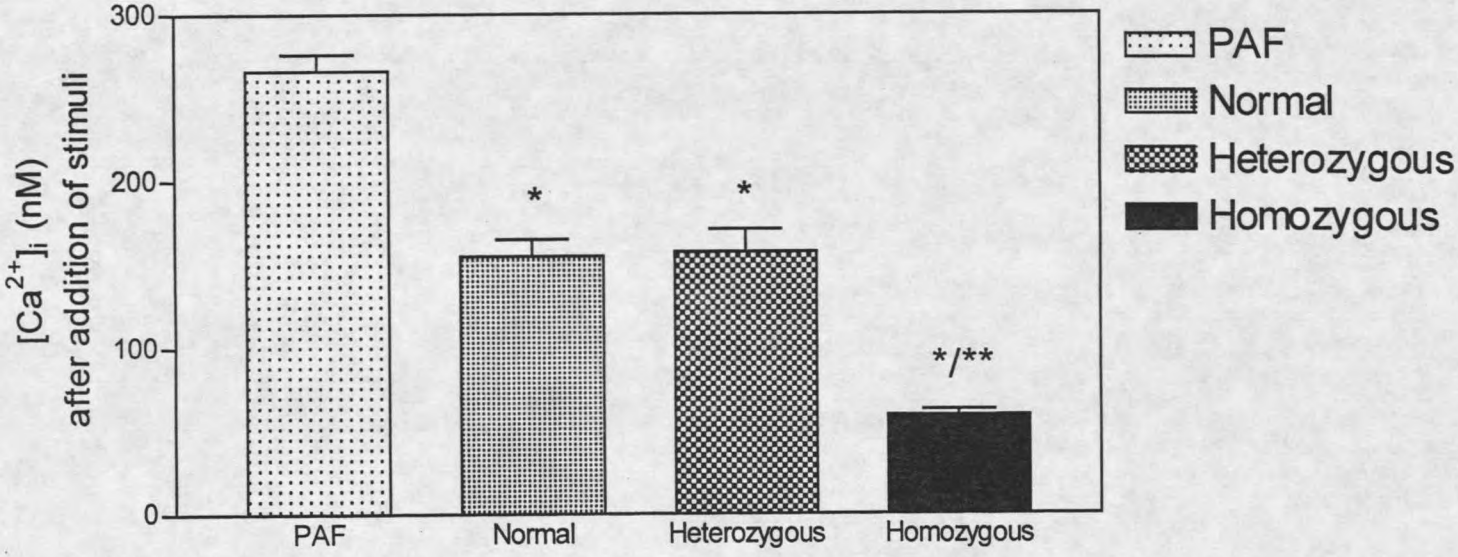

Figure 3.2 Changes in Intracellular Free Calcium in Response to CD18 Cross-linking. Primary antibody-treated bovine neutrophils were incubated with the fluorescent dye Fura 2/AM and then stimulated with 10^{-7} M PAF (normal bovine neutrophils) or cross-linking mAb (normal, heterozygous, and homozygous BLAD neutrophils) as described under Materials and Methods. Intracellular free calcium concentrations are expressed as mean concentration ± SEM of three separate experiments. *P<0.001 as compared to PAF, **P<0.001 as compared to normal and heterozygous.

Surface Antigen Expression

To determine if β_2 integrin cross-linking altered bovine neutrophil surface antigen expression, we examined cell surface expression of L-selectin and CD18 in treated cells. Neutrophils from normal and heterozygous BLAD cattle showed a time-dependent increase in staining of cell surface L-selectin (DREG-56) after CD18 cross-linking (Figure 3.3). Neutrophils from normal cattle showed significant ($P<0.01$) increased staining at 25 minutes, while heterozygous BLAD neutrophils showed a similar increase in staining at an earlier time point (10 minutes). In contrast, neutrophils isolated from homozygous BLAD cattle showed very little increase in L-selectin staining over the entire 60 minutes tested.

To rule out non-specific binding of the FITC-conjugated mAbs, the L-selectin study was repeated with a FITC-conjugated isotype specific control (anti-TCR) or blocking with 10% mouse serum prior to addition of the anti-L-selectin antibody. At 30 minutes post cross-linking, both blocked and unblocked neutrophils had a comparable increase in L-selectin staining (129% & 120%, respectively), whereas the isotype control showed no staining above background (Data not shown). These controls demonstrate the specificity of our data showing up-regulation of L-selectin in response to β_2 integrin cross-linking.

After addition of the cross-linking mAb, cell surface expression of CD18 was up-regulated on neutrophils isolated from normal cattle, again showing a significant increase ($p<0.01$) at ~25 minutes (Figure 3.4). Neutrophils from heterozygous BLAD cattle also showed a significant increase in staining at an earlier time ($P<0.05$ at 15 minutes and

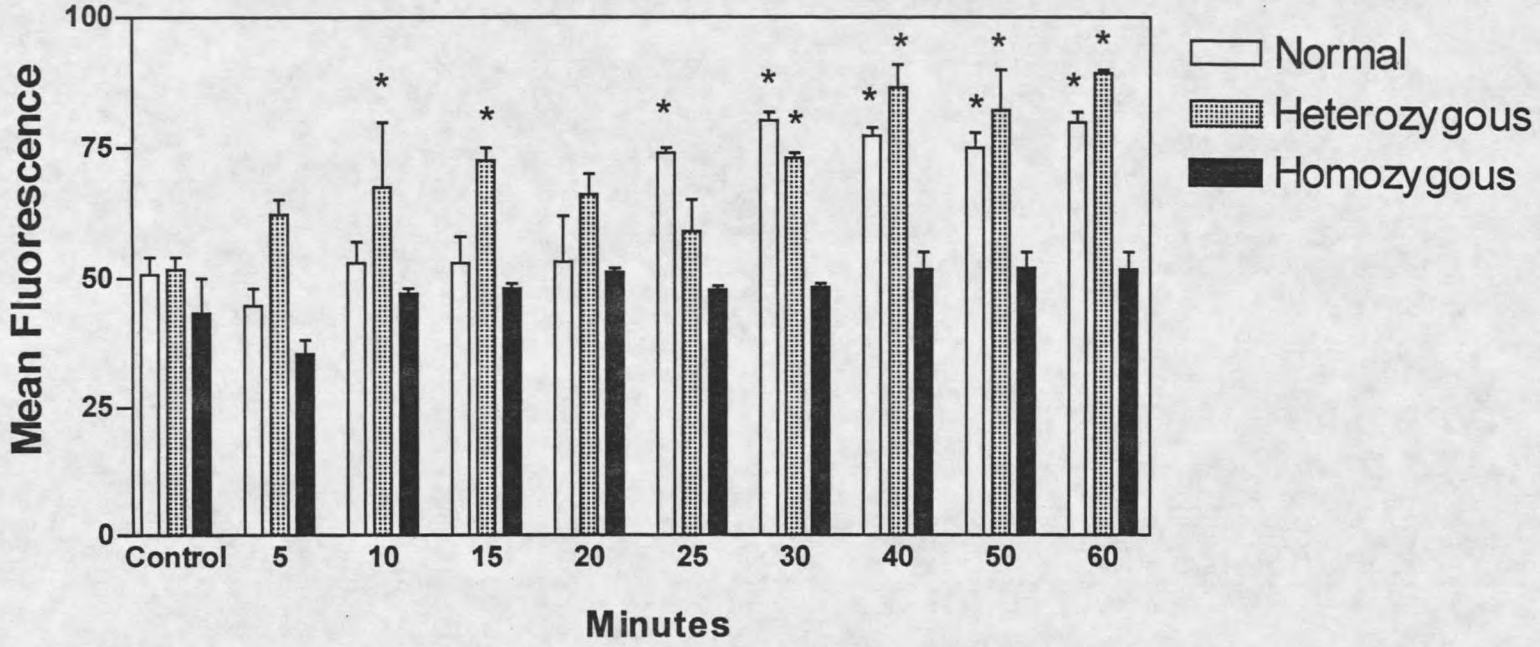

Figure 3.3 Staining of Cell-surface L-selectin in Response to CD18 Cross-linking. Flow cytometric analysis of the expression of L-selectin as detected with FITC-conjugated DREG-56 (dilution 1:400). MHM23 antibody-treated normal, heterozygous, and homozygous BLAD neutrophils were cross-linked at 37°C for the times indicated. Results are expressed as mean fluorescence ± SEM, N=3. Control represents primary antibody-treated neutrophils without secondary cross-linking mAb at 37°C for 60 minutes. *P<0.001 compared to control.

$P<0.01$ at 20 minutes). Consistent with the absence of the β_2 integrin molecules on the surface of the neutrophils from the homozygous BLAD cattle, surface staining of CD18 remained at background levels throughout the testing (Figure 3.4).

Degranulation of neutrophil specific granules was analyzed by measuring the change in the relative amount of lactoferrin on the cell surface at various times after CD18 cross-linking. Neutrophils from both normal and heterozygous BLAD cattle showed increased staining ($P<0.01$) of cell surface lactoferrin 25 minutes after cross-linking CD18 (Figure 3.5). In contrast, homozygous BLAD neutrophils showed very little increase in cell surface lactoferrin staining.

Redistribution of Proteins Induced
by CD18 Cross-linking

As shown in Figure 3.6, antibody to $p58^{fgr}$ detected a 58kD protein in both membrane and cytosol fractions of human neutrophils and bovine membrane samples. In contrast, anti-$p58^{fgr}$ also detected a smaller (50kD) protein in the cytosol fraction from bovine neutrophils (Figure 3.6). To determine whether CD18 cross-linking on bovine neutrophils causes a redistribution of intracellular proteins, we cross-linked CD18 on suspended cells for various times and, analyzed $f58^{fgr}$ redistribution to the Triton X-100 insoluble fraction. In CD18 cross-linking studies on normal and heterozygous BLAD bovine neutrophils, $p58^{fgr}$ redistributed from the Triton X-100 soluble fraction (50kD) to the insoluble fraction (58kD) at ~30 minutes (Figure 3.7; Normal data not shown). This redistribution was not seen in cell fractions from similarly treated neutrophils isolated

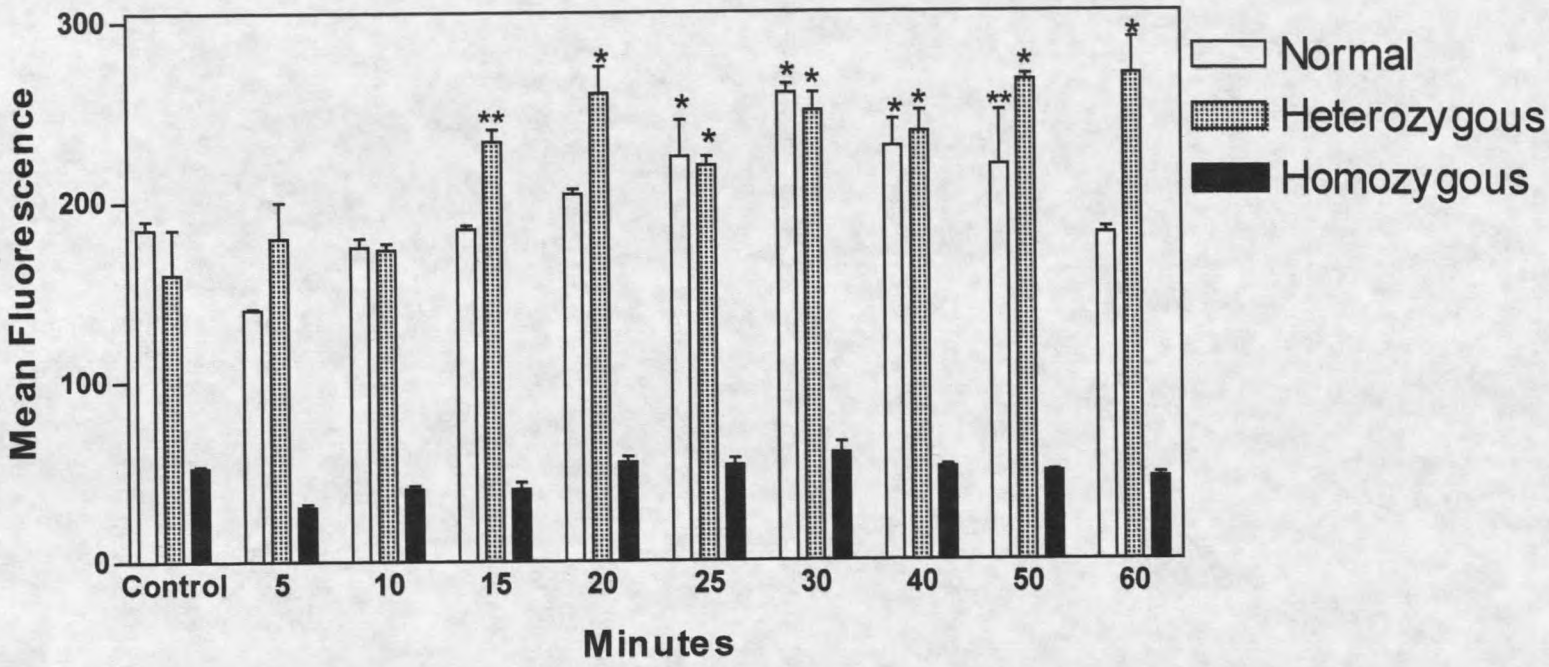

Figure 3.4 Staining of Cell-surface CD18 in Response to Cross-linking. Flow cytometric analysis of the expression of CD18 as detected with FITC-conjugated IB4 (50 μg/ml). MHM23 antibody-treated normal, heterozygous and homozygous BLAD neutrophils were cross-linked at 37°C for the times indicated. Results are expressed as mean fluorescence ± SEM, N=3. Control represents primary antibody-treated neutrophils without secondary cross-linking mAb at 37°C for 60 minutes. *P<0.01 compared to control, **P<0.05 compared to control.

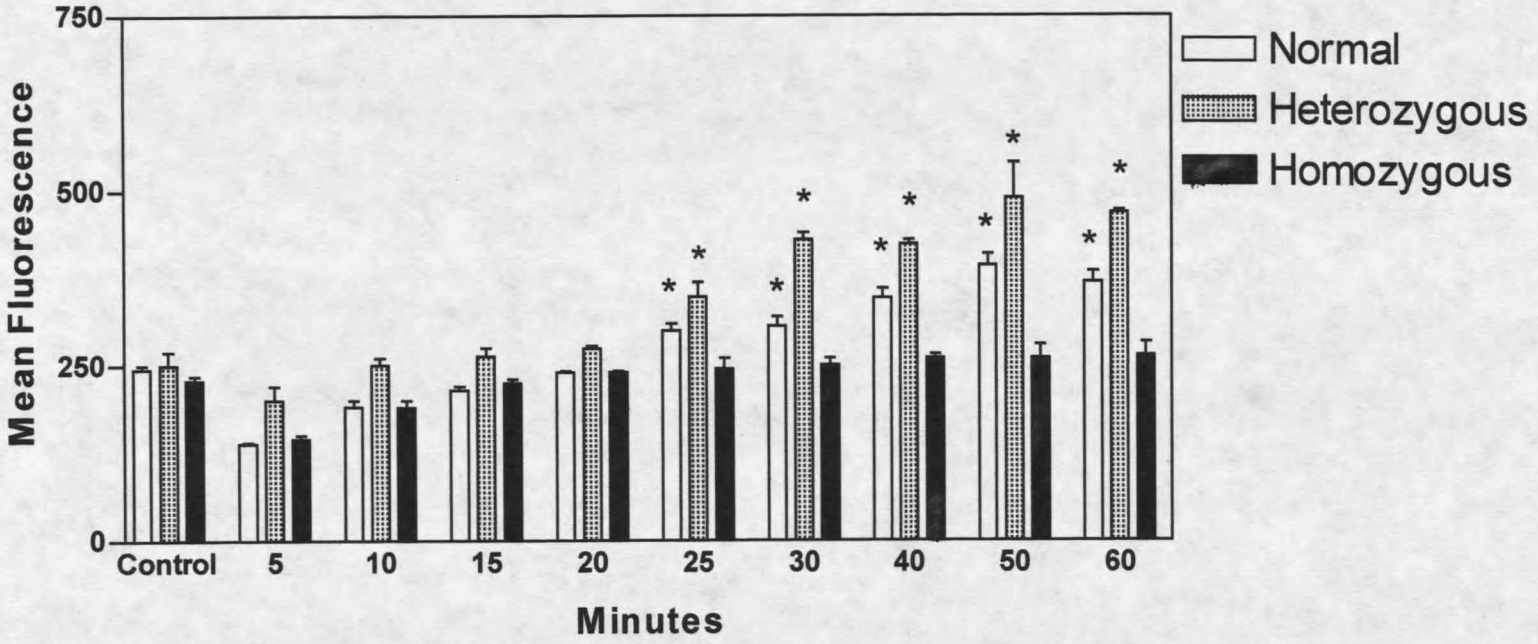

Figure 3.5 Staining of Cell-surface Lactoferrin in Response to CD18 Cross-linking. Flow cytometric analysis of neutrophil cell-surface lactoferrin as detected with FITC-conjugated BL97 (50 μg/ml). MHM23 antibody-treated normal, heterozygous and homozygous BLAD neutrophils were cross-linked at 37°C for the times indicated. Results are expressed as mean fluorescence ± SEM, N=3. Control represents primary antibody-treated neutrophils without secondary cross-linking mAb at 37°C for 60 minutes. *P<0.01 compared to control.

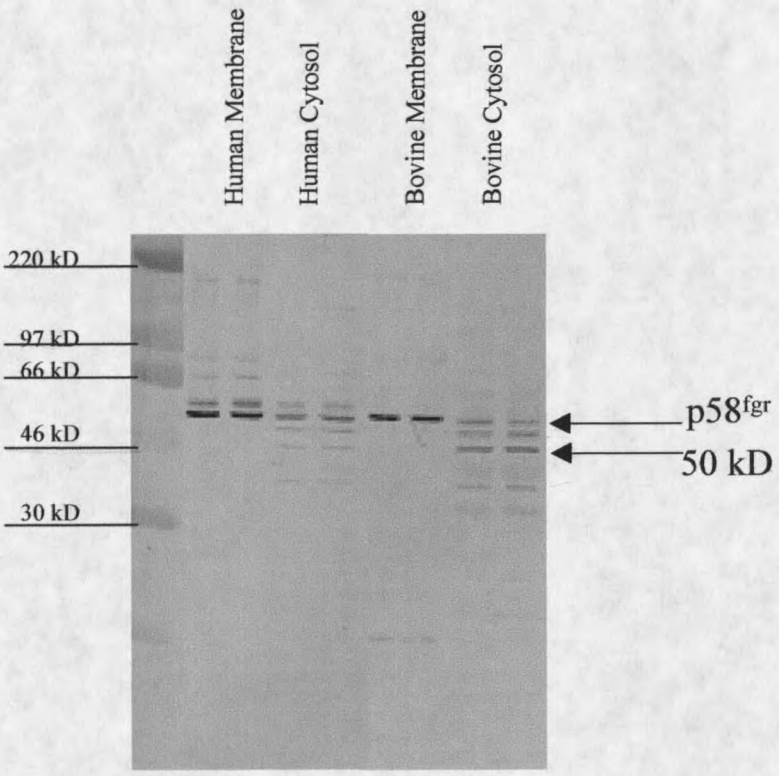

Figure 3.6 Western Blot of Neutrophil Membrane and Cytosol Fractions with Anti-p58^{fgr}. Membrane and cytosol fractions from unstimulated neutrophils isolated from human or clinically normal bovine blood were immunoblotted with anti-p58^{fgr}. Representative of immunoblots from three separate experiments. Samples were run in duplicate.

from an animal homozygous for BLAD (Figure 3.7). Unlike $p58^{fgr}$, the cytoskeletal protein, α-actinin, remained consistently distributed in both the Triton X-100 insoluble and soluble fractions throughout the cross-linking studies in neutrophils from animals heterozygous or homozygous for BLAD (Figure 3.8).

Western Blots

To identify anti-β_2 integrin antibodies that detect the β_2 integrin subunits in bovine neutrophil membranes, we tested 12 antibodies on Western blots of human and bovine neutrophil cell membranes as described in Materials and Methods. In human neutrophil membranes, CD11b (170 kD) was detected with 5 of the antibodies (R7928a, 13.22, TS1, 44a, and Dako 11b), CD11a (180 kD) was detected with 1 antibody (Immunotech), and CD18 (95 kD) was detected with one antibody (13.22) (Figure 3.9). In bovine neutrophil membranes, only one antibody (R7928a) detected CD11b at ~160 kD (Figure 3.9). The higher band at ~230 kD appears to be a non-specific band.

Previous reports have shown that the β chain (CD18) of the β_2 integrins redistributes to the Triton X-100 insoluble fraction upon adherent-dependent activation of human neutrophils with peak redistribution at 30 minutes (68). In contrast, we did not detect a redistribution of CD11b (~160 kD) to the Triton X-100 insoluble fraction of normal or heterozygous BLAD bovine neutrophils upon CD18 cross-linking (Figure 3.10; Normal data not shown). Immunoblots of cross-linked bovine neutrophils from calves heterozygous for BLAD with the antibody R7928a indicated that CD11b remained in the Triton X-100 soluble fraction throughout the experiment (Figure 3.10). Due to the

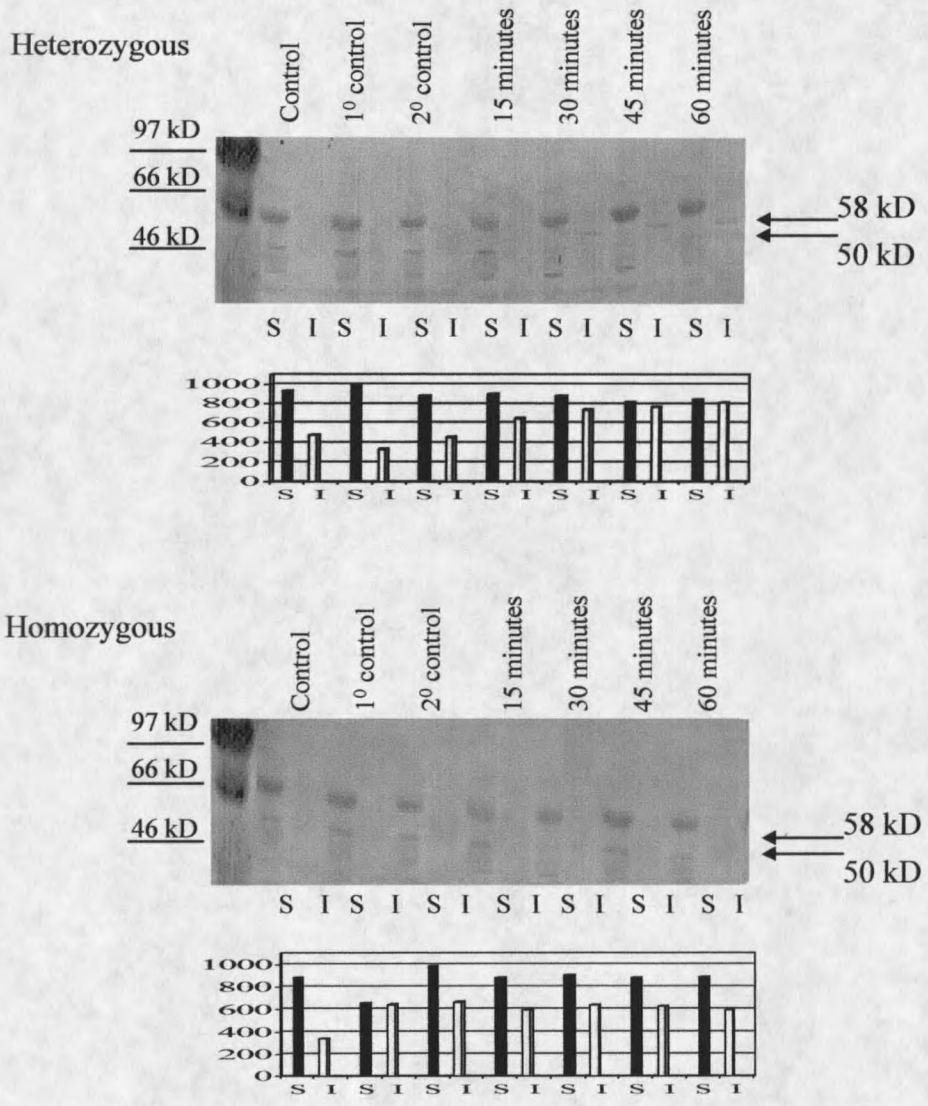

Figure 3.7 Relocation of p58^{fgr} in Response to β_2 Integrin Cross-linking. MHM23 antibody-treated or untreated heterozygous or homozygous BLAD neutrophils were cross-linked at 37^{0}C for the times indicated. Triton X-100 soluble (S) and insoluble (I) fractions were immunoblotted for the detection of p58^{fgr}. Graph represents the integrated density value of each lane with the background subtracted. Control is untreated cells at 37^{0}C for 60 minutes. Primary control is without cross-linking antibody. Secondary control is without primary antibody. Data are representative of three separate experiments.

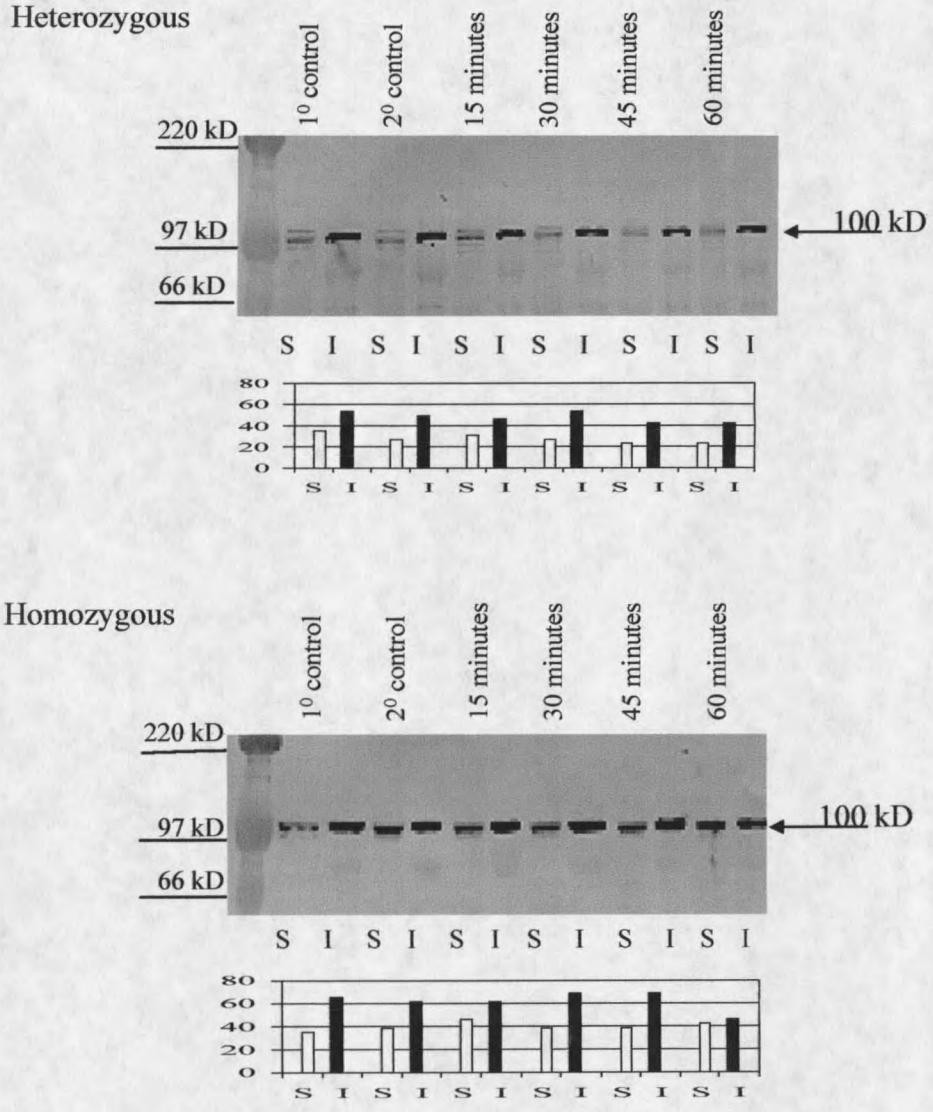

Figure 3.8 Detection of α-actinin in Response to β_2 Integrin Cross-linking. MHM23 antibody-treated or untreated heterozygous or homozygous BLAD neutrophils were cross-linked at 37°C for the times indicated. Triton X-100 soluble and insoluble fractions were immunoblotted for the detection of α-actinin. Graph represents the integrated density value of each lane with the background subtracted. Primary control is without cross-linking antibody. Secondary control is without primary antibody. Data are representative of three separate experiments.

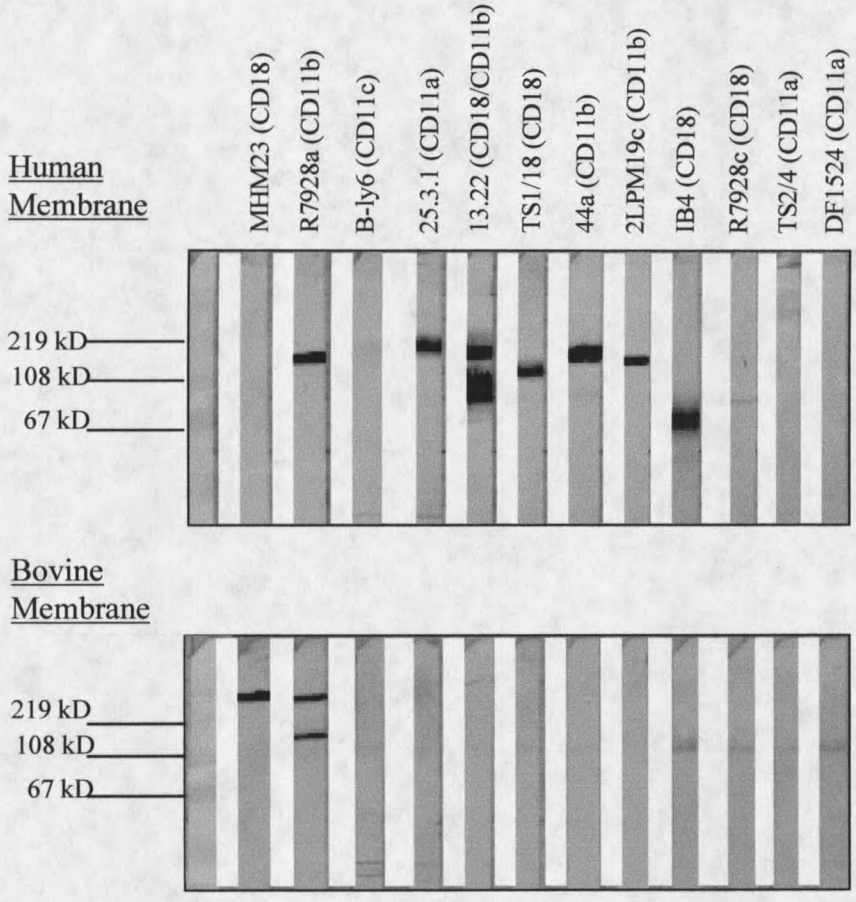

Figure 3.9 Immunoblot Detection of the β_2 Integrin Subunits. Octyl glucoside-membrane fractions from unstimulated neutrophils isolated from human and clinically normal bovine blood were immunoblotted with 12 antibodies specific for the various subunits of the β_2 integrins. Representative of 3 (positive antibodies) or 2 (negative antibodies) separate experiments.

lack of CD11b in BLAD neutrophils, there was not a 160 kD protein band detected in either the soluble or insoluble fractions of similarly treated neutrophils from an animal homozygous for BLAD (Figure 3.10).

Immunoprecipitation

To identify anti-β_2 integrin antibodies that immunoprecipitate the β_2 integrin subunits from bovine neutrophil lysates, we immunoprecipitated bovine neutrophil lysates with several anti-β_2 integrin antibodies (Figure 3.11). Silver staining of the 68-5A5 (RDI-CD18) immunoprecipitate revealed three bands corresponding to the expected molecular weights of the three α subunits of the β_2 integrins (~150 kD–180 kD) and one band at the expected molecular weight of the β subunit of the β_2 integrins (~97 kD) (Figure 3.11). Silver staining of the 3.9 (RDI-CD11c) immunoprecipitate revealed one protein band at ~180 kD which corresponds to the molecular weight of CD11c (Figure 3.11). Western blots on the immunoprecipitations with the anti-CD11b antibody R7829a, confirmed that one of the three higher molecular weight bands in the 68-5A5 (RDI-CD18) immunoprecipitate from the normal bovine neutrophils was indeed CD11b (Figure 3.11). As expected, the ~180 kD band in the 3.9 (RDI-CD11c) immunoprecipitate was not detected with the anti-CD11b antibody R7928a (Figure 3.11).

To possibly identify the other bands in the β_2 integrin immunoprecipitate, immunoprecipitations were also done on cell lysates of neutrophils isolated from a homozygous BLAD calf. Interestingly, immunoprecipitations with 68-5A5 (RDI-CD18) on lysates from neutrophils from an animal homozygous for BLAD revealed two higher

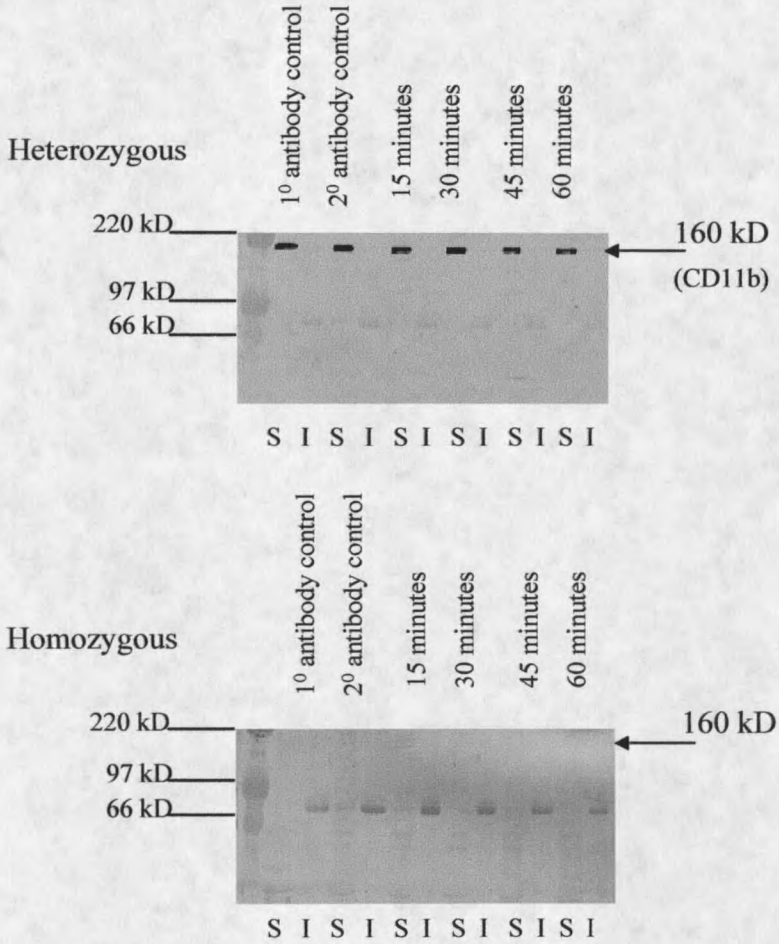

Figure 3.10 Detection of CD11b in Response to β_2 Integrin Cross-linking. MHM23 antibody-treated or untreated heterozygous or homozygous BLAD neutrophils were cross-linked at 37°C for the times indicated. Triton X-100 soluble and insoluble fractions were immunoblotted with R7928a (anti-CD11b). Primary control is without cross-linking antibody. Secondary control is without primary antibody. Representative of three separate experiments.

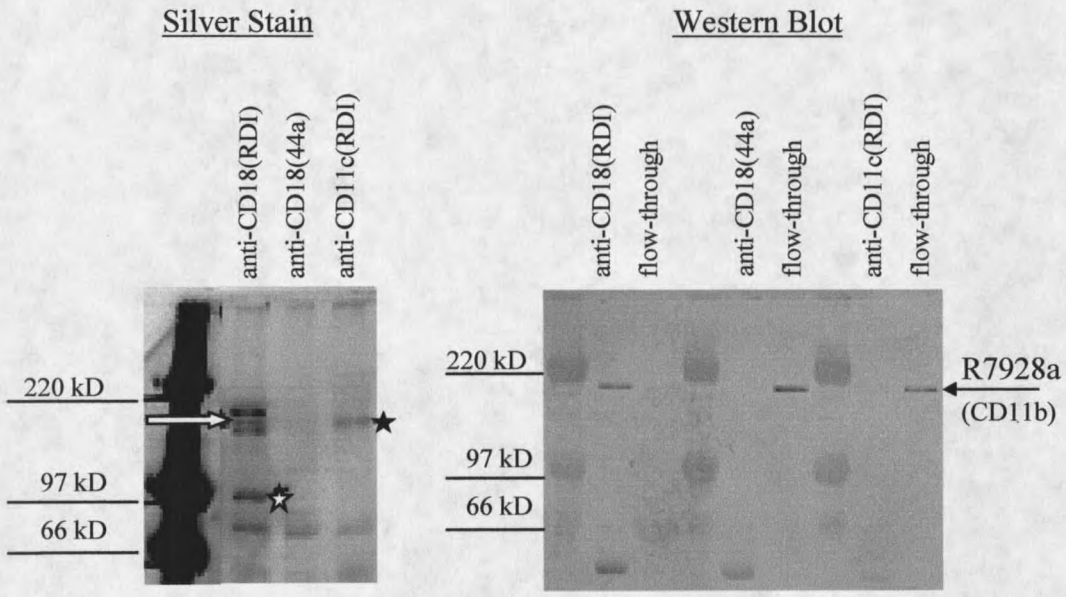

Figure 3.11 Immunoprecipitation of Bovine Neutrophil Lysates with Antibodies to the β_2 Integrin Subunits. Triton X-100 soluble neutrophil lysates were immunoprecipitated with two mAb to the β_2 integrin subunits, 68-5A5 (RDI–CD18) and 3.9 (RDI–CD11c), or one control mAb (44a). The three α subunits, CD11a,b,c (white arrow), and the β subunit CD18 (white star) were immunoprecipitated with 68-5A5. CD11c (black star) was immunoprecipitated with 3.9. Immunoprecipitates were visualized by silver staining. CD11b was detected by immunoblotting with R7928a. Representative of three separate experiments.

molecular weight protein bands (~150 kD–180 kD) and another band at ~97 kD. (Figure 3.12). Similar to the normal bovine sample, immunoprecipitation of homozygous BLAD neutrophil lysates with 3.9 (RDI-CD11c) revealed one protein band at ~180 kD with silver staining (Figure 3.12). Western blot analysis of the immunoprecipitates (68-5A5 and 3.9) from the normal and homozygous BLAD neutrophil lysates with the anti-CD11b antibody R7928a again revealed that the α subunit, CD11b, was one of the proteins immunoprecipitated with 68-5A5 (RDI-CD18) from the normal bovine neutrophil lysates (Figure 3.12), but none of the bands immunoprecipitated from the homozygous BLAD neutrophil lysates were recognized by R7928a (Figure 3.12), suggesting they were different than normal CD11b.

Discussion

It is well established that the β_2 integrins are involved in numerous neutrophil functions (1-12). In the present study, we show that ligation of CD18 on bovine neutrophils through mAb cross-linking causes a transient increase in $[Ca^{2+}]_i$, upregulation of cell surface L-selectin and CD18, degranulation of specific granules, and redistribution of the cytosolic tyrosine kinase $p58^{fgr}$. In addition, we identified two mAb that immunoprecipitate one or more of the β_2 integrin subunits from bovine neutrophil immunoprecipitated lysates. Surprisingly, in neutrophil lysates from the homozygous animal, these two mAb bands corresponding to three of the β_2 integrin subunits. In all species where it has been studied, including human and bovine, isolated neutrophils exhibit an increase in $[Ca^{2+}]_i$ in response to platelet activating factor (PAF) (44,67). This

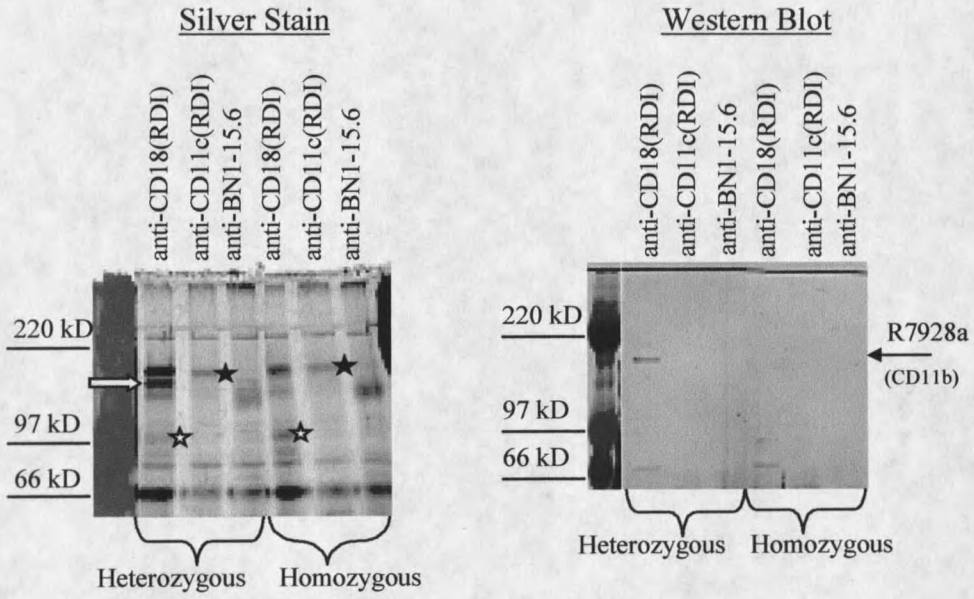

Figure 3.12 Immunoprecipitation of Heterozygous and Homozygous BLAD Neutrophil Lysates with Antibodies to the β_2 Integrin Subunits. Triton X-100 soluble neutrophil lysates from calves heterozygous or homozygous for BLAD were immunoprecipitated with two mAb to the β_2 integrin subunits, 68-5A5 (RDI–CD18) and 3.9 (RDI–CD11c), or one control mAb (BN1-15.6). In the heterozygous sample, the three α subunits, CD11a,b,c (white arrow), and the β subunit CD18 (white star) were immunoprecipitated with 68-5A5. CD11c (black star) was immunoprecipitated with 3.9. In the homozygous sample, two higher molecular weight bands and the lower molecular weight band were immunoprecipitated with 68-5A5. The single CD11c band was also immunoprecipitated in the homozygous sample. Immunoprecipitates were visualized by silver staining. CD11b subunit was detected by immunoblotting with R7928a. Representative of three separate experiments.

response in bovine and human neutrophils is both measurable at similar concentrations of PAF (10^{-7} M, and a similar amplitude of $[Ca^{2+}]_i$ is observed (approximately 220 nM) (44,69). With PAF stimulation, the increase in $[Ca^{2+}]_i$ peaks within 20 seconds and declines back to baseline within 5 minutes (44) after a second smaller but prolonged peak (67). We have shown that the rise of $[Ca^{2+}]_i$ induced by CD18 cross-linking in normal bovine neutrophils was dependent on the concentration of the secondary mAb (Figure 3.1). This increase in $[Ca^{2+}]_i$ is smaller in size but delayed as compared to stimulation with 10^{-7} M PAF (44). The calcium flux in bovine neutrophils corresponds with the second calcium peak induced with PAF. This supports the theory that cell signaling through the β_2 integrins involves a PLC pathway (39), specifically the activation of 5-lipoxygenase and the subsequent activation of LTB_4 (67).

The increase in $[Ca^{2+}]_i$ observed after cross-linking CD18 on neutrophils isolated from heterozygous BLAD animals is comparable to that seen in normal bovine neutrophils (Figure 3.2). As expected, the $[Ca^{2+}]_i$ increase seen in similarly treated neutrophils isolated from homozygous BLAD cattle is significantly reduced. These findings suggest that the observed response is, in part, specific for CD18. The small $[Ca^{2+}]_i$ response seen in the homozygous BLAD neutrophils may be due to the interaction of the neutrophil Fc receptor with the Fc portion of the primary or secondary mAb. Nagahata et al. (70) reported enhanced expression of Fc receptors (FcR) on neutrophils from calves with BLAD, but Fc receptor-mediated responses were lower than or equivalent to those of normal animals (10), supporting the hypothesis that the β_2 integrins are needed for correct FcR-mediated neutrophil function (10,70).

Activating agents typically cause shedding of L-selectin on neutrophils (40,71,72), although Burton et al. (73) reported that bovine neutrophil L-selectin shows only a slight decrease with PAF stimulation. The slight increase in L-selectin that we show as a result of cross-linking the β_2 integrins (Figure 3.3) may be due to the up-regulation of the DREG-56 epitope on L-selectin. One possible explanation is that DREG-56 may bind with higher affinity after cross-linking of the β_2 integrins. Another possibility explaining the increased staining of surface L-selectin upon β_2 integrin cross-linking would be a quantitative increase of the L-selectin molecules from granule stores. It has been reported that the shedding of L-selectin is regulated in a more restricted manner than other neutrophil responses (40). Loss of L-selectin on human neutrophils following CD18 cross-linking occurs to a lesser extent than the loss of L-selectin seen with other stimuli like fMLP or PMA, also suggesting a more complex regulation of L-selectin shedding (40).

Increased staining for CD18 has been previously reported after β_2 integrin cross-linking in human neutrophils (40) and upon PAF (44) and PMA stimulation (62) in bovine neutrophils. Neutrophil stimulation activates β_2 integrin-dependent functions by inducing conformational changes in the integrins (70). In addition, β_2 integrin expression is up-regulated upon neutrophil activation and priming by exocytosis of specific granules, which are storage sites for CD11b,c/CD18 (44,74). In our studies, the increased CD18 staining of cross-linked neutrophils from normal and heterozygous BLAD animals could be due to a conformational change in the CD18 molecule or exocytosis of the specific granules. Walzog et al. (40) has shown that the binding of FITC-conjugated MHM23

(anti-CD18) on human neutrophils was reduced to almost background levels by pretreatment with unlabeled IB4 mAb (antibody specificity). Consistent with their findings, out data also suggests that the increased expression of CD18 on the normal and heterozygous neutrophil surface after cross-linking represents an increase in the number of CD18 molecules on the cell surface due to exocytosis of granule stores. This conclusion is supported by our data showing concomitant increases in cell surface lactoferrin. Lactoferrin release is a common marker for specific granule degranulation in both human and bovine neutrophils (44,75). Recent studies on human neutrophils show that trace amounts of lactoferrin are present on the neutrophil cell membrane and this amount increases with activation by PMA, fMLP, and TNF-α (76,77). This has also been shown in bovine neutrophils stimulated with PAF or PMA (44). We show that exocytosis of the specific granules, as measured by lactoferrin release, was similar in normal and heterozygous BLAD neutrophils after CD18 cross-linking. This response was significant around 25 minutes after cross-linking, which corresponds to the change in the other surface molecules we studied. Consistent with the absence of β_2 integrins on homozygous cells, little increase in surface lactoferrin on similarly treated BLAD neutrophils occurred after the addition of cross-linking antibody

Upon adherence-dependent β_2 integrin cross-linking or mechanical integrin loading, tyrosine phosphorylated proteins become physically anchored to the cytoskeleton (36,78). The tyrosine kinase, $p58^{fgr}$, which is specifically expressed in granulocytes, monocytes, and natural killer cells (25) is associated with FcγRII and is involved in FcγRII-mediated signal transduction pathways in human neutrophils (40,79).

$p58^{fgr}$ is associated with the plasma membrane and, during release of secondary granules, is translocated from a granule location to the plasma membrane (40,80,81). Tyrosine kinase $p58^{fgr}$ is not required for myeloid cell development but is critical for β_2 integrin-mediated signaling events (82). During direct β_2 integrin signaling, $p58^{fgr}$ is activated in the absence of kinase activity directly associated with the β_2 integrin molecules and is redistributed to detergent-insoluble cell fractions of human neutrophil lysates (36,40). In this study, we have shown that bovine $p58^{fgr}$ appears to have a membrane associated molecular weight of ~58 kD and a cytosolic molecular weight of ~50 kD (Figure 3.6). On Western blots of Triton X-100 soluble and insoluble bovine neutrophil fractions from time-dependent CD18 cross-linking studies, the protein detected with anti-Fgr antibody redistributes to the Triton X-100 insoluble fraction and shifts from the cytosolic molecular weight of ~50 kD to the membrane-associated molecular weight of ~58 kD. This was observed in studies on neutrophils from both clinically normal animals (Data not shown) and an animal heterozygous for BLAD (Figure 3.7). This redistribution and shift in molecular weight was not detected in Western blots of similarly treated neutrophils from a calf homozygous for BLAD (Figure 3.7). The amino acid sequence for bovine Fgr has yet to be determined, and further studies on this protein may be important in the understanding of this protein and its involvement in neutrophil cell signaling.

It has been suggested that cytoskeleton rearrangement, induced by β_2 integrin ligation, occurs in two distinct steps. The first is a transmembrane clustering of cytoskeletal and signaling proteins and the second being actin polymerization (36).

During CD18 ligation with immobilized mAb, the redistribution of the cytoskeletal protein α-actinin and $p58^{fgr}$ is not affected by tyrosine kinase inhibitors, suggesting that the activation of tyrosine kinases follows their redistribution (36). Redistribution of α-actinin to the Triton X-100 insoluble fraction has been observed in human neutrophils adhered to immobilized anti-CD18 (36). Our studies involving CD18 cross-linking on suspended bovine neutrophils show that the majority of α-actinin is present in the Triton X-100 insoluble fraction throughout the cross-linking. A similar distribution was observed in neutrophils from both clinically normal cattle (Data not shown) and a calf heterozygous for BLAD (Figure 3.8). In neutrophils isolated from a calf homozygous for BLAD, α-actinin was evenly distributed between the Triton X-100 soluble and insoluble fractions (Figure 3.8). Although the distribution of α-actinin between the soluble and insoluble fractions did not change due to cross-linking, the difference in α-actinin distribution seen in the heterozygous and homozygous animal may be due to the association of α-actinin with the β_2 integrins.

In resting human neutrophils, α-actinin was recovered mainly in the Triton X-100 soluble fraction (36,41). Upon ligation of CD18 on human neutrophils, α-actinin redistributed to the insoluble fraction (36). Different stimuli induce association of the cytoskeletal protein α-actinin with CD18 in human neutrophils (13). To determine if this association is present in the bovine neutrophil, we looked for antibodies that would detect the bovine β_2 integrin subunits on Western blots. As shown in Figure 3.9, only one (R7928a) out of twelve antibodies tested immunoblotted one of the β_2 integrin subunits in bovine membrane fractions. In contrast, six out of the twelve antibodies detected β_2

integrin subunits in human membrane fractions (Figure 3.9). Using the antibody R7928a on Western blots identical to those used in the α-actinin study, we determined that CD11b remained in the Triton X-100 soluble fraction throughout the cross-linking study on suspended bovine neutrophils from a calf heterozygous for BLAD (Figure 3.10). This disproved our hypothesis that the difference in distribution between the Triton X-100 soluble and insoluble fraction of α-actinin in neutrophils from cattle heterozygous and homozygous for BLAD was due to the association of α-actinin with the β_2 integrins. Further studies on bovine neutrophil cytoskeletal activation and assembly may help understand this process.

Previous studies on the membrane associated NADPH heterodimer gp91-*phox*/p22-*phox* have shown that in certain forms of human chronic granulomatous disease (CGD) a mutation in the gene for one of the proteins hinders the stable expression of the other protein even though the gene for that protein appears to be normal (83). More recently, Porter *et al* (84) reported that autosomal recessive/cytochrome *b*-negative (no p22-*phox* expression) patients express an incompletely glycosylated gp91-*phox* precursor in patient-derived B-cell lines. This same gp91-*phox* precursor was also detected in the X-linked form of the disease caused by mutations in the gp91-*phox* gene (84). Based on these observations, it is not unreasonable that the cattle homozygous for BLAD, due to a genetic mutation in the CD18 gene, may be expressing altered forms of the β_2 integrin subunit molecules that are not able to assemble correctly and, therefore, are not expressed on the cell surface..

To further investigate this possibility, we studied two mAb that immunoprecipitated what we believe are the β_2 integrin subunits from clinically normal bovine neutrophil lysates (Figure 3.11 & 3.12). Antibody 68-5A5 (RDI-CD18) immunoprecipitated three protein bands with the molecular weights between 150-180 kD, and one protein band with the apparent molecular weight ~97 kD, and antibody 3.9 (RDI-CD11c) immunoprecipitated one protein band with a molecular weight of ~180 kD. Western blots of the immunoprecipitations and the corresponding flow-through with the antibody R7928a (CD11b) confirmed that one of the bands immunoprecipitated with 68-5A5 (RDI-CD18) was the β_2 integrin subunit CD11b (Figure 3.11 & 3.12). In an attempt to identify the other protein bands as β_2 integrin subunits, immunoprecipitations were performed on lysates of neutrophils from homozygous BLAD calves. Interestingly, immunoprecipitations with these two mAb on cell lysates of neutrophils isolated from a calf homozygous for BLAD revealed two higher molecular weight bands (~150-180 kD) and one band at ~97 kD (Figure 4.7). Western blots on these immunoprecipitations and the corresponding flow-through with the antibody R7928a (CD11b) did not detect the β_2 integrin subunit CD11b in any of the cell preparations (Figure 4.7). Further investigation into antibodies that will either recognize the β_2 integrin subunits on Western blot or in immunoprecipitation is warranted. Likewise, obtaining the genetic sequence of bovine CD11a, CD11b, and CD11c may shed light on the expected molecular weights of those proteins and aid in the study of the association of the β_2 integrin subunits in the formation of their heterodimers and their possible presence in cattle homozygous for BLAD.

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CHAPTER 4

SUMMARY

The leukocyte β_2 integrins play key roles in neutrophil adhesion, chemotaxis, phagocytosis, and receptor signaling. The absence of these surface adhesion molecules severely impairs neutrophil adherent-dependent functions and results in compromised mucosal immunity (1,2,3). Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive disease of Holstein dairy cattle involving a single mutation in CD18 resulting in the absence of β_2 integrin expression on leukocytes (1,4,5). The purpose of the current studies was to determine if priming and activation of bovine neutrophils can be modulated by β_2 integrins. We were able to utilize neutrophils from calves heterozygous or homozygous for BLAD to investigate bovine neutrophil response to β_2 integrin cross-linking.

With the exception of the β_2 integrins, surface molecule expression on leukocytes from calves heterozygous or homozygous for BLAD were comparable to those of clinically normal cattle (Chapter 2). Further analysis with antibodies directed towards the β_2 integrins revealed only a few antibodies that recognized normal bovine β_2 integrins in surface fluorescence staining, on immunoblot or in immunoprecipitation (Chapter 3). Although the bovine CD18 gene has 82.4% identity with the human CD18 gene, the sequences for the α subunits, CD11a, b, and c, have not yet been determined.

Functional assays revealed that neutrophils from heterozygous calves phagocytosed and killed *C. albicans* as well as those from clinically normal calves,

demonstrating that the heterozygous genotype likely does not have an effect on adhesion-dependent functions of leukocytes (Chapter 2). In contrast, and as previously reported, neutrophils from homozygous calves were not able to efficiently phagocytose yeast (1,6). Even though neutrophils from homozygous calves were unable to phagocytose yeast, they still maintained approximately 30% of the capacity of clinically normal or heterozygous cells to kill *C. albicans* (Chapter 2). One explanation for these results is that neutrophils from calves homozygous for BLAD, which cannot phagocytose, might use oxygen-dependent, nonphagocytic mechanisms to attack small organisms such as yeast blastoconidia (7,8). Indeed, we found that neutrophils from homozygous animals generated approximately twice the amount of O_2^- as cells from clinically normal and heterozygous calves (Chapter 2). Thus, the ability of neutrophils from calves homozygous for BLAD to kill yeast without phagocytosing them indicates that other host defense capabilities are functional and may even be upregulated.

The increase in oxidase activity in neutrophils from calves homozygous for BLAD might result from enrichment of a subpopulation of cells that have a higher respiratory burst capacity. Differential counts did reveal a significant increase in the number of band neutrophils in blood from homozygous animals (Chapter 2). However, the presence of greater numbers of band neutrophils may not be sufficient to explain the enhanced oxidative burst (9), and the variability of the oxidase response between BLAD genotypes may also reflect differences in prior in vivo exposure of the cells to inflammatory stimuli (10,11).

We examined a variety of cell responses in neutrophils isolated from normal,

heterozygous and homozygous BLAD cattle in order to define β_2 integrin-dependent effects of CD18 cross-linking. In the studies reported here, we have shown that the rise in $[Ca^{2+}]_i$ induced by CD18 cross-linking in normal bovine neutrophils was dependent on the concentration of the secondary antibody (Chapter 3). This increase in $[Ca^{2+}]_i$ is smaller in size and delayed as compared to stimulation with PAF (12). The calcium flux in bovine neutrophils corresponds with the second calcium peak induced with PAF. This supports the theory that cell signaling through the β_2 integrins involves the PLC pathway (13,14). As expected, the $[Ca^{2+}]_i$ increase in similarly treated neutrophils isolated from homozygous BLAD cattle was significantly reduced (Chapter 3). CD18 cross-linking also induced a redistribution of the cytosolic tyrosine kinase $p58^{fgr}$ in normal bovine and heterozygous neutrophils (Chapter 3). Again, this response was not seen in similarly treated neutrophils from homozygous BLAD calves (Chapter 3).

Cell-surface antigen up-regulation has been previously reported for stimulated human neutrophils (12,15-19). We have shown that C18 cross-linking on normal bovine neutrophils, or neutrophils from calves heterozygous for BLAD, increases the expression of cell surface L-selectin, up-regulates surface CD18 expression, and increases the amount of surface lactoferrin (Chapter 3). Consistent with the absence of β_2 integrins on homozygous cells, little increase in L-selectin staining, CD18 up-regulation, and lactoferrin staining occurred after the addition of cross-linking antibody (Chapter 3).

Immunoprecipitations of homozygous BLAD neutrophil lysates with anti-β_2 integrin antibodies revealed three protein bands (Chapter 3); however, none of our β_2 integrin antibodies recognized these proteins on immunoblots. Our results suggest the

exciting possibility that residual forms of β_2 integrin proteins may be present in cells from BLAD animals. Previous studies on the membrane associated NADPH heterodimer gp91-*phox*/p22-*phox* have shown that in certain forms of human chronic granulomatous disease some patients express an incompletely processed gp91-*phox* in their endoplasmic reticulum (20). Based on these observations, it is not unreasonable that the cattle homozygous for BLAD, due to a genetic mutation in the CD18 gene, may be producing altered forms of the β_2 integrin subunit molecules that are not expressed on the cell surface. Further investigation into antibodies that will either recognize the β_2 integrin subunits on Western blot or in immunoprecipitation is warranted. Likewise, obtaining the genetic sequence of bovine CD11a, D11b, and CD11c may shed light on the expected molecular weights of these proteins and aid in the study of the association of the β_2 integrin subunits in the formation of their heterodimers and their possible presence in cattle homozygous for BLAD.

Overall, my studies provide new information related to the role of β_2 integrins in neutrophil function and may lead to a better understanding of the pathogenic mechanisms leading to leukocyte adhesion deficiency in these animals.

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