Characterization of equine neutrophil surface antigens with an anti-β-integrin-like and two anti-CD18 monoclonal antibodies and effect of lipopolysaccharide stimulation.

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ABSTRACT

The surface presentations of CD18 and β-integrin-like neutrophil antigens were evaluated in six clinically normal horses twice at seven to 30 day intervals. The monoclonal antibodies (MAb) 60.3 and #38 recognize portions of CD18, the β_2-subunit of heterodimeric integrins LFA-1 (i.e. CD11a/CD18), Mac-1 (i.e. CD11b/CD18) and p150,95 (i.e. CD11c/CD18). Monoclonal antibody #25 putatively recognizes a β-integrin-like surface antigen. Neutrophils were isolated from whole blood and incubated with either Hanks' balanced salt solution or lipopolysaccharide (LPS, No. L7261 from *Salmonella typhimurium*) and then with one of three primary MAbs (60.3, #38 or #25). Cells were then incubated with the secondary MAb [fluorescein isothiocyanate (FITC)-conjugated affinipure $F(ab')_2$ fragment-goat antimouse (GAM) IgG], which acted as a label for fluorescence activated cell sorting. Cell viability measurements were performed pre- and post-incubation; and cell type was confirmed.

Results indicate that unstimulated equine neutrophils expressed CD18 cell surface adhesion molecules almost constitutively ($p < 0.05$) using MAbs 60.3 and #38. Unstimulated cells incubated with MAb #25 had a labeling percentage of 87.67%, indicating that most equine neutrophils express a β-integrin-like antigen on their surface. The labeling percentages, and mean and peak channel numbers (i.e. indicators of fluorescence intensity) were significantly greater ($p < 0.05$) in neutrophils incubated with MAbs 60.3, #38, and #25 in comparison to control cells (i.e. not incubated with primary MAb). Some autofluorescence was evident in control neutrophils; however, non-selective fluorescence was minimized by use of a secondary MAb composed of $F(ab')_2$. Monoclonal antibody 60.3 labeled significantly more ($p < 0.05$) neutrophils than MAb #38 and had greater fluorescence intensity. Conversely, LPS-stimulated cells incubated with MAbs 60.3 and #38 showed significant decreases ($p < 0.05$) in the percentage of CD18 moieties labeled compared to unstimulated cells. However, there was no significant alteration in percentage labeling with MAb # 25. Mean and peak channel numbers tended to increase after LPS-stimulation in cells incubated with MAbs 60.3 and # 38; however, no significant differences could be ascribed. This data showed that whilst fewer neutrophils were labeled for CD18 after LPS-stimulation, the neutrophils had a higher density of labeling indicative of quantitative up-regulation. Qualitative up-regulation may also have occurred as the number of cells labeled decreased. Viability pre- and post-

incubation ranged from 94 to 100% and was not different, indicating that MAb incubation did not adversely effect equine neutrophils. It was concluded that unstimulated neutrophils from horses almost constitutively express important integrin cell surface antigens, which are crucial to adhesion, interactive communication, and the immune response. Lipopolysaccharide stimulation of neutrophils causes quantitative up-regulation, and may facilitate qualitative alterations in CD18 moieties. Also MAb 60.3 appears superior to MAb #38 in its ability to label CD18 subunit of equine neutrophils. These MAb modalities could be used to manipulate certain diseases, exacerbated by excessive neutrophil numbers and degranulation (eg. ischemia/reperfusion and respiratory distress syndromes).

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CHAPTER 1

Introduction and Objectives

The neutrophil is responsible for a large proportion of the early immunological response in the horse. The function of neutrophils and other immune cells, and their interaction with cells and intercellular matrix are partially dependent upon adhesion molecules of various types (Springer et al. 1984). Cell surface adhesion molecules (i.e. types of surface antigens) facilitate cell to cell and cell to intercellular matrix anchorage, immigration and interactive communication. Neutrophil adhesion factors include the β-integrins, which are termed CD11/18 by World Health Organization Nomenclature (Reinherz 1986).

Astounding advances in knowledge regarding surface antigens have been made and in some cases have allowed diagnosis of certain diseases eg. leukocyte adhesion deficiency of humans (LAD) and bovine leukocyte adhesion deficiency (BLAD). A similar condition has not been diagnosed in the horse.

Cell surface antigen number, density, configuration, and function may alter in response to immune stimulation, however, in general qualitative upgrading (Rothlein et al. 1986; Rothlein and Springer 1986; Springer 1990) via conformational changes may be more important than quantitative upgrading (Reichner, Whiteheart and Hart 1988; Bochsler, Slauson and Neilsen 1990).

The immune system is vast and complex. Limited work has been performed on equine neutrophil surface antigens, some of which may function as adhesion molecules (Bochsler et al. 1990; Tumas et al. 1994). Studies reported in this thesis were designed to further characterize equine neutrophil surface antigens of the β-integrin family using an anti-β-integrin-like (#25) and two anti-CD18 (60.3 and #38) monoclonal antibodies. Further studies were undertaken to assess effect of static lipopolysaccharide (LPS) incubation on stimulation of neutrophils, using monoclonal antibody (MAb) and fluorescence activated cell sorting (FACS) techniques.

The neutrophil was evaluated, because it is crucial to early inflammation in many species, with manifestations including response to endotoxemia in the horse and the role of ischemia-reperfusion-induced vascular injury in the small intestine (Kurtel, Tso and Granger 1992) and lungs (Cooper, Lo and Madik 1988).

The objectives of the studies reported herein were as follows:

a) to investigate the effect of incubation of equine neutrophils with one anti-β-integrin-like and two anti-CD18 MAbs. These MAbs included:

- #25 = DH59B, a murine "heinz" IgG_1, which recognizes an antigen of Mr 96,000, which may be distinct from CD18. Putatively it recognizes a β-integrin-like surface antigen on neutrophils, macrophages and other immune cells.
- 60.3, a mouse antihuman $IgG_{2\alpha}$, which recognizes CD18 multimeric cell surface glycoprotein complex.
- #38 = H20A, a mouse antihuman ("heinz") IgG_1, which also recognizes CD18 cell surface complex.

A secondary MAb: FITC $F(ab')_2$-GAM (i.e. fluorescein isothiocyanate $F(ab')_2$ - goat antimouse) was used to measure the percentage of fluorescent labeling.

b) to establish if a difference exists in the ability of the anti-β-integrin-like and two anti-CD18 MAbs in labeling neutrophils.

c) to document variation (i.e. quantitative up - or down-regulation) of percentage labeling and fluorescence intensity after external stimulation of neutrophils with LPS.

1.1 List of Abbreviations

Before commencing the thesis proper, it was considered important to include a comprehensive list of abbreviations used throughout it.

LPS = lipopolysaccharide (endotoxin)

MAb = monoclonal antibody

60.3 = monoclonal antibody (mouse antihuman $IgG_{2\alpha}$), which recognizes CD18 multimeric cell surface glycoprotein complex. This MAb has no workshop number

#38 = monoclonal antibody H20A, which recognizes CD18 cell surface antigen

#25 = monoclonal antibody DH59B, which recognizes a Mr 96,000, a β-integrin-like surface antigen distinct from CD18

FITC-GAM = fluorescein isothiocyanate - goat antimouse

FITC$(Fab')_2$GAM = fluorescein isothiocyanate $(Fab')_2$ - goat antimouse

CD = cluster designation

EqCD = equine cluster designation

CAM = cellular adhesion molecule

VLA = very late antigen

β integrins = CD18 containing integrins:

- CD11a/CD18 = LFA-1 = leukocyte function antigen = lymphocyte function - associated antigen 1
- CD11b/CD18 = Mac-1 = macrophage 1 = OKM1 = Mo-1Ag = monocyte 1
- CD11c/CD18 = p150,95 = Leu M5

ICAM 1 = intercellular adhesion molecule 1 = CD54

ICAM 2 = intercellular adhesion molecule 2

gp = platelet glycoprotein

C3 = third component of complement cascade (C3a and C3b)

C3bi = inactive form of C3b (= ligand for Mac-1 = CD11b/CD18)

pmn = polymorphonuclear cell = neutrophil = segmented granulocyte

NK = natural killer cell

mono = monocyte

T-cell = T lymphocyte

B-cell = B lymphocyte

PBMC = peripheral blood mononuclear cells

Ag = antigen

Ab = antibody

Ig = immunoglobulin

Da = daltons

S = Svedberg unit of sedimentation constant, used in determining relative molecular weight of antibodies

GGFHS = gamma globulin free horse serum

PMA = Phorbol 12-myristate 13-acetate, which is an activator of protein kinase C

ZAS = zymosan A-activated serum

HBSS = Hanks' Balanced Salt Solution

CFMH = Calcium Free Modified Hanks' Solution

AA = animo acid

FACS = Fluorescence activated cell sorting

CHAPTER 2

Review of the Literature on Neutrophils, Integrins and their Ligands, Leukocyte Adhesion Deficiency, Antibodies, Lipopolysaccharide and Flow Cytometry

2.1 Introduction

This literature review covers the major areas investigated in this thesis, ranging from neutrophils, cell surface antigens (with more specific data relating to the integrins and their ligands) and leukocyte adhesion deficiency. Other topics include lipopolysaccharide, antibodies, monoclonal antibody usage and flow cytometry (i.e. the method of fluorescence activated cell sorting (FACS).

2.2 Neutrophils

2.2.1 General

The immune response relies on two sources of complementary phagocytic cell systems:

- myeloid
- mononuclear - phagocytic.

The most predominant cell type of the former system is the neutrophil (i.e. polymorphonuclear cell, pmn). The neutrophil is formed in the bone marrow and then migrates to the circulatory system in which it spends < 1 hour to 48 hours (Elgert 1990) (mean = 6 hours (Ganong 1993)) before migrating, via adhesion to endothelia, to the tissues (Dustin and Springer 1988). Hence, neutrophil action is rapid, but prolonged activity is not possible due to the short life span.

Neutrophils possess two distinct types of granules (Tizard 1982):

- primary granules = lysosomes
- secondary granules = specific granules

Over 60 enzymes are contained within the granules. These include proteolytic enzymes (i.e. cathepsins, collagenase, elastase, acid phosphatase etc.), enzymes acting on lipids (i.e. phospholipases, aryl sulfatase), enzymes catalyzing carbohydrate metabolism (i.e. lysozyme, glucosidases, galactosidases, hyaluronidases, neuraminidases), nucleases (i.e. acid ribonuclease, acid deoxyribonuclease) and enzymes of the respiratory burst (i.e. catalase, superoxide dismutase and myeloperoxidase (Wallace et al. 1992)) (Tizard 1982).

Phagocytosis occurs in four main stages including:

- chemotaxis (i.e. directed movement, involving many facets of the adhesion molecule network, which allows extravasation and diapedesis of neutrophils)
- adherence to foreign particle(s)
- ingestion
- digestion.

Chemotactants include bacterial products, factors released by damaged cells (Wexler, Nelson and Cleary 1983) and other inflammatory mediators. These substances induce margination of neutrophils against the blood vessel wall (Butcher 1991; Lawrence and Springer 1991). This is followed by adhesion and extravasation into the surrounding inflamed/infected tissue (Hynes and Lander 1992). It is crucial that the zeta potential (i.e. net negative charge causal in repulsion between neutrophils and foreign particles in body fluids) is neutralized in order for adherence and thus phagocytosis to take place. Diminution of the zeta potential occurs by opsonization of the foreign particle with positively charged proteins (eg. antibody molecules and C3), thus enabling electrostatic contact and subsequent adherence. Neutrophils possess specific receptors for the crystallizable fraction (i.e. Fc = tail) (Fig. 2.1) of antibodies and for the third component of complement (C3). The protein coating of the foreign particle also assists in transformation and phagocytosis by rendering the particle more hydrophobic than the neutrophil.

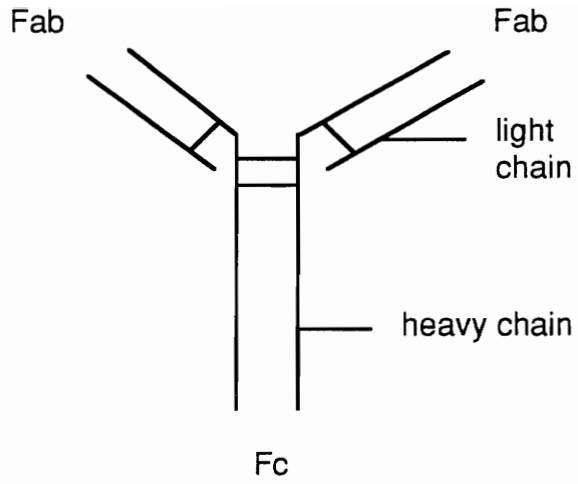


Figure 2.1 A schematic representation of an antibody (immunoglobulin G) molecule, showing the two antigen binding fractions (Fab), and the crystallizable fraction (Fc), which makes up the tail of the immunoglobulin. The structures of light chains, heavy chains and disulfide bonds (—) are also presented.

Once engulfment of the foreign particle occurs, the phagosome is united with neutrophilic granules forming a phagolysosome, ensuring contact between the foreign particle and granular enzymes, thereby aiding digestion.

Destruction is enhanced by an increasing concentration of lactic acid through promotion of glycolysis (Tizard 1982) and by optimizing the action of the hexose monophosphate shunt with increased oxygen cytoconsumption (i.e. the "respiratory burst") (Fig. 2.2). The "respiratory burst" results in enhanced turnover of reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) and results in the generation of highly reactive and destructive oxygen metabolites (i.e. H_2O_2, superoxide anion (O_2^-), singlet oxygen, hydroxyl radicals, chloramines and aldehydes).

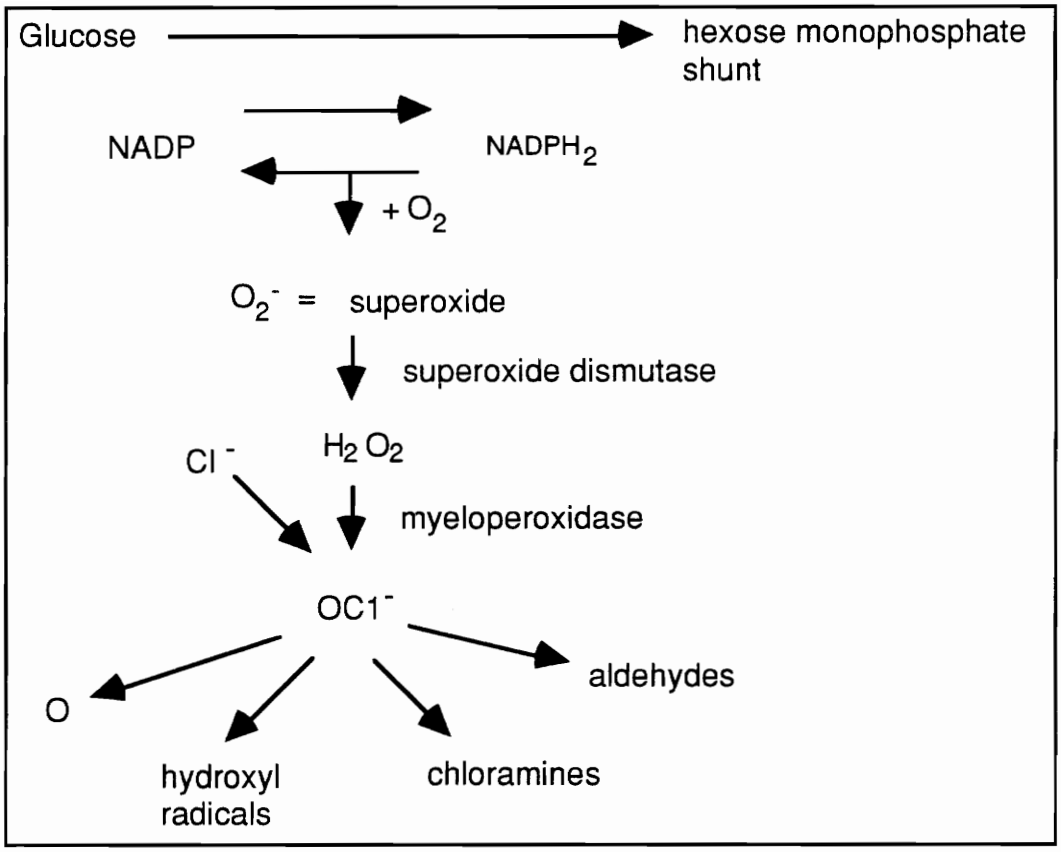


Figure 2.2 Respiratory Burst Flow diagram

Cluster designation (CD) antigens of human polymorphonuclear cells (pmn) are shown in Table 2.1. These have been used extensively in identification of cells and elaboration of different functional capacities of cytological subsets.

Table 2.1 Cluster designation antigens (CD) on human polymorphonuclear cells.

Cluster Designation Antigens on Human Polymorphonuclear Cells		
CD	Synonym	Leukocyte Type
CD11a	LFA-1 (α chain)	All leukocytes (except some tissue MØ)
CD11b	Mac-1 (α chain) = CR3 = M01	pmn, NK, mono/MØ, some B and T-cells
CD11c	pl50, 95 (= p150,94), LEU-M5 (α chain)	pmn, mono/MØ, some B and T-cells
CD18	β-chain of CD11a, b, c	All leukocytes (except some tissue MØ)
CDW12	—	pmn, monos, platelets
CD13	MY7, aminopeptidase	pmn, monos
CD14	Leu-M2, M02	pmn, monos/MØ
CD15		pmn, monos
CD16	—	pmn, MØ, NK
CDW17	Fc receptor (Fcγ RIII), Leu-11	pmn, monos, platelets
CD31	—	pmn, monos/MØ, B-cells, platelets
CDW32	—	pmn, monos, B-cells
CD33	Fc Receptor (Fcγ R11)	pmn, mono precursor, mono/MØ
CD35	MY9	pmn, monos, B-cells
CD43	CR1	pmn, T-cells, monos, brain
CDW65	—	pmn, monos
CD66	—	pmn
CD67	—	pmn
	—	

Leukocytes, probably including the pmms also have the following cluster designation antigens: CD44 = Pyp1, CD45 = LCA = T200, CD46, CD48, CDW50, CDW52, CD53, CD55 = DAF and CD58 = LFA-3.

2.2.2 Equine neutrophils

Leukocyte counts are influenced by age (Jeffcott 1971; Miller and Campbell 1983). As an animal ages, there is a decrease in the equine lymphocyte count (Schalm 1984, Jain 1986), which results in a relative increase in neutrophils.

In equine neutrophils, nuclear lobulation is distinct and nuclear chromatin is markedly plaqued causing a more irregular outline than in other domestic animals (Jain 1986). Mature neutrophil counts in hot-blooded horses range from approximately 2,700/µl of blood to 7,200/µl of blood (Jain 1986), but laboratories differ (e.g. VMRCVM clinical pathology laboratory values for equine mature neutrophils range from 3,000/µl to 6,000/µl).

2.2.3 Adhesion molecules of neutrophils

The distribution and type of adhesion molecules of human neutrophils have been documented by many researchers (Arnaout et al. 1983; Berg et al. 1991; Polley et al. 1991; Springer and Lasky 1991).

Some important receptors or adhesion molecules identified on human neutrophils include:

- LFA-1 = CD11a/CD18 (Krensky et al. 1983; Springer et al. 1984; Arnaout, Lanier and Faller 1988)
- Mac-1 = CD11b/CD18 (OKM1 = Mo-1 Ag) (Springer et al. 1984; Arnaout et al. 1985; Wallis et al. 1986; Arnaout et al. 1988)
- p150.95 = CD11c/CD18 (= Leu M5) (Arnaout et al. 1982; Springer et al. 1984; Lanier et al. 1985; Springer, Miller and Anderson 1986; Arnaout et al. 1988)
- L-selectin (Schleiffenbaum, Ortini and Tedder 1992)

- carbohydrate structure of Sialyl-Lewis X a terminal structure on the cell-surface glycoprotein and glycolipid groups (Phillips et al. 1990; Berg et al. 1991; Etzioni et al. 1992).

2.3 Integrins and their ligands

Neutrophil adhesion to endothelial cells is essential for extravasation to areas of inflammation (Harlan 1985). This is partially dependent on the integrin protein family and the immunoglobulin superfamily, however, integrins of various leukocytes also bind with selectins and complement fragments. Integrins are present on platelets, leukocytes and other cells. Differential surface expression of some integrin cell surface antigens has been shown during granulocyte maturation (Lund-Johansen and Terstappen 1993). Development of competence by mononuclear phagocytes and granulocytes is accompanied by increases in cell to cell interaction and avidity (Strassman et al. 1985).

2.3.1 Integrins

The integrin family of cell surface adhesion molecules, as described for human cells, include the β_1-integrins (i.e. very late antigens (VLA)), the β_2-integrins (i.e. CD11/CD18) and the novel integrins. The β_2-integrins include:

- CD11a/CD18
  = LFA-1 = lymphocyte function associated antigen-1
  = α_L β_2
- CD11b/CD18
  = Mac-1 = macrophage antigen-1
  = CR3 = Mo-1 = OKM-1
  = α_M β_2
- CD11c/CD18
  = p150,95 = Leu M5 = CR4
  = α_X β_2

Some novel integrins (eg. VNR (CD51/CD61) and platelet glycoprotein (gp) IIb/IIIa (CD41/CD61)) guide cell localization in both embryological morphogenesis and wound healing (Kishimoto et al. 1989) by adhesion to intercellular matrix. They

appear to be related on an evolutionary scale to the leukocyte (β_2) integrins, which are responsible for leukocyte localization during inflammation (Kishimoto et al. 1989). Expression of the three leukocyte β_2-integrins is restricted to immune cells (Kishimoto et al. 1989). CD11a/CD18 is expressed by nearly all immune cells (Kürzinger and Springer 1982; Krensky et al. 1983) except for most tissue macrophages (Strassmann et al. 1985). CD11b/CD18 has been documented on neutrophils, monocytes, macrophages, large granular lymphocytes and $CD5^+$ and mature B cells (de la Hera et al. 1988). CD11c/CD18 expression simulates that of CD11b/CD18, although the former is also expressed on subpopulations of activated lymphocytes and is a marker for the entity "hairy-cell leukemia" (Schwarting, Stein and Wang 1985).

These integrin glycoproteins are associated with numerous recognition functions (Strassman et al. 1985). The β_2-integrins share common structural and functional properties. CD11a/CD18 appears to be more selectively expressed on mononuclear phagocytes than CD11b/CD18, in that expression is confined to primed and activated murine mononuclear phagocytic cells (Strassman et al. 1985). Adhesion of cytotoxic T-cells to targets appears to be due in part to CD11a/CD18 mechanisms (Springer 1982; Strassman et al. 1985).

CD11b/CD18 is a functional adhesion molecule and has been shown to promote attachment of cells in a temperature- and divalent cation (eg.Mg^{2+})-dependent fashion (Wright et al. 1987).

The β_2-integrins probably function as transiently fixed sites in the membrane, thus connecting the cytoskeleton (and cytosol) to the extracellular environment.

2.3.1.1 Structure of Integrins

Members of the integrin group contain two subunits:

- α - comprising approximately 1,100 amino acids
- β - containing approximately 750 amino acids

In the human, homology is moderately conserved in the α-subunit (i.e. 25-63% of the chain has identical amino acid sequence) (Corbi et al. 1987; Suzuki et al. 1987; Arnaout et al. 1988) and in the β-subunit, in which 37-45% of the amino acids are in identical sequence (Tamkun et al. 1986; Kishimoto et al. 1987; Law et al. 1987). The α-and β-subunits are non-covalently bonded (Springer 1990), which is important as

structure and function appear to be related. In some integrins (eg. β_1-integrins: VLA-3, VLA-5, VLA-6), there is cleavage of the a-subunit upon reduction (Kishimoto et al. 1989), yet others appear to be in a "hinged" state upon activation.

The structural domains of many integrins bind to ligands by cross-linking to peptides, containing the arginine-glycine-aspartic acid amino acid (AA) sequence (i.e. Arg-Gly-Asp or RGD in single letter AA nomenclature): C3bi a ligand of CD11b/CD18 and CD11c/CD18 contains a peptide fragment with the RGD sequence. This peptide may be responsible for the binding of C3bi to macrophages, which is inhibited by anti-Mac-1 MAb. The RGD residue putatively occupies a small region of a larger, conserved segment, recognized by adhesion molecules (Wright et al. 1987). However, the main ligand of CD11a/CD18 does not contain RGD (Simmons, Makgoba and Seed 1988) and confirmation that this recognition sequence is the critical portion for binding of the leukocyte β_2-integrins does not exist (Kishimoto et al. 1989). The leukocyte β_2-integrins are α_1 β_2 heterodimers (Kürzinger and Springer 1982) with non-covalent association between α and β-subunits. The three α-subunits are discrete entities (ie. α_L, α_M, α_X), whereas the β-subunit with a molecular weight of approximately 95K is identical in all three protein surface antigens (Trowbridge and Omary 1981; Kürzinger and Springer 1982). The four subunits have polypeptide backbones of varying weights. After deglycosylation α_L, α_M, α_X and β_2 weighed 149 kDa, 137kDa, 132 kDa and 78 kDa respectively. A subset of β_2-integrins on neutrophils have a unique lacto-N-fucopentaose II oligosaccharide moiety, although the function is not known.

Binding of VLA-4 (a β_1-integrin) and fibronectin occurs via the recognition of CS-1 (i.e. connecting segment 1) sequence in fibronectin (Garcia-Pardo et al. 1990; Guan and Hynes 1990), however this does not appear to be pertinent to β_2-integrins.

2.3.1.2 The B_2-integrins

These comprise CD11a/CD18, CD11b/CD18 and CD11c/CD18.

2.3.1.2.1 Structure of CD11/CD18

The distinct cDNA types encoding the three α-subunits of β_2-integrins have been cloned and classified in the human (Corbi et al. 1987; Arnaout et al. 1988; Larson et al. 1989). There appear to be three domains to each a-subunit:

- the long extracellular domain (α_L = 1063 AA, α_M = 1092 AA, α_X = 1081 AA)
- the hydrophobic transmembrane domain (α_L = 29 AA, α_M = 26 AA, α_X = 26 AA)
- the short cytoplasmic domain (α_L = 53 AA, α_M = 19 AA, α_X = 29 AA)

The number of N-glycosylation regions varies, which may cause some disparity in the apparent molecular weight of the individual proteins, despite the polypeptide backbones being essentially identical (Kishimoto et al. 1989).

The α-subunits contain triplets of divalent cation binding sites, which cause dependence on Mg^{2+}, seen in β_2-integrin adhesion. An I-domain (i.e. inserted/inactive domain) also exists in the a-subunits of the β_2-integrins. This 200 AA segment is present in the extracellular domain and is homologous to the "A" domain of von Willebrand factor, a domain of protein C2 (i.e. complement cascade protein) and to two domains in CMP (cartilage matrix protein) (Arnaout et al. 1988; Kishimoto et al. 1989). However, the significance of the I-domain is not known.

The chromosome encoding CD11a, CD11b, and CD11c in somatic cell hybrids is number 16 (Marlin et al. 1986; Corbi et al. 1988).

The cDNA encoding the common β_2 subunit has been characterized in the human (Law et al. 1987) and the mouse (Wilson, O'Brien and Beaudet 1989). The β_2 subunit has a 769 AA sequence, comprising an integral membrane, extracellular protein (i.e. 677 AA) with six possible N-glycosylation sites; a transmembrane domain (i.e. 23 AA) and a cytoplasmic domain of 46 amino acids (Kishimoto et al. 1987). It has a large number of cystein-rich repeats with a 7.4% total cystein content (Kishimoto et al. 1989), which probably confers rigidity to the tertiary structure of the β_2-subunit. Kishimoto et al. (1987) confirmed that a single gene encodes the β_2 subunit, irrespective of the α-subunit type with which it is associated. This gene has been mapped to band 21q22 (Corbi et al. 1988) of chromosome 21 (Marlin et al. 1986).

2.3.1.2.2 Spatial distribution of CD11/CD18

High-resolution field emission scanning electron microscopy and backscatter electron imaging of immunogold have been used by Erlandsen, Hasslen, and Nelson (1993) to detect the spatial distribution of CD11b/CD18 on the surface of unactivated and activated human neutrophils. In unactivated neutrophils, CD11b/CD18 was detected predominantly on the cell body membrane domain as singletons or in small clusters. This contrasted with findings in surface activated neutrophils, in which CD11b/CD18 appeared to be distributed in a ubiquitous manner and found in clusters on the membrane of the cell body and on surface microvilli and ruffles (Erlandsen et al. 1993). This qualifies as quantitative up-regulation.

In 1984, Todd et al. reported that Mo1 (i.e. CD11b here) was located principally in specific granules of the neutrophil, with a small portion positioned in the plasma membrane where it could bind anti-Mo1 MAb. During degranulation of neutrophils, translocation of glycoprotein from specific granules to the plasma membrane occurred, causing up to a ten fold increase in surface expression (Todd et al. 1984). Translocation of specific granules only occurred after incubation with the calcium ionophore A23187 for 25 minutes in a shaking water bath.

2.3.1.2.3 Functional stimulation (up regulation) of B_2-integrins

Modulation of adhesion is very complex. It may occur via either qualitative alterations in the short term (Springer 1990), perhaps by reversible phosphorylation mechanisms (Hibbs et al. 1991) or by quantitative glycoprotein sialylation, which requires *de novo* biosynthesis of glycoprotein (Reichner et al.1988), which takes much longer (i.e. approximately 12 to 24 hours) (Springer 1990). However, controversy and conflicting results exist.

Evidence suggesting the importance of qualitative changes include that no increase in surface expression of CD11a/CD18 or ICAM-1 was seen when phorbol ester stimulation was used to cause inter-lymphocyte adhesion, through CD11a/CD18 and ICAM-1 mechanisms (Rothlein et al. 1986; Rothlein and Springer 1986). Avidity of CD11a/CD18 in lymphocytes appears to peak within 10 minutes and decline to basal levels within 30 minutes (Springer 1990). Dustin and Springer (1989) also reported that stimulation of resting T cells can increase the avidity of LFA-1 without altering cell

surface density. T cell receptor contact with cells expressing specific antigen causes configurational (i.e. qualitative) conversion of CD11a/CD18 to a temporary high avidity state.

Cell migration may be assisted by two factors:

- Local changes in integrin avidity, so high and low avidities are produced at the leading and trailing edges of cells respectively (Dustin and Springer 1989, Springer 1990).
- Cytoskeletal tension generation (Springer 1990).

Diamond et al. (1991) hypothesized that the extent of glycosylation on ICAM-1 may regulate the cellular adhesion, mediated by CD11a/CD18 and CD11b/CD18. There are two distinct binding sites on ICAM-1 for CD11a/CD18 and CD11b/CD18 respectively, which may mean that the unicellular globular heads of these two β_2-integrins could bind a single ICAM-1 molecule (Diamond et al. 1991) on an endothelial cell. This dual binding may be significant if attachment of a single integrin versus dual integrins transmit specific and distinct signals to the cell (Diamond et al. 1991). Only a small portion of CD11b resides on the plasma membrane of unstimulated human neutrophils, however, this still allows exposure to the extracellular environment (Todd et al. 1984).

Phosphorylation of the β-subunit may activate a specific protein (eg. protein kinase C), that then alters CD18 avidity (Hibbs et al. 1991), however, phosphorylation may not be involved in the avidity up-regulation of CD18 integrins. Much speculation exists. Regulation of adhesiveness due to CD11a/CD18 was proposed to be a function of a site defined by Hibbs et al. (1991). This site consisted of five amino acids (threonine 758-760, Phe 766 and serine 756) and is highly conserved in other integrins (i.e. B_1, B_3 and B_7). The major site phosphorylated in response to PMA stimulation is serine 756 of the CD18 cytoplasmic domain, yet this does not appear to affect avidity (Hibbs et al. 1991) and therefore cannot be crucial to up-regulation. Serine 756 is also unlikely to be a substrate for protein kinase C, which has been promoted as a phosphorylation stimulator, with subsequent augmentation of CD11a/CD18 avidity (Hibbs et al. 1991). However, it remains possible that phosphorylation of one of the three threonines identified in CD18 by Hibbs et al. (1991) may be important in avidity changes. The cytoplasmic domain of CD18 may also be involved in avidity changes (Hibbs et al. 1991). Conversely, phorbol esters can stimulate phosphorylation of CD18 on neutrophils and mononuclear cells with

subsequent increases in adhesiveness (Hibbs et al. 1991). However, controversy surrounds stimulation by the chemoattractant fMLP, which may cause either transient phosphorylation (Chatila, Geha and Arnaout 1989) or no phosphorylation (Buyon et al. 1990) of CD18.

2.3.2 Ligands

Ligands are molecules that bind to complementary shaped sites of surface antigens (i.e. in this case the integrins).

Ligands identified for the β_1-integrins include the extracellular matrix glycoproteins: collagen, laminin and fibronectin (Shimizu and Shaw 1991). Fibronectin, fibrinogen, vitronectin and von Willebrand factor are ligands for various other novel integrins including gpIIb/IIIa and VNR (Kishimoto et al. 1989).

Ligands specific to β_2-integrins are imperative for immune cell adherence and the effects of inflammation.

2.3.2.1 Ligands to CD11a/CD18

The ligands for LFA-1 (i.e. CD11a/CD18) are ICAM-1 and intercellular adhesion molecule-2 (ICAM-2) (Staunton, Dustin and Springer 1989), both of which are members of the immunoglobulin superfamily and are present on a variety of cells. The ICAM-1 molecule has been identified on leukocytes including transformed B-cells, myeloid cell lines and T lymphocyte blast cells (Dustin et al. 1986). Endothelial cells, thymic epithelium, dendritic cells and cultured fibroblasts, synovial cells and keratinocytes also express ICAM-1 (Smith et al. 1988; Dustin et al. 1988; Mentzer et al. 1988). The ICAM-1 molecule is heavily glycosylated, approximately 76 to 114 KDa (Kishimoto et al. 1989) and has been mapped to chromosome 19 (Makgoba et al. 1988).

ICAM-1 expression on non-hematopoietic cells is usually low, but is up-regulated by numerous cytokines (i.e. IL-1, TNF and Interferon-γ) (Dustin and Springer 1988, Kishimoto et al. 1989). It is widely distributed and becomes prominent in inflammation on cytokine-activated endothelial cells (Carlos and Harlan 1990, Springer 1990). CD11a/CD18 binds to the first immunoglobulin repeat of ICAM-1, which differs from CD11b/CD18 (Zimmerman, Prescott and McIntyre 1992). Apparently glycosylation of ICAM-1 is not involved in binding CD11a/CD18, which

contrasts with CD11b/CD18-ICAM-1 receptor-ligand binding (Diamond et al. 1991). There may also be another binding site for CD11a/CD18.

Smith et al. (1988) demonstrated that anti-ICAM-1 MAbs inhibited transendothelial migration of human neutrophils by at least 85%. However, support exists for the possibility that ICAM-1 may not be sufficient for CD18-dependent neutrophil attachment and transendothelial migration (Smith et al. 1988). ICAM-1 has five immunoglobulin domains, but no RGD sequence (Simmons et al. 1988).

The intermembrane distance necessary for interaction of cells using CD11a/CD18 and ICAM-1 mechanisms is approximately 36 nm (Springer 1990). This is often selectin-mediated.

Another ligand for CD11a/CD18 has been identified (i.e. ICAM-2) and cloned. This molecule has two immunoglobulin-like domains, which appear to be 35% homologous to the first two domains of ICAM-1. This difference in domains explains the fact that CD11a/CD18 can bind ICAM-2 (i.e. via the first Ig-like domain), yet CD11b/CD18 can not (i.e. requires the third Ig-like domain, which is only present in ICAM-1) (Diamond et al. 1991; Kishimoto et al. 1989). ICAM-2 is not upregulated via cytokines. A third ligand, fibrin, has also been identified as a ligand for CD18 (Cooper et al. 1988).

2.3.2.2 Ligands to CD11b/CD18

The most widely accepted ligand to CD11b/CD18 is the complement fragment C3bi (Micklem and Sim 1985; Anderson et al. 1986; Kishimoto et al. 1989). The complement system is a series of 9 plasma proteins, activated by antigen antibody (Ag-Ab) complexes, which react in a sequential cascade, effecting the destruction/lysis of foreign substances and cells (Elgert 1990). Component C3 is involved in the amplification of the classical complement pathway.

Complement factors B, D, C3, the magnesium ion and an activator result in C3b formation (Schreiber, Götze and Müller-Eberhard 1976). C3bi is formed from C3b and this may occur endogenously in endothelium (Marks, Todd, and Ward 1989). The "i" at the end of a complement abbreviation indicates that this particular fragment is "inactive" and has no biological activity (eg. C3bi) (Ross and Lambris 1982). In 1982,

Beller, Springer and Schreiber demonstrated that anti-Mac-1 MAb selectively blocked the rosetting that occured between C3bi-coated red blood cells and murine macrophages or human neutrophils. Endothelial complement fixation at inflammatory sites may elucidate an endothelial-restricted signal for neutrophil adherence (Marks et al. 1989).

It is not known whether the RGD sequence is essential for CD11b/CD18-C3bi binding, even though the RGD sequence has been isolated in a peptide fragment of C3bi (Wright et al. 1987).

There are numerous other putative ligands for CD11b/CD18 (Kishimoto et al. 1989), including fibrinogen (Wright et al. 1988), coagulation protein X and the third Ig domain of ICAM-1 (Zimmerman et al. 1992). Glycosylation of ICAM-1 is important in CD11b/CD18 binding, but not for CD11a/CD18 (Diamond et al. 1991).

2.3.2.3 Ligands to CD11c/CD18

CD11c/CD18 binds to C3bi (Micklem and Sim 1985). Zimmerman et al. (1992) suggested that CD11c/CD18 also recognizes ICAM-1, and perhaps other endothelial cell ligands.

2.4 Leukocyte Adhesion Deficiency

Normal neutrophils have a critical role in cell adherence. The pathological importance of abnormalities of cell adherence have been increasingly recognized as leukocyte adhesion deficiency (LAD, LAD1) in humans (Crowley et al. 1980; Abramson et al. 1981; Arnaout et al. 1982; Anderson et al. 1984; Springer et al. 1984), cattle (Kehrli et al. 1990; Shuster et al. 1992; Gilbert et al. 1993; Olchowy et al. 1993), and dogs (Giger et al. 1987). Leukocyte adhesion deficiency 2 (LAD2) (Etzioni et al. 1992) has been recognized and defined in humans, and should not be confused with LAD (i.e. LAD1).

2.4.1 Human Leukocyte Adhesion Deficiency

Patients with a clinical syndrome characterized by recurrent, severe bacterial and fungal infections have been identified since the late 1970s (Crowley et al. 1980;

Arnaout et al. 1982; Bowen et al. 1982; Anderson et al. 1984; Dana et al. 1984; Springer et al. 1984). Some of the common symptoms recognized include:

- persistent granulocytosis (Springer et al. 1984)
- impaired pus formation (Anderson et al. 1984)
- skin and perirectal anus abscessation (Arnaout et al. 1982)
- recurrent otitis media (Arnaout et al. 1982)
- progressive periodontitis (Springer et al. 1984)
- pneumonia (Arnaout et al. 1982)
- delayed and impaired wound healing (Springer et al. 1984)
- delayed umbilical cord separation (Hayward et al. 1979; Bissenden et al. 1981; Anderson et al. 1984).

This syndrome was described in children (Crowley et al. 1980; Arnaout et al. 1982; Anderson et al. 1984), whose neutrophils demonstrated deficiencies in adherence, chemotaxis, aggregation, antibody-dependent cytotoxicity, CR3-mediated adherence and phagocytosis (Crowley et al. 1980; Anderson et al. 1984; Beatty et al. 1984). Dysfunction corresponded with deficiency of granulocyte membrane glycoproteins (i.e. LFA-1, Mac-1, p150/95) (Arnaout et al. 1982; Springer et al. 1984). Recurrent soft tissue infections, secondary to both bacteria and fungi, are related to dysfunction of neutrophils (Springer et al. 1984), although at times other cells in the patient may also be affected.

Arnaout et al. (1982) described the defect as an absence of the normal granulocyte surface membrane glycoprotein (gp 150), which in this paper refers to the CD11a subunit of LFA-1. Phagocytosis of opsonized-oil red 0, -zymosan, IgG-coated red 0 or IgG-coated sheep erythrocytes by neutrophils from patients was markedly impaired, yet neutrophils from patients' parents exhibited normal phagocytosis.

Severe deficits in leukocyte mobilization in these patients have been identified using the "Rebouck skin window" *in vivo* and "Boyden chamber assay" *in vitro* (Springer et al. 1984). Gel electrophoresis and galactose oxidase labeling (Anderson et al. 1984) and immunofluorescence flow cytometry (Springer et al. 1984) have all been used in attempts to clarify this syndrome. Gel electrophoresis studies showed severe deficiencies of a protein complex with a molecular weight of 138kD in patients' (< 5% of control) and maternal (< 20% of control) neutrophils (Anderson et al. 1984).

The features of this disease (i.e.LAD, LAD1) are attributable to the deficiency and even total absence of the cell surface expression of a family of functionally and structurally related glycoproteins. These include:

- = CD11a/CD18
- = CD11b/CD18
- = CD11c/CD18 (Sanchez-Madrid et al. 1983).

Interestingly, Arnaout et al. in 1982 perceived that C3bi receptor (i.e. Mac-1) was not involved because patient neutrophils rosetted normally with C3b, C3bi and IgG coated erythrocytes. However, this was later proved incorrect (Anderson et al. 1984).

These glycoproteins have identical β subunits of M_r = 95,000 (i.e. CD18) (Sanchez-Madrid et al. 1983: Anderson and Springer 1987), yet CD11a/CD18, CD11b/CD18, and CD11c/CD18 have different functions in leukocyte adhesion. CD11a/CD18 is important in antibody-dependent killing by granulocytes, but also participates in lymphocyte and monocyte adhesion to cells and natural killing. CD11b/CD18 is identical to the complement receptor 3 (i.e. CR3), which binds the ligand C3bi (Beller et al. 1982). Beller et al. (1982) and Anderson et al. (1986) found that anti-Mac-1 MAbs inhibit binding and phagocytosis of C3bi-opsonized particles by granulocytes and monocytes. Even CD11c/CD18 may bind C3bi (Micklem and Sim 1985); however, the functional importance of this glycoprotein as a cell surface C3bi receptor has not been demonstrated (Lanier et al. 1985).

In unstimulated granulocytes, CD11b/CD18 and CD11c/CD18 are present in intracellular, vesicular compartments, as well as on the cell surface (Springer et al. 1984; Anderson et al. 1985; Anderson and Springer 1987). CD11b/CD18 has been localized in neutrophil secondary granules (Todd et al. 1984).

Researchers have found, using specific MAbs, that patients with LAD are deficient in all 4 subunits (i.e. CD11a, CD11b, CD11c, and CD18) (Springer et al. 1984; Anderson et al. 1985; Springer et al. 1986). Interestingly, patient granulocytes and monocytes have decreased intracellular storage pools and surface expression of the CD11/CD18 complexes (Anderson and Springer 1987). Selective deficiencies have not been shown, instead the deficient state appears to encompass all subunits

(Anderson et al. 1985; Anderson and Springer 1987). However, there appears to be quantitative variation in the extent of α and β subunit deficiency. Consequently two phenotypes of affected individuals have been identified:

- severe
- moderate (Springer et al. 1984; Anderson et al. 1985).

The severe phenotype has < 0.3% of the normal amount of expression of all three αβ complexes (i.e. CD11a/CD18, CD11b/CD18, CD11c/CD18) on the neutrophil, whereas the moderate phenotype usually expresses 2.5 to 6% of the three αβ complexes (Anderson and Springer 1987). The third class is composed of carriers, in which clinically normal individuals are heterozygous carriers of LAD. This group appears to have approximately 50% expression (Arnaout et al. 1982; Springer et al. 1984; Anderson et al. 1985). Leukocyte adhesion deficiency appears to be present as a result of inheritance of a recessive autosomal gene, although X-linked inheritance was proposed in one family (Crowley et al. 1980; Arnaout et al. 1984).

The initial recruitment of neutrophils to sites of inflammation occurs with the "rolling" of neutrophils onto endothelium (Lawrence and Springer 1991; Hynes and Lander 1992), with subsequent adhesion and extravasation into the tissues by different mechanisms. "Rolling" of neutrophils is mediated by members of the selectin family (Lawrence and Springer 1991; Ley et al. 1991). Endothelial leukocyte adhesion molecule-1 (ELAM 1, E-selection) is expressed on activated endothelial cells, and is produced within two to four hours of cytokine induction (Cotran et al. 1986). Cell adhesion by ELAM-1 is mediated by a neutrophil carbohydrate ligand, which is a terminal structure on neutrophil surface glycoprotein and glycolipid carbohydrate groups (Phillips et al. 1990). This ligand has been identified as sialylated lacto-N-fucopentaose III, which is termed the sialyl-Lewis X antigen (SLe^X, sialyl Le^X) (Phillips et al. 1990; Berg et al. 1991).

Recurrent severe infections including pneumonia, otitis media, periodontitis, and localized cellulitis with poor pus formation also occur in children with distinctive phenotypes (i.e. mental retardation, short stature, distinctive face appearance and the Bombay (LL) blood phenotype) (Etzioni et al. 1992). Etzioni et al. (1992) described two unrelated children with leukocytosis and markedly diminished neutrophil mobility. This syndrome is due to the absence of SLe^X (Etzioni et al. 1992) and has officially

been designated leukocyte adhesion deficiency type 2 (i.e. LAD2) to distinguish it from the integrin deficiency (LAD, LAD1).

2.4.2 Bovine Leukocyte Adhesion Deficiency

A syndrome of granulocytopathy, recurrent infections, and delayed wound healing in young Holstein cattle has been reported (Hagemoser et al. 1983; Nagahata et al. 1987; Takahashi et al. 1987; Kehrli et al. 1990).

Calves appear healthy at birth (Kehrli et al. 1990; Gilbert et al. 1993; Ackermann, Kehrli, and Morfitt 1993), but often have leukocytosis at an early age (e.g. 7 days (Kehrli et al. 1990)), which steadily increases to approximately 100,000 wbc/µl. The leukocytosis is usually characterized by severe granulocytopathy with hypersegmentation and a modest monocytosis. Approximately 50 percent of affected individuals have band cells (Gilbert et al. 1993). These calves usually die between two and seven months of age (Kehrli et al. 1990; Anon 1991), although Nagahata et al. (1987) and Hagemoser et al. (1983) reported on a 15 month old heifer. Symptoms such as fever, inappetence, weight loss or decreased average daily gain, ventral dermatitis and vasculitis (Ackermann et al. 1993), lymphadenopathy, gingivitis, diarrhea and pneumonia have been described, despite intensive antimicrobial therapy (Hagemoser et al. 1983; Kehrli et al. 1990; Shuster et al. 1992; Ackermann et al. 1993; Gilbert et al. 1993).

Assessment *in vitro* has identified abnormalities of neutrophil motility, phagocytosis and oxidative function, all of which contribute to the *in vivo* immunological deficits (Hagemoser et al. 1983; Nagahata et al. 1987; Takahashi et al. 1987). Kehrli et al. (1990) found that a heifer calf affected with this syndrome - bovine leukocyte adhesion deficiency (BLAD) - had numerous neutrophil function defects. These included decreased endocytosis and killing of Ig-opsonized *Staphlococcus aureus*, diminished chemiluminescence, decreased superoxide production and myeloperoxidase-catalyzed iodination, which are associated with phagocytosis and the respiratory-burst pathway. Abnormalities of random migration were occasionally detected. The CD11b/CD18 complex of the affected calf was negligible compared to the neutrophil lysates of control holsteins. The quantity of CD18 expressed by dam, sire, and 8/15 paternal half-siblings' neutrophil surfaces was diminished, consistent

with the heterozygous carrier state (Kehrli et al. 1990) of an autosomal recessive condition.

Predictably, necropsies on calves with BLAD have shown large numbers of neutrophils intravascularly and sequestered in the spleen, with a paucity of neutrophils in infected and inflamed tissues (Kehrli et al. 1990; Kerhli et al. 1992).

Bone marrow examination has revealed diffuse myeloid hyperplasia (Kehrli et al. 1990). Unfortunately, Gilbert et al. (1993) do not document the complete histological reports on the 14 cases of BLAD calves in their retrospective study to confirm the original findings of Kehrli et al. (1990). However, Ackermann et al. (1993), showed chronic lymphoplasmocytic and histiocytic perivascular and ulcerative dermatitis in a 54 day old BLAD calf reared initially in a gnotobiotic isolator for 43 days, and then transferred to a "decontaminated" room with straw bedding. This calf deteriorated markedly upon removal from the gnotobiotic environment, so euthanasia was performed. At necropsy, infiltration of macrophages and lymphocytes were seen in the epidermis and dermis, whilst the blood vessels contained neutrophils (Ackermann et al. 1993).

Results of a variety of different tests for bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, parainfluenza-3 virus, bovine immunodeficiency virus, bovine leukemia virus, coronavirus and rotavirus have been negative (Kehrli et al. 1990; Ackermann et al. 1993; Gilbert et al. 1993). This is important as leukocytosis caused by viral infection may have been important (Kehrli et al. 1990) in some of the calves, thereby confounding the situation.

Takahashi et al. (1987) and Kehrli et al. (1990) provided information on Holstein pedigrees from the affected calves, which suggested that BLAD was an heritable condition with a simple autosomal recessive mode of inheritance. Their works have identified some popular bulls including:

- Osborndale Ivanhoe
- Penstate Ivanhoe Star
- Carlin-M Ivanhoe Bell

(Anon 1991), which have subsequently tested positive as heterozygous carriers.

Shuster, Bosworth and Kehrli (1992) and Shuster et al. (1992) sequenced the entire CD18 coding region in BLAD calves and in normal cattle via reverse transcribed

poly (A)$^+$ RNA. Two point mutations were identified within the gene encoding bovine CD18, one of which causes an aspartic acid to glycine substitution at amino acid 128 (D128G) and the other is silent (Shuster et al. 1992). The D128G mutation occurs in the highly conserved extracellular region of this glycoprotein (Shuster et al. 1992) and is similar to the region, in which human LAD mutations are seen. Shuster et al. (1992) have also shown the carrier frequency in the United States amongst Holstein cattle (i.e. 15% -bulls, 6% - cows). This syndrome has also been identified in cattle of American descent (Takahashi et al. 1987) in other countries (Nagahata et al. 1987).

Bovine leukocyte adhesion deficiency is an important heritable disease. DNA evaluation via polymerase chain reaction technology is now available for identification of affected homozygotes, carrier heterozygotes and normal homozygotes (Shuster et al. 1992, Gilbert et al. 1993). Currently the Holstein Association and the National Association of Animal Breeders are implementing a control program, whereby carriers are identified by DNA probe (Anon 1991). This will allow the elimination of matings between daughters of carriers bulls with other carrier bulls, and will decrease the carrier artificial insemination pool.

2.5 Antibodies

2.5.1 General

Antibodies (Ab) (or immunoglobulins (Ig)) are protein molecules produced by plasma cells after interaction between an Ag, Ag-processing and presenting cells and B-lymphocytes, whereby B-lymphocytes are converted to plasma cells. Antibodies have a specific pathophysiologic function to increase the destruction/elimination of foreign Ags. Antibodies are present in most bodily fluids, however, their greatest concentration is found in serum.

Classification of Ab molecules has traditionally been performed on the basis of four factors:

- solubility
- electrostatic charge
- molecular weight
- antigenic structure (Tizard 1982)

Immunoglobulin G (IgG) is one of the most important opsonins. It constitutes approximately 80 percent of the Ab in serum (Elgert 1990). The structure of the IgG is shown in Figure 2.1. It consists of two light polypeptide chains (i.e. either K or λ and two heavy polypeptide chains (i.e. γ). Disulfide bonds hold the four chains together covalently. Its molecular weight is approximately 150,000.

The structure of γ-globulins was further elucidated by Porter, Edelman and Nisonoff in the 1950s (Elgert 1990). Subunits were defined by breaking Ab molecules into different fragments using papain, dinitrothreitol, and pepsin respectively. Edelman's experiments showed the presence of heavy and light chains. The summarized findings of Porter and Nisonoff are presented in Figure 2.3. Two fragment Ag binding portions (Fab) confer a protective function on Abs. Only a very small portion of the AA residues of Fab contact the antigenic determinants of an Ag. The Fab portion consists of an entire light chain and a part of a heavy chain. Each has a molecular weight of approximately 50,000. The fragment crystallizable portion (Fc) (i.e. crystallizes in the cold) also has a molecular weight of approximately 50,000 and is made up of heavy chains. It mediates placental transfer and other cytophilic properties. There is a paucity of information on the structure and function of Fc (Dorrington and Klein 1987). Numerous cells have various Fc receptors. The different types of Fc receptors include:

- CD64 = Fc receptor (FcRI) found on monocytes.
- CDw32 = Fc receptor (FcγRII) found on granulocytes, monocytes, and B-cells.
- CD23 = Fc receptor ($Fc_{\varepsilon}RII$ = IgE receptor = found on some B-cells, activated macrophages and natural killer (NK) cells and possibly mast cells and basophils (Elgert 1990)).
- CD16 = Fc receptor (Fcγ RIII) = Leu-11 found on granulocytes, macrophages, and NK cells.

Cells with Fc receptors may participate in Ab-dependent cellular cytotoxicity (Tizard 1982), which causes lysis of target cells through the action of Ab combined with either neutrophils, macrophages, or null cells (e.g. NK cells). Macrophages and B-cells have the largest number of Fc receptors, followed by neutrophils and eosinophils (i.e. both types of granulocytes), NK cells, and there are some suppressor T cells with a small number of Fc receptors.

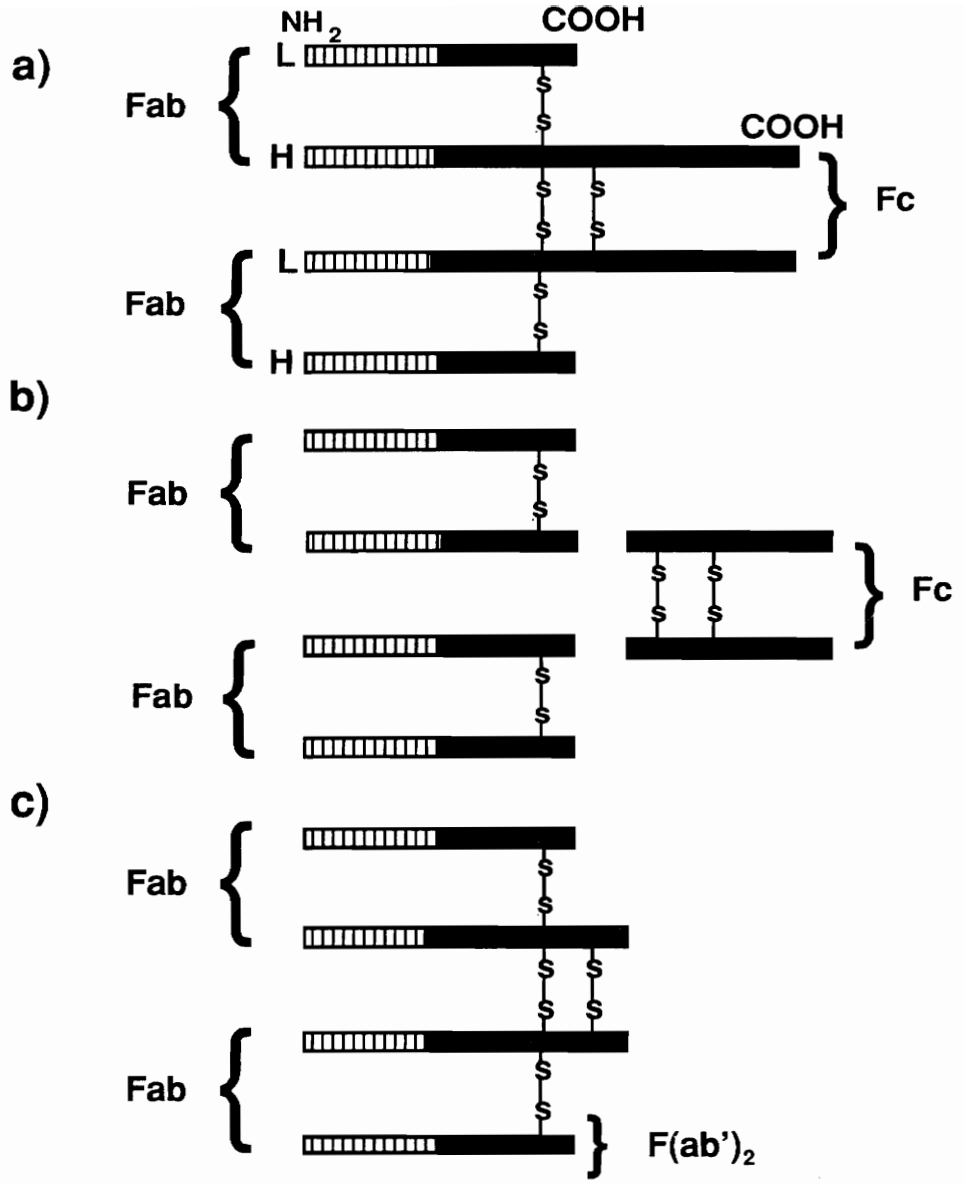


Figure 2.3

a IgG molecule
b Papain fragmentation (3 fragments = Fc + 2 x Fab)
c Pepsin fragmentation (1 main fragment $F(ab')_2$ with numerous low molecular weight fragments)

= constant portion of heavy/light chain
= variable portion of heavy/light chain
H = heavy chain
L = light chain
Fab = fragment antigen-binding
Fc = fragment crystallizable
$F(ab')_2$ = $(Fab')_2$ = Fab sites available but no Fc tail

numerous low molecular weight fragments

When pepsin (Figure 2.3) degrades the heavy chains at the carboxyl-terminal end of the inter-heavy chain disulfide bonds, then $F(ab')_2$ is formed along with numerous small fragments of heavy chain. The $F(ab')_2$ is made up of two intact light chains and two portions of heavy chains bound via disulfide bonds. Consequently it has two antigen binding fragments, but no Fc tail. This effectively stops any binding of cells to the Fc position. This theory (Fig. 2.3c) can then be used practically in indirect immunofluorescence.

2.5.2 Monoclonal Antibodies

Monoclonal antibodies (MAb) are artificially-produced (Anon 1984), pure Abs with single antigenic determinant specificities. The need for monoclonal Abs arose due to the variability of experimental results gathered using even highly specific antisera (i.e. contain Abs of widely different specificities, affinities, and cross-reactivities) (Elgert 1990). Development of hybridization techniques (Kohler and Milstein 1975, 1976) allowed the production of a single type of antibody by separate immortal B-cell derived hybridoma clones (Anon 1984; Elgert 1990).

The production of large amounts of homogenous specific antibodies (i.e. monoclonal) has advanced immunological research assay capabilities, and has application in clinical pharmacology as MAbs may be used as: 1) immunosuppressants in transplantation, autoimmune reactions and ARDS (i.e. adult/acute respiratory distress syndrome), 2) targeting vehicles for neoplasia therapy, 3) immunosuppressants for ischemia/reperfusion injury (Vedder et al. 1988) (eg. equine colic). Monoclonal Abs can be used as probes to specifically inhibit their target cells functions, thereby linking specific molecular structure to specific function (Springer 1982). However, MAbs have numerous disadvantages:

- it is difficult to use MAbs *in vivo* in many species (eg. human, horse) because of the difficulty of cloning human/equine hybridomas.
- individual species of MAbs do not readily activate complement or agglutinate Ags *in vivo*.
- the affinity of the MAb for the specific Ag may be low.

The specificity, affinity of binding, titer, specific activity, stability, Ig class and subclass, monoclonality and immunochemical identification of Ag detected by a MAb should be established (Zola and Brooks 1982).

2.5.2.1 Monoclonal antibody 60.3

Monoclonal antibody 60.3 does not have a workshop number from the First International Workshop on Equine Leukocyte Ags (Kydd and Antczak 1991). In 1983, Beatty et al. used MAb 60.3 to define a novel cell surface Ag common to human leukocytes. The MAb is a mouse antihuman $IgG_{2\alpha}$, which recognizes CD18-multimeric cell surface glycoprotein complex. This has allowed investigation into LFA-1 (i.e. CD11a/CD18), Mac-1 (i.e. CD11b/CD18) and p150,95 (i.e. CD11c/CD18).

Immunofluorescent labeling techniques have demonstrated that MAb 60.3 binds to resting and unstimulated equine granulocytes (Bochsler et al. 1990), probably by binding EqCD18 (Lunn 1993). Immunoreactivity using MAb 60.3 revealed the surface orientation, and fluorescence intensity in equine granulocytes previously stimulated with PMA and Zymosan A-activated serum (ZAS) (Bochsler et al. 1990).

The MAb 60.3 has been selected for numerous leukocyte characterization, adherence, aggregation and emigration investigations in various species including the rabbit (Thomas et al. 1992; Winn and Harlan 1993; Longdale et al. 1993; Mileski et al. 1993), the dog (Loughran, Deeg, and Storb 1993), the bovine (Bochsler et al. 1990), the horse (Bochsler et al. 1990), the monkey (Mileski et al. 1990) and the human (Carlos et al. 1990).

Monoclonal antibody 60.3 precipitates at least three distinct polypeptides (i.e. the CDw18 complex) (Beatty et al. 1983; Wallis et al. 1986). Numerous studies with MAbs 60.3, 60.1 and anti-Mo1 suggest that epitopes mediating CR3 function and adherence-dependent functions are either closely spatially linked or may be identical.

2.5.2.2 Monoclonal antibody H20A = #38

Monoclonal antibody H20A (i.e. #38 via workshop nomenclature) is a mouse antihuman/ "heinz" IgG_1, which recognizes CD18 and possibly CD11a. Recognition is by a highly conserved determinant found in multiple species ("heinz") (Davis et al. 1987; Davis W.C. - personal communication).

2.5.2.3 Monoclonal Antibody DH59B = #25

Monoclonal antibody DH59B (i.e. #25 via workshop nomenclature) is a murine MAb identifying a pan-granulocyte/monocyte surface molecule in the horse

(Davis W.C. - personal communication*; Tumas et al. 1994). Its specificity has been shown using immunofluorescent microscopy and 2-color immunofluorescent flow cytometry (Tumas et al.). Hoffman et al. (1980) defined a subpopulation of human monocytes/macrophages of peripheral blood mononuclear cells (PBMC) using this MAb and light-scattering properties. In the study by Tumas et al. 1994, forward and orthogonal light scatter properties identified that PBMC reacting with MAb #25 were a discrete subset. The same group showed that MAb #25 reacted with 100% of peripheral blood granulocytes in five horses (Tumas et al.1994).

Equine tissue distributions of leukocytes have been studied using indirect immunohistochemistry. Monoclonal antibody #25 reacted with:

- all granulocytes and most mononuclear cells in subcapsular and medullary portions of lymph nodes
- some histiocytes in cortical and medullary portions of lymph node
- granulocytic and histiocytic mononuclear cells in the spleen (i.e. red pulp of splenic cords, white pulp of the marginal zone and in the periarteriolar lymphatic sheaths)
- Kupffer cells in the liver (Tumas et al. 1994)

Western blot analyses have determined the molecular weight of the cell surface marker recognized by MAb #25. Equine granulocytes and monocytes identified by MAb #25 under reducing and non-reducing conditions, have a solitary band of 96 kDa. Monoclonal antibody #25 immunoprecipitated a complex having components of 168, 95, and 48 kDa (Tumas et al. 1994). It appears that the 95-96kDa pan-granulocyte/monocyte surface molecule, identified by MAb #25, may be a subunit of the integrin family of heterodimer leukocyte adhesion molecules (Tumas et al. 1994), although Davis (personal communication) suggests that the pattern of expression differs and it is likely to be a different β-integrin.

As MAb #25 also cross reacts with human, bovine, caprine, ovine/buffalo, porcine, and owl monkey leukocytes (Davis et al. 1987; Davis, Larsen, and Monaghan 1990; Smith et al. 1992), it is probable that this MAb identifies an evolutionary conserved surface antigen. Conservation of the leukocyte differentiation molecules of humans appears to occur in numerous species (Ledbetter et al. 1981; Davis et al. 1987; MacKay, Maddox and Brandon 1987).

* Davis W. C. Washington State University - personal communication

Monoclonal antibody #25 has been used in investigations detailing allograft injury (Tsao et al. 1993), trafficking patterns of lymphocytes in response to Interleukin-2 administration (Campos et al. 1992), and delineation of leukocyte types in different regions of bovine intestinal mucosa (Nagi and Babiuk 1989).

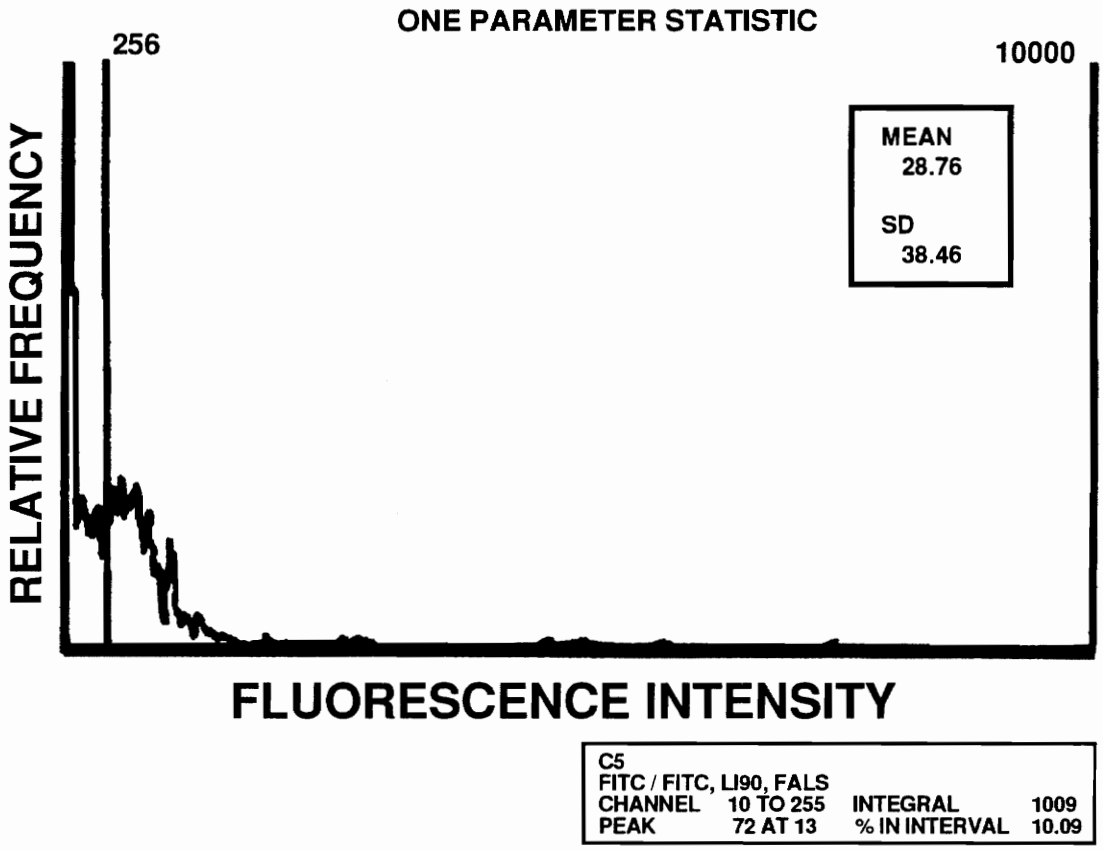
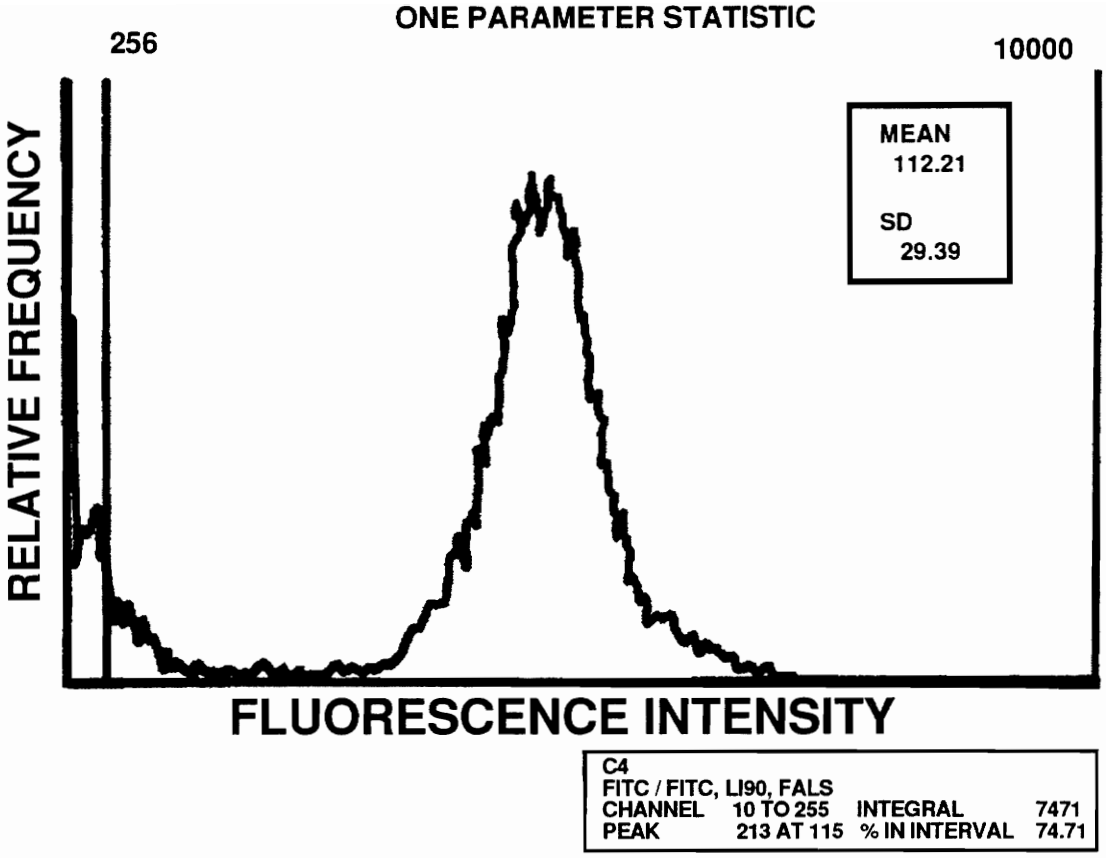


Figure 2.4

Fuorescence histograms showing one parameter statistics: (a) an histogram from equine negative control neutrophils (i.e. no primary MAb incubation), in which there is only minor background autofluorescence shown by 72 events at peak channel 13 (i.e. 72 at 13); (b) an histogram from equine neutrophils labeled with MAb #25 (i.e. DH59B) and FITC-GAM $(Fab')_2$. Gating is from channel 10 to 255. The relative frequency is 213 events at channel 115, and the mean (±SD) channel is 112.21 ± 29.39. The labelling percentage was 74.71%, which means that approximately 3/4 of these neutrophils have the specific β-integrin-like Ag.



KEY TO FIGURE 2.4

Mean	=	mean channel no.
Channel No.	=	0-256
256	=	no. of data points (i.e. channels) (x-axis).
10000	=	no. of cells counted.
Channel	=	fluorescence intensity (x-axis) (e. g. 10-255 via gating
Peak	=	peak channel.
	=	channel no. at which largest no. of events occurs (eg. 213 events at peak channel 115 - Fig. 2.4b).
	=	relative frequency.
Mean	=	mean channel no.
% labeling	=	no. of cells responding in gate 10-255 / total no. of cells counted (i.e. 10000)
	=	specific fluorescence.
	=	% of cells responding.

2.6 Flow Cytometry

Flow cytometry is a laser powered technique (Campbell 1991) used for determination of physical parameters of cells by automatic and quantitative means (Zola 1988). Subjectivity is diminished, large numbers of cells are counted and the technique is very rapid. Cells flow in a single stream through a light beam allowing interaction and information processing by optical sensors. Each specific cell emits a scatter signal, which is relayed and detected by a photodiode in direct line with the incidental light beam. Scattered light pulses trigger the counting system, ensuring that there is enumeration of approximately 10,000 to 25,000 cells (Zola 1988). Any light given off at 90 degrees to the direction of the light beam will be detected by photomultipliers. Filters can be used to remove incidental light wavelength, so only fluorescence emission will be recorded. This enables enumeration of cells with specific monoclonal antibody attachment of fluorescent dyes. Fluorescence activated cell sorting (FACS) is responsible for recording two signals for each cell:

1. light scatter
2. fluorescence.

Low angle scatter signals allow cell by cell enumeration and estimation of cell size. Conversely, right angle scatter intensity relates to membrane contours of the cell. Right angle scatter intensity increases if cells have rough membranes or many organelles (Zola 1988). The combination of this data allows differentiation of many cells.

Fluorescent tagging of certain cell surface antigens by MAb usage increases the usefulness of flow cytometry greatly. Fluorescence activated cell sorting can indicate whether there is a strong signal from a small population or a poor signal from a large population, which is advantageous compared to live-cell ELISA. However, FACS does not adequately localize Ag distribution, and is therefore inferior to immunocytochemical assays in this respect (Campbell 1991).

The monochromatic emission of the laser from the FACS instrument will not excite all dyes capable of fluorescence (Campbell 1991). The primary excitation wavelength varies with the ion of the laser. The most commonly used is the argon ion laser, which has a wavelength of 488 nm. The most common fluorochrome used for FACS is probably fluorescein isothiocyanate (FITC). This has an excitation range from

400-530 nm with an emission maximum of 530 nm (Campbell 1991). It fluoresces green in low density staining and yellow in high density staining. It is easily bound to proteins. Other fluorochromes used in FACS include: R-phycoerythrin (used extensively in two color FACS analysis), Texas red (not compatible with the argon ion lasers), Rhodamine (not compatible with the argon ion lasers), Propidium iodide and 7-amino Actinomycin D (Campbell 1991).

Fluorescence histogram interpretation may be complex (Fig. 2.4 a & b) (Fletcher and Seligmann 1985). Bimodal and unimodal frequency distributions require different interpretations. "Gating" (i.e. the setting of an analytical gate to exclude some data) and setting thresholds to look at particular subsets of cells are crucial. By selecting thresholds on the FACS instrument, one eliminates inter-observer variance (Zola 1988).

2.7 Lipopolysaccharide

Lipopolysaccharide (LPS) is part of the lipid bilayer outer membrane of the cell wall of gram negative bacteria. This complex constitutes phospholipid A, oligosaccharide R-specific core and polysaccharide portions (Berry 1977; Henry and Moore 1990). The polysaccharide is termed "O specific antigen." Release of LPS occurs secondary to bacteriolysis (Burrows 1981) and during the rapid phase of bacterial multiplication.

Endotoxemia caused by circulating LPS is a destructive and serious syndrome accompanying numerous disease entities in the horse. Lipopolysaccharide is directly and indirectly responsible for severe hemodynamic, hematologic and metabolic alterations (Duncan et al. 1985; Henry and Moore 1988, 1990). Lipopolysaccharide directly activates the alternative complement pathway, with subsequent anaphylotoxin (i.e. C3a and C5a) production causing vasodilation and chemotaxis respectively (Henry and Moore 1990). The complement fragment C3b is probably not involved in LPS pathogenesis.

Leukocytes play a critical role in the pathophysiology of endotoxic/endotoxemic shock due to their elaboration of multiple vasoactive mediators (eg. procoagulant activity (Henry and Moore 1988), platelet activating factor (Doebber et al. 1985; Issekutz and Szpejda 1986), Interleukin 1 (IL-1) , tumor necrosis factor, colony stimulating factor and interferon). Unfortunately, the role of these factors has been examined only minimally in the horse. Interferon-γ (INF-γ) may have specific

amplification (eg. INF-γ amplification of IL-1 production from monocytes/macrophages stimulated with LPS (Gerrard et al. 1987)) and suppressive (Schindler, Ghezzi and Dinarello 1990) effects on inflammatory reactions, mediated by LPS.

Lipopolysaccharide also functions to exacerbate glomerular injury in type III hypersensitivity reactions (Karkar et al. 1992). Toxicity of LPS is assumed in part to relate to its ability to activate neutrophils. Low toxic LPS (eg. *Bordetella pertussis* LPS) has potential as an antitumor agent (Abe et al. 1992). This low toxin LPS is probably less noxious than other LPS, because of its inability to activate neutrophils (Abe et al. 1992).

CHAPTER 3

Materials and Methods

3.1 Experimental Plan

Blood was collected from six horses on two different occasions at seven- to thirty-day intervals. Eleven tubes of whole blood (i.e. EDTA vacutainer tubes) and a tube of heparinized blood (i.e. heparin vacutainer tube) were collected each time for neutrophil isolation, complete blood count (CBC), and biochemical profile.

3.1.1 Animals

Six mixed-bred geldings aged from three to six years were used for this investigation. Details of their identification codes, breeds, sex and ages are given in Table 3.1. Horses were currently vaccinated for tetanus, eastern equine encephalomyelitis and western equine encephalomyelitis, herpes virus type 1 and influenza virus ("Encevac TC-4", Miles Inc., Kansas, 66201, USA; "Rhinomune", SmithKline Beecham Animal Health, PA, 19380, USA) and were dewormed at 90 day intervals. Physical examinations were conducted on the horses prior to the investigation. A complete blood count and biochemical profile were performed at each sampling period.

Table 3.1 The identification code, breed, sex and age of experimental horses. (MN = male neuter, ASB = American Saddlebred, TB = Thoroughbred, AND = Andulusian, MISS = Missouri Foxtrotter, QH = Quarterhorse, X = cross).

Identification Code	Breed	Sex	Age (Years)
1	ASB-TB	MN	3
2	TB-X	MN	3
3	Holsteiner	MN	6
4	AND-TB	MN	4
5	MISS	MN	5
6	QH-TB	MN	4

Horses (except horse 6) were accommodated in open yards (~100 x 50 m) with six horses per yard. Diets consisted of pasture, which was minimal, free choice grass hay, and approximately 2 kg of corn/soybean meal mixture twice daily. Horses were fed in groups. Horse 6 was accommodated in an 11 acre pasture with one other horse. Horse 6 was on a diet consisting of pasture, free choice mixed clover / alfalfa / fescue hay and 0.45 kg/day mixed grain / pellet ration (Southern States, USA).

3.2 Methods

3.2.1 Neutrophil isolation and confirmation of cell type

Percoll gradients were prepared with 59 and 75 percent Percoll. Ten ml of 59 percent Percoll was placed into a centrifuge tube, then by using a 25 gauge spinal needle and a Harvard compact infusion pump (model 975), ten mL of 75 percent Percoll was layered underneath the 59 percent Percoll. The two Percoll solutions were produced using isosmotic Percoll and calcium free modified Hanks solution (CFMH). The specific gravities of the 59 and 75 percent Percoll solutions were 1.015 and 1.044 respectively.

Whole blood was centrifuged at 1000 rpm for 15 minutes at 19-24° C (66.2 - 75.2° F) with an optimum of 22.5° C (72.5° F) on a IEC HN-S11 centrifuge (Damon/IEC Division, USA). This procedure caused formation of the buffy coat. Superfluous fluid was removed down to the level of the leukocyte-rich buffy coat. The buffy coat layer was collected and diluted. This suspension was then layered onto the Percoll gradient. The centrifuge was balanced, and tubes containing buffy coat suspension layered onto Percoll gradient were spun at 2200 rpm for 45 minutes. After centrifugation three different bands could be perceived in the tube. Neutrophils sedimented to the interface of the 59 percent and 75 percent Percoll layers, whilst mononuclear cells remained on top of the 59 percent. Percoll solution and erythrocytes sedimented to the bottom of the 75 percent Percoll layer (Bryant T. - personal communication*).

The band of mononuclear cells and 59 percent Percoll were carefully removed to a level approximately 5 mm above the neutrophil band, using the water vacuum. Then a glass Pasteur pipette was used to siphon the neutrophil band from the Percoll gradient. Fifty µl of the cell suspension was combined with 450 µl of Hanks Balanced Salt Solution (HBSS) and neutrophil concentration was determined using the Coulter Counter Model ZF (Coulter, FL, USA). Cells were then diluted to 1×10^7 cells/ml. The cell suspension was immediately sampled to evaluate cell type and viability (i.e. pre-incubation sample). The trypan blue exclusion method was used to assess the latter. Twenty µl of the cell suspension (i.e. neutrophil isolate) was mixed with 20 µl of 0.4 percent trypan blue. Ten µl of the mixture was dispensed onto an hemocytometer and 200 neutrophils were counted to determine the percentage of viable cells, which excluded trypan blue.

3.2.2 Monoclonal antibodies and their concentrations

Three primary monoclonal antibodies were used in this investigation, including:

- 60.3 (Bristol-Myers Squibb, Seattle, WA). This is a mouse antihuman $IgG_{2\alpha}$ MAb, which recognizes CD18 multimeric cell surface glycoprotein complex (Beatty et al. 1983). This MAb does not have an equine workshop number.

* Bryant T. Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University - personal communication

- H20A = #38 (Kydd and Antczak 1991) is a mouse antihuman "heinz" IgG_1 MAb, which recognizes the CD18 integrin subunit, and possibly the CD11a subunit, by recognition of a highly conserved determinant identified in multiple species. The term "heinz" refers to the ability of #38 MAb to cross react with numerous species, other than human (i.e. equine, bovine, porcine, etc.)
- DH59B = #25 (Kydd and Antczak 1991) is a mouse antidog "heinz" IgG_1 MAb, which appears to recognize a CD18-like integrin subunit (Tumas et al. 1994). This MAb cross-reacts with many species (Davis et al. 1987).

The secondary MAb was fluorescein isothiocyanate - goat antimouse $F(ab')_2$: (FITC-GAM $F(ab')_2$) (Accurate Chemical and Scientific Corp., Westbury, NY, USA). This fluorescent-tagged secondary MAb was produced in a goat host as $F(ab')_2$ and has affinity to the murine IgG (H + L) (i.e. heavy and light chain whole molecule), so that it can recognize primary MAbs and fluorescently label them. This secondary MAb was isolated from antiserum by a combination of pepsin digestion (see Fig. 2.3) and immunoaffinity chromatography, using Ags coupled to agarose beads. Whole IgG molecules and Fc fragments were removed from the product.

All MAbs were diluted to a concentration of 10 µg/ml (i.e. 1:100 dilution from a 1 mg/ml stock solution) using sterile technique.

3.2.3 Centrifugation speed and duration

The speed and duration of 96 well plate centrifugation resulting in the highest yield of viable cells was documented prior to the commencement of this investigation (Savage C.J. and Wilcke J.R. - personal communication*). A speed of 1100 rpm for ten minutes was used for centrifugation of 96 well plates.

3.2.4 Lipopolysaccharide stimulation, monoclonal labeling and flow cytometry

Peripheral blood neutrophils were obtained from six geldings (Table 3.1) on two occasions. Isolated cells were suspended in HBSS with 1% gamma globulin free

* Savage C. J. and Wilcke J. R. - personal communication

horse serum (GGFHS) (Catalogue no. H-9764, Sigma Cell Culture Reagent, Sigma Chemical Company, St. Louis, MO, USA) and adjusted to a concentration of 1 x 10^7 cells/ml. Aliquots of 100 µl of neutrophil solution were placed into 16 wells of 96 well microtiter plates (i.e. 1 x 10^6 cells/well) for centrifugation. Sedimented neutrophils were then either stimulated with 100 µl of 1 mg/ml Lipopolysaccharide (LPS) (No. L7261 Lipopolysaccharide from *Salmonella typhimurium* TCA Extract, Sigma Chemical Company, St. Louis, MO, USA) or incubated only with 100 µl of HBSS and 1% GGFHS). The plates were incubated for one hour in a Napco model 5300 CO_2 incubator (Napco, USA) with 5% CO_2 at 98.6°F (37°C).

Plates were centrifuged for ten minutes at 1100 rpm and the supernatant was removed, thus leaving the cells at the base of the wells. One hundred µl of MAb solution (i.e. 60.3, #38 or #25) was added to wells containing 1 x 10^6 cells and incubated in darkness on ice within the refrigerator at 4° C for 20 minutes. After centrifugation supernatant was discarded and cells washed (i.e. suspended, balanced and re-centrifuged) twice in HBSS with 1% GGFHS.

The cell pellet was then reconstituted in 100 µl of the secondary MAb. FITC-GAM F(ab')$_2$), mixed and incubated on ice in the refrigerator at 4° C for 20 minutes.

Cells were washed twice, supernatant removed and cells reconstituted in 200 µl of HBSS with 1% GGFHS. Flow cytometry was used to characterize the cells. Cells were also examined using light microscopy for evidence of activation.

3.2.5 Fluorescent activated cell sorting (FACS)

Fluorescence intensity (i.e. percentage of cells with fluorescence), relative frequency (i.e. cells counted) at peak channel number and mean channel number were determined by an argon ion flow cytometry unit (Coulter Epies 752, Coulter, FL, USA) until enumeration of 10,000 cells. Gating of channels was from 10 to 245-255 and maximum cells counted per channel was 269, although the capacity of each channel was much greater. The argon ion laser had a wavelength of 488 nm, whilst the wavelength of fluorescein in FITC-GAM F(ab')$_2$ was approximately 530 nm (Campbell 1991).

3.2.6 Cell viability

Cells were examined for viability after isolation and FACS, using trypan blue exclusion. This technique is described in Chapter 3.2.1. The trypan blue stain is excluded by live cells. Viability was expressed as follows:

$$\text{Viability (\%)} = 100 - \frac{\text{no. dead cells x } 100}{\text{cells counted}}$$

3.2.7 Statistical Methodology

Fluorescent labeling percentage, peak channel number (i.e. channel number at which greatest number of neutrophils (relative frequency) were counted) and mean channel number are presented as means ($\bar{X}$). Numerous variance stabilization transformations did not solve the problem of non-normal data with unequal variances, which consequently caused violation of two assumptions of the parametric analysis of variance statistical test. Therefore, the non-parametric Friedman test with appropriate multiple comparisons (Conover 1980) was chosen to assess differences ($p < 0.05$). Analysis was performed on ranked data using "PROC GLM" (Procedure General Linear Models) and "SAS" (Statistical Analysis Systems).

3.2.8 Discussion

Isolation of essentially pure neutrophils increased confidence in results. Neutrophil activation can occur in systems in which their is contamination with macrophages and/or T- and B-cells, due to their production of cytokines (i.e. IL6, IL1, TNF) (Bevilacqua et al. 1985; Robinson 1994). In the study on equine neutrophils by Bochsler et al (1990), FITC-GAM $F(ab')_2$ was not used, thereby increasing non-specific fluorescence. This omission was rectified in the study described herein. Studies have been performed confirming the action of $F(ab')_2$ (Arfors et al. 1987). Primary monoclonal antibodies, not specific for the horse, (i.e. 60.3, #25, #38) were used here, which was less than optimal. The microscopic technique used to confirm degranulation of neutrophils after LPS-stimulation was also suboptimal, and lysozyme (Lopez et al. 1986) and myeloperoxidase techniques should be pursued. Fluorescence activated cell sorting does not adequately localize antigen (i.e. CD18 and β-integrin-like) and results should be confirmed with immunocytochemical techniques (Campbell 1991).

CHAPTER 4

Characterization of CD18 and β-integrin-like surface antigen of equine neutrophils using three monoclonal antibodies

4.1 Introduction

Immunocompetence is of paramount importance to the survival of animals. Neutrophils are important in the acute defense mechanism (Issekutz and Movat 1980), and deficiencies or dysfunction of neutrophils cause grave pathological sequelae.

Recent progress in the immunology of humans and horses has been astounding, although only one "International Workshop on Equine Leukocyte Differentiation Antigens"* has been held. Here terminology (eg. "equine cluster designations" (eq CD)) and nomenclature (eg. classification of monoclonal antibodies by number: H20A = #38) for antigen and monoclonal tools were defined (Kydd and Antczak 1991).

Neutrophil surface antigens have been the focus of much development and progress in the human field, especially with the recognition of LAD (Crowley et al. 1980). This has also been beneficial for the bovine industry, in that BLAD (Hagemoser et al. 1983; Kehrli et al. 1990) has subsequently been characterized rapidly. Syndromes like these have not been recognized in the horse, however, the characterization of the surface presentation of Ags acting as adhesion molecules is crucial to understand mechanisms of immunity in the horse. The equine industry often relies on knowledge inferred by extrapolation of studies in different species (eg. human, rodent), which is often inappropriate. The neutrophil can also accumulate in tissues in excessive numbers, thereby causing and exacerbating tissue damage. This has been proposed in ARDS and ischemia-reperfusion processes, and may be blocked by anti-β_2-integrin monoclonal antibodies, although this appears to be site and stimulus-dependent (Carlos and Harlan 1990). The lung may express inducible unique ligands allowing alveolar neutrophil accumulation by additional CD18-independent mechanisms (Carlos and Harlan 1990). This has not been determined in horses.

It is clear that β_2-integrin expression on neutrophils exists without the occurrence of adhesion to endothelial cells, but equine studies have only been undertaken recently (Bochsler et al. 1990) and little is known in the horse.

CD11/CD18 is not necessary for the initial "tethering" of the neutrophil to the endothelial cell. If endothelial cells are activated (eg. by thrombin), then co-expression of platelet activating factor (PAF) and p-selection occurs to which neutrophil receptors (i.e. ligands) bind. This initial binding procedure requires neither functional nor quantitative upgrading of CD11/CD18 heterodimer on the neutrophil (Zimmerman et

* International Workshop on Equine Leukocyte Differentiation Antigens. Equine Vet. J. Suppl. 12, 1991

al. 1992), because it is primarily "selectin" mediated. However, PAF may enhance subsequent CD11/CD18 dependent adhesion (Zimmerman et al. 1992). If neutrophils lack CD11/CD18 (eg. LAD, BLAD), then PAF binding occurs, but is too weak (Lorant et al. 1991) to ensure adhesion and migration.

The aim of this experiment was to determine the surface presentation of the CD18 subunit of β_2-integrins and β-integrin-like Ags of equine neutrophils. Two anti-CD18 MAbs (i.e. 60.3 and #38) and an anti-β-integrin-like MAb (i.e. #25) were used. The latter recognizes a peptide of 95 kDa, which may be CD18 (Tumas et al. 1994), but appears to differ from CD18 because the peptide has a different pattern of expression (Davis W.C. - personal communication). Two of the three MAbs were described for use in the equine by Kydd and Antczak (1991) (i.e. H20A = #38 and DH59B = #25) and MAb 60.3 was used for studies in the horse by Bochsler et al. (1990).

A secondary aim was to assess and compare the degree of labeling of neutrophils with the three different MAbs (i.e. MAb 60.3, MAb #38, MAb #25).

4.2 Results

In this portion of the investigation:

- the labeling percentage (i.e. number of cells responding within gated region X 100/ total number of cells counted (i.e. 10000)

- the peak channel number (i.e. indicator of fluorescence intensity and sometimes density)

- the mean channel number (i.e. indicator of fluorescence intensity)

- were examined in neutrophils, which had no prior stimulation (see Fig 2.4).

4.2.1 Expression of CD18 Surface Antigen on Equine Neutrophils using Monoclonal Antibody 60.3

Neutrophils of six horses, labeled with anti-CD18 MAb 60.3 and FITC-GAM $F(ab')_2$ had a mean labeling percentage of 97.17%. This was significantly greater ($p = 0.0001$) than neutrophils from the same horses acting as a negative control (i.e. those cells that were not incubated with a primary MAb) (Fig. 2.4a and Table 4.4). Mean labeling percentage of the negative control neutrophils was 10.37%. These results are presented in Table 4.1.

The mean number of events (i.e. neutrophils counted with fluorescence) was 259.96 at the peak channel (i.e. fluorescence intensity channel with largest number of labeled neutrophils) of 143.63 (Table 4.2). The mean channel number, indicating mean fluorescence intensity was 141.99 (Table 4.3).

4.2.2 Expression of CD18 Surface Antigen on Equine Neutrophils using Monoclonal antibody #38.

The mean labeling percentage of neutrophils from six horses labelled with MAb #38 and FITC-GAM $F(ab')_2$ was 94.76%. This was significantly different ($p=0.0001$) from negative control neutrophils (Tables 4.1 and 4.4). The mean number of labeled neutrophils counted at the peak channel of 121.29 was 206.63 (Table 4.2). The mean channel number was 120.38 (Table 4.3).

4.2.3 Expression of β-integrin-like Surface Antigen on Equine Neutrophils using Monoclonal antibody #25.

Neutrophils from the six horses labelled with MAb #25 and FITC-GAM $F(ab')_2$ had a mean labeling percentage of 87.67% (Table 4.1), which was significantly different from the control group ($p=0.0001$) (Table 4.4). The mean number of labeled neutrophils counted was 269.26 at the peak channel of 107.00 (Table 4.2). Mean channel number was 103.84 (Table 4.3).

Table 4.1 Percentage labeling of non-stimulated neutrophils within the gated region.

PERCENTAGE LABELING OF NON-STIMULATED NEUTROPHILS %			
60.3	#38	#25	Negative Control
97.17	94.76	87.67	10.37

Table 4.2 Mean number of labeled, non-stimulated neutrophils at the peak channel number (i.e. fluorescence intensity). This refers to the relative frequency of peak fluorescence intensity of non-stimulated neutrophils. Different letters (i.e. a-d) indicate significant differences ($p < 0.05$) between groups.

Group	RELATIVE FREQUENCY OF FLUORESCENCE INTENSITY OF NON-STIMULATED NEUTROPHILS	
	No. of Events ($\bar{X}$)	Peak Channel No. ($\bar{X}$)
60.3	259.96	143.63^{abcd}
#38	206.63	121.29^{abcd}
#25	269.26	107.00^{abcd}
Negative Control	48.96	17.58^{abcd}

Table 4.3 Mean channel number of non-stimulated neutrophils incubated with monoclonal antibodies 60.3, #38 (i.e. H20A) and #25 (i.e. DH59B); and a negative control (i.e. no monoclonal antibody). Different letters (i.e. a-d) indicate significant differences ($p < 0.05$) between groups.

Group	Mean Channel No.
60.3	141.99^{abcd}
#38	120.38^{abcd}
#25	103.84^{abcd}
Negative Control	78.61^{abcd}

4.2.4 Comparison of monoclonal antibody labeling

The results of percentage labeling of non-stimulated neutrophils incubated with three different monoclonal antibodies (i.e. 60.3, #38, #25) and FITC-GAM $F(Ab')_2$, or FITC-GAM $F(Ab')_2$ alone (i.e. no primary MAb = negative control) are shown in Table 4.4. Tables 4.2 and 4.3 also show the differences between groups (i.e. 60.3, #38, #25 and negative control) in peak channel number (reflecting the fluorescence intensity at which the greatest number of neutrophils were counted, and mean channel number (indicative of mean fluorescence intensity). No standard deviations or standard errors are presented, because statistics were performed using the non-parametric Friedman's test with appropriate multiple comparisons (i.e. a ranking system, in which means and standard deviations/standard errors are not pertinent).

Table 4.4 The p-values associated with differences in labeling percentages. Differences were tested with the non-parametric Friedman's test and multiple comparisons (i.e. a ranking system) of non-stimulated neutrophils incubated with primary monoclonal antibodies (i.e. 60.3, #38, #25).

DIFFERENCE IN PERCENTAGE LABELING OF NON-STIMULATED NEUTROPHILS		
Groups	Percentage Labeling	P-Value*
Negative Control vs 60.3	10.37 vs 97.17	p = 0.0001
Negative Control vs #38	10.37 vs 94.76	p = 0.0001
Negative Control vs #25	10.37 vs 87.67	p = 0.0001
60.3 vs #38	97.17 vs 94.76	p = 0.006
60.3 vs #25	97.17 vs 87.67	p = 0.0001
#38 vs #25	94.76 vs 87.67	p = 0.0013

*Ranked data

These results show that MAb 60.3 labels significantly more neutrophils and has significantly greater fluorescence intensities (indicated by mean and peak channel numbers) compared with MAb #38, which labels significantly more and has greater fluorescence intensity than MAb #25. All three MAbs cause significantly greater labeling percentages and have greater fluorescence intensities than negative control group neutrophils.

In addition, it can be seen that the negative control group (i.e. no incubation with primary MAb) has only limited autofluorescence (Tables 4.1, 4.2, 4.3).

4.2.5 Viability of Neutrophils

Viability of neutrophils pre-and post-incubation with LPS, primary MAbs and secondary MAb was measured using trypan blue exclusion. The percentage of viable neutrophils pre-incubation ranged from 94% to 100% ($\overline{X}$ = 97%). After incubation, neutrophils from each group were assessed. Viability ranged from between 95% to 100% (i.e. 60.3 $\overline{X}$ = 96%; #38 $\overline{X}$ = 98%; #25 $\overline{X}$ = 97%; negative control $\overline{X}$ = 96%). These were not significantly different ($p > 0.05$) either between groups or after incubation. There was no significant difference between viability of cells after incubation with either HBSS with 1% GGFHS ($\overline{X}$ = 98%) or LPS ($\overline{X}$ = 96%).

4.3 Discussion

Adhesion of neutrophils to endothelial cells early in the inflammatory process is a crucial and complex mechanism involving numerous proadhesive and adhesion molecules.

The β_2-integrins are an important class of adhesion molecules. In activated granulocytes, the CD11b/CD18 surface antigen complex mediates binding to endothelia (Harlan et al. 1985); although CD11a/CD18's importance has also been described (Forsyth and Levinsky 1989). Co-expression of GMP-140 and PAF occurs on activated endothelia, leading to cooperative function (Lorant et al. 1991). The GMP-140 tethers neutrophils independently of β_2-integrins, yet PAF upregulates CD11/CD18 on granulocytes (Lorant et al. 1991).

Results from this study showed that CD18 is almost constitutively expressed on unstimulated equine neutrophils. Using MAbs 60.3 and #38, it was clear that >94% of these neutrophils express surface CD18. The MAb #38 may also recognize a determinant of CD11a (Davis W.C. - personal communication)* . It is possible that constitutive expression of β_2-integrins occurs and that MAbs 60.3 and #38 have distinct and discrete optimal incubation conditions. It is well recognized that most MAb systems require incubation at $\leq 4°$ C for 10 to 30 minutes. Specific conditions were not tested for MAbs 60.3 and #38, however, labeling percentage was uniformly high. This, however, does not preclude that MAb #38's ability to recognize CD18 ($\pm$ CD11a) may have been hampered by lack of appropriate temperature control or sufficient time, thereby causing significantly less ($p < 0.05$) labeling than MAb 60.3. These MAbs probably recognize epitopes of the integral membrane protein, including the extracellular position of the β_2 subunit. Consequently surface expression is common in the unstimulated cell. This is interesting as this means that quantitative up-regulation (Bochsler et al. 1990) may be less important than functional qualitative alterations to the β_2 subunit, which subsequently cause a type of stimulatory up-regulation (Wright and Meyer 1986; Phillips et al. 1988; Schwartz and Harlan 1989).

In this study, increased labeling percentage of non-stimulated equine neutrophils was demonstrated when MAb 60.3 was used compared to MAb #38 ($p=0.006$) and MAb #25 ($p=0.0001$) (Table 4.4). The percentage labeling refers to the percentage of neutrophils enumerated with at least one fluorescent label. It does not

* Davis W. C., Washington State University - personal communication.

reflect fluorescent density, and is a poor measure of quantitative up-regulation if expression is almost constitutive. Similarly the fluorescence intensity was also significantly greater in neutrophils incubated with MAb 60.3 (Tables 4.2, 4.3). MAb 60.3 appeared to be repeatedly superior in its binding to CD18 than MAb #38. The mean and peak channel numbers merely indicate static fluorescence intensity, without reflection of fluorescence density per neutrophil. The latter can only be expressed when there is dynamic drift in FACS histograms for the same MAb. This is demonstrated in Chapter 5. Both MAbs 60.3 and #38 appear superior to MAb #25 in aspects of percentage labelling and fluorescence intensity; however, Tumas et al. (1994) found that MAb #25 identified 100% of peripheral blood granulocytes in five horses. It appears that MAb #25 recognizes a β_2-integrin-like surface antigen of equine neutrophils, but this has not been definitively clarified (Davis W.C. -personal communication)* . It is unlikely that MAb #25 is specific for CD18. Consequently, direct comparison between the two anti-CD18 MAbs and MAb #25 has minimal relevance, except to demonstrate that the degree of expression of β_2-integrin like surface antigen is less than for CD18 and is not constitutive, although this is not in agreement with findings by Tumas et al. (1994). Specific incubation conditions are not known for MAb #25.

The low level of autofluorescence in the negative control group occurs secondarily to the use of FITC-GAM $F(ab')_2$, as there is no Fc fragment (Figs. 2.3 and 2.4) to elicit non-selective binding. However, autofluorescence is not completely abolished.

* Davis W. C., Washington State University - personal communication.

CHAPTER 5

The effect of lipopolysaccharide stimulation on equine neutrophil CD18 and β-integrin-like surface antigen expression.

Page No.

5.1 Introduction

Lipopolysaccharide (i.e. LPS) induces neutrophil activation (Abe et al. 1992), release of chemotactic factors (Collins et al. 1989) and subsequent adhesion and extravasation. Conversely, LPS has been shown to depress bovine granulocyte oxidative metabolism and ingestion (Kehrli 1985).

Neutrophils contain lysosomal enzymes, one of which, acycloxyacyl hydrolase, is instrumental in deacylation and detoxification of LPS. This has been reported in peripheral and milk granulocytes of the bovine (McDermott, Merrill and Fenwick 1991). Severity of endotoxemia in coliform mastitis may be due to a complex interaction of numerous factors including neutrophil acycloxyacyl hydrolase, a non-protein inhibitory substance and the inherent ability of milk to deacylate LPS inherently (McDermott et al. 1991). Endothelial cells may be damaged directly by LPS, although this can be ameliorated by granulocyte activity (Breider, Kumar and Corstvet 1991; Breider, Kumar and Corstvet 1991a).

Lipopolysaccharide may directly stimulate leukotriene production in neutrophils and cause their sequestration (Meyruk and Brigham 1983; Bottoms et al. 1985). It has also been demonstrated that eicosanoid elaboration occurs secondary to LPS stimulation through its direct effects on granulocytes and endothelial cells (Bottoms et al. 1985).

Numerous equine diseases manifest with endotoxemia, caused by circulating LPS and its subsequent cascade of cytokine-and eicosanoid-induced effects. Deleterious responses have been demonstrated in hemodynamic, procoagulant, hematologic and metabolic system alterations in the horse (Duncan et al. 1985; Henry and Moore 1988, 1990). Toxicity of LPS appears to relate in part to its ability to activate neutrophils, however, the effects of circulating cytokine and eicosanoid concentration and function also appear crucial for the onset of endotoxemia. Conversely, LPS may cause activation of neutrophils, which initially marginate in blood vessels, manifesting as neutropenia. Subsequent activation of CD11/CD18 causes extravasation to tissues. Thus LPS appears to cause activation of neutrophils, which are necessary to combat disease, yet on over-zealous response may actually result in further symptoms of disease.

Most immune cells must adhere to different cell and intercellular substance ligands for functional integrity. Without adhesion, neutrophils can neither enhance inflammatory function, nor arrive at their target tissues (eg. LAD and BLAD

syndromes). However, neutrophils are not innocuous and in times of excess can actually manifest in a deleterious manner (eg. ARDS, LPS-induced pneumonia (Udeze, Latimer and Kadis 1987), mastitis (Kehrli 1985) and ischemia-reperfusion injuries (Vedder et al. 1988)).

The β_2 integrins are critical in neutrophil adhesion in times of injury or inflammation. One may expect to see increased expression of C11/CD18 complexes per cell with an increased number of cells elaborating CD11/CD18 adhesion molecules. Rieu et al. (1992) reported the induction of extensive CD11b up-regulation by LPS. However, quantitative down-regulation has also been reported (Dustin and Springer 1989; Carlos and Harlan 1990; Lorant et al. 1991; Zimmerman et al. 1992) and is assumed to be associated with qualitative up-regulation.

5.2 Results

In this study, LPS-stimulated equine neutrophils were examined using FACS, and comparisons to non-stimulated neutrophils were made.

5.2.1 Effect of lipopolysaccharide stimulation on CD18 and β-integrin-like surface presentation assessed with monoclonal antibodies 60.3, #38 and #25

Lipopolysaccharide-stimulated neutrophils of six horses labeled with the anti-CD18 MAb 60.3 and FITC-GAM $F(Ab')_2$ had a mean labeling percentage of 93.17%. This labeling percentage was significantly greater ($p=0.0002$) than that of LPS-stimulated neutrophils from the same horses acting as a negative control group. Mean for negative control neutrophils was 16.87% (Table 5.1). The percentage of LPS-stimulated neutrophils labelled with MAb #38 was 89.16%, which was also significantly greater ($p=0.0001$) than the control group (Tables 5.1 and 5.2). The percentage of LPS-stimulated neutrophils labeled with MAb #25 was 85.77%, which was significantly greater ($p=0.0001$) than the control group (Tables 5.1 and 5.2).

Table 5.1 Percentage labeling of LPS-stimulated neutrophils within the gated region.

Percentage Labeling of LPS-Stimulated Neutrophils (%)			
60.3	#38	#25	Negative Control
93.17	89.16	85.77	16.87

Table 5.2 The p-values associated with differences in labeling percentages of LPS-stimulated neutrophils incubated with primary monoclonal antibodies (i.e. 60.3, #38, #25). Differences were tested with the non-parametric Friedman's test and multiple comparisons (i.e. a ranking system).

Difference in Labeling Percentage of LPS-Stimulated Neutrophils		
Groups	Percentage Labeling	p-value*
Negative Control vs. 60.3	16.87 vs 93.17	p = 0.0001
Negative Control vs. #38	16.87 vs 89.16	p = 0.0001
Negative Control vs. #25	16.87 vs 85.77	p = 0.0001
60.3 vs. #38	93.17 vs 89.16	p = 0.0047
60.3 vs. #25	93.17 vs 85.77	p = 0.0003
#38 vs. #25	89.16 vs 85.77	p = 0.36

* Ranked data

The results of the number of events ($\bar{X}$) at the peak channel ($\bar{X}$) are tabulated in Table 5.3. The peak channel number was highest in LPS-stimulated neutrophils labeled with primary MAb 60.3 (153.35), then MAb #38 (125.54), then MAb #25 (106.88). These were all significantly different ($p < 0.05$) from the negative control LPS-stimulated neutrophils (27.17), which were not incubated with primary MAb.

Mean channel numbers, reflecting mean fluorescence intensity were greater in LPS-stimulated neutrophils incubated with MAb 60.3 (148.46) compared with MAb #38 (120.80) and MAb #25 (105.97). Negative control group showed minimal mean autofluorescence intensity (65.53).

Table 5.3 Mean number of neutrophils counted (events) at the peak channel number (i.e. fluorescence intensity); these refer to the relative frequency of LPS-stimulated neutrophils.

Group	Fluorescence Intensity of LPS-Stimulated Neutrophils	
	No. of Events ($\bar{X}$)	Peak Channel No. ($\bar{X}$)
60.3	242.61	153.35
#38	153.92	125.54
#25	229.29	106.88
Negative Control	52.79	27.17

5.2.2 Effect of lipopolysaccharide stimulation on CD18 and β-integrin-like surface presentation in comparison with non-stimulated neutrophils

There was no difference ($p > 0.05$) in the labeling percentage of neutrophils in the two negative control groups of neutrophils irrespective of stimulation status (i.e. non-stimulated vs. LPS-stimulated), because of a similar degree of autofluorescence (Table 5.4). Nor did the peak channel number alter significantly ($p > 0.05$) (i.e. non-stimulated control: 17.58 vs. LPS-stimulated control : 27.17) (Tables 4.2 and 5.3).

Lipopolysaccharide-stimulated neutrophils labeled with MAb 60.3 did not show an increase in labeling percentage. In fact, there was a significant ($p = 0.002$) decrease in the percentage of neutrophils labeled for CD18 surface antigen, compared to non-stimulated pmns labeled with MAb 60.3. This was calculated using the ranking system. Neutrophils labeled with MAb #38, which had been stimulated with LPS also did not demonstrate an increase in labeling percentage. There was a significant ($p = 0.002$) decrease in the number of neutrophils with CD18 moeities labeled, using the ranking system. Stimulation of neutrophils with LPS, subsequently labeled with MAb #25 revealed no difference ($p = 0.41$) in β-integrin-like surface antigens labelling percentage by FACS, compared to non-stimulated neutrophils.

Table 5.4 The p-values associated with differences in labeling percentage tested by non-parametric Friedman's test and multiple comparisons (i.e. a ranking system) between non-stimulated and LPS-stimulated neutrophils.

Non-stimulated Neutrophil vs. LPS Stimulated Neutrophil			Labeling Percentage	p-value*
Negative control	vs.	Negative control/LPS	10.37 vs 16.87	0.46
60.3	vs.	60.3/LPS	97.17 vs 93.17	0.002
#38	vs.	#38/LPS	94.76 vs 89.16	0.002
#25	vs.	#25/LPS	87.67 vs 85.77	0.41

* ranked data

Mean and peak channel numbers of these groups (i.e. non-stimulated vs LPS-stimulated) are tabulated in Tables 5.5 and 5.6. No p-values could be generated for mean and peak channel numbers, as there was no significant interaction between LPS and group type in the mean and peak channel numbers, using the Friedman's analysis of variance (i.e. non parametric test). Consequently individual MAb treatments (i.e. groups) could not be compared on a non-stimulated to LPS-stimulated basis. However, as Tables 5.5 and 5.6 demonstrate there were increases in fluorescence intensity (i.e. mean and peak) with LPS-stimulation in neutrophils labeled with MAbs 60.3 and #38, and an increase in mean fluorescence intensity in LPS-stimulated neutrophils incubated with #25. However, there was a decrease in peak fluorescence intensity in LPS-stimulated neutrophils labeled with #25.

Table 5.5 The peak channel number values ($\bar{X}$) for non-stimulated and LPS-stimulated neutrophils, labeled with monoclonal antibodies 60.3, #38 and #25.

Peak Channel Number		
Group	Non-stimulated Neutrophils	LPS-stimulated Neutrophils
60.3	143.63	153.35
#38	121.29	125.54
#25	107.00	106.88
Negative Control	22.52	27.17

Table 5.6 The mean channel number values ($\bar{X}$) for non-stimulated and LPS-stimulated neutrophils, labeled with monoclonal antibodies 60.3 #38 and #25.

Mean Channel Number		
Group	Non-stimulated Neutrophils	LPS-stimulated Neutrophils
60.3	141.99	148.46
#38	120.38	120.80
#25	103.84	105.97
Negative Control	78.61	65.53

Neutrophil viability after incubation did not vary with LPS-stimulation in comparison to HBSS with 1% GGFHS incubation (Section 4.2.5).

5.3 Discussion

Equine neutrophils stimulated with LPS and labeled with primary MAb 60.3 had a lower labeling percentage than non-stimulated, 60.3-labeled neutrophils. This did not concur with works by Lo et al. (1989) and Vedder and Harlan (1988), who demonstrated up-regulation of surface expression of CD11/CD18 via the CD11b and CD11c subunits after neutrophil activation with a variety of stimulants. Bochsler et al. (1990) reported up-regulation of equine neutrophil surface expression of CD18, using MAb 60.3 after LPS-pre-incubation; however, greatest enhancement of expression occurred with LPS and a neutrophil agonist (i.e. zymosan-activated serum or phorbol 12-myristate 13-acetate). In this study, LPS-stimulation caused CD18 density alterations on neutrophils incubated with MAbs 60.3 and #38. There was no increase (i.e. actually significant decrease) in labeling percentage, indicating no increase in the number of neutrophils expressing at least one CD18 molecule on the surface counted. Yet, these neutrophils actually increased in fluorescence intensity, as shown by increases in mean and peak channel numbers with LPS-stimulation (Tables 5.5 and 5.6). This indicated that there was an increase in density on the LPS-stimulated neutrophil surface, because there was no increase in labeling percentage, yet an increase in fluorescence intensity per cell. This dynamic drift after LPS-stimulation demonstrated a type of intra-cell quantitative up-regulation. However, these investigations failed to reveal quantitative up-regulation of percentage labeling, in fact significant decreases were measured. These results possibly indicate qualitative alterations in some neutrophils, although this was not proven as neutrophil activation function tests were not performed. It is interesting to speculate that quantitative and qualitative up-regulation of CD18 cell surface antigen, as measured by MAbs 60.3 and #38, may exist together. It is well documented that increased surface expression and neutrophil adherence are not necessarily causally related. In one study, stimulation of human neutrophils with various factors caused up-regulation of CD11b/CD18 without promoting adherence (Lopez et al. 1986). Conversely, activated neutrophils with their CD11b/CD18 surface expression blocked by the anion channel-blocker: 4', 4'-diisothiocyanostilbene-2,2'-disulfonic acid (i.e., DIDS), demonstrated increased adherence to endothelial cells (Vedder and Harlan 1988).

A crucial study by Schwartz and Harlan (1989) revealed that the stimulating agent, dithiothreitol, augmented neutrophil adhesion by CD11b/CD18 dependent mechanisms, without alteration in surface expression of these molecules. It appears that increased surface expression is neither necessary nor sufficient for promotion of adherence to endothelium. Research indicates that increased adherence occurs secondary to conformational changes in CD11/CD18; and theories as to whether phosphorylation, proteolytic cleavage, or other biochemical mechanisms are involved abound (Carlos and Harlan 1990).

It is interesting that LPS did not result in upregulation of labeling percentage of stimulated equine neutrophils in this study. Zimmerman and McIntyre (1988) have shown that different agonists of neutrophil adherence differ in their requirements for CD18 activity (i.e. agonists LTC_4 and thrombin do not absolutely require CD18 mechanisms for adherence, whereas agonists LTB_4, N-formyl-methionyl-leucyl-phenylalanine do).

Lipopolysaccharide *in vivo* is an indirect neutrophil stimulant through the activation of the alternate and classical complement pathways with elaboration of C3a and C5a in humans and probably horses (Webster et al. 1980, Henry and Moore 1990). The enhanced expression reported by Bochsler et al. (1990), when equine neutrophils were stimulated with ZAS in addition to LPS-preincubation may have manifested through the alternate complement pathway. However, in this study LPS-activated equine neutrophils showed a decrease in labeling with MAbs 60.3 and # 38, and no significant difference (with a tendancy to decrease) with MAb #25. Monoclonal antibody #25 probably does not recognize CD18, but a β-integrin-like surface antigen, distinct from CD18. There was no significant interaction between LPS and MAb in the analyses of mean and peak channel numbers, using Friedman's analysis of variance, so it was not possible to compare individual MAb responses of non-stimulated neutrophils to LPS-stimulated neutrophils. However, fluorescence intensity (i.e. peak and mean channel numbers) tended to increase in cells marked with MAb 60.3 and #38, and some use this as the criterion for quantitative upregulation of CD18. If there is dynamic drift of fluorescence intensity with static or decreased percentage labeling, then density of fluorescence per cell must be increased, indicating quantitative up-regulation on some cells.

Bochsler et al. (1990) demonstrated moderate binding increases of MAb 60.3 to equine LPS-stimulated neutrophils, when determined by fluorescence intensity. Here, the peak channel number of MAb 60.3 neutrophils tended to increase from 143.63 (i.e. non-stimulated neutrophils) to 153.35 (i.e. LPS-stimulated neutrophils). The mean channel number for LPS-stimulated neutrophils incubated with MAb 60.3 also showed an increase (i.e. 148.46) over non-stimulated (i.e. 141.99). However, the percentage of cells responding by showing specific fluorescence (i.e. labeling percentage) after LPS-stimulation decreased significantly ($p = 0.002$) indicating an actual down regulation of this CD18 moiety across all cells. This could be related to actual qualitative upregulation, but in this study no adherence studies were performed to confirm this. Quantitative upgrading of LPS-stimulated equine neutrophils in studies by Bochsler et al. (1990) may have been secondary to and dependent on agitation during the 30-minute incubation period. However, quantitative up-regulation in the investigation by Bochsler et al. was determined only by fluorescence intensity alterations (i.e. channel numbers or mean number of events at the mean peak channel number) and did not take into consideration the percentage of cells labeled after LPS-stimulation, which actually decreased in the study described herein. No agitation was used during incubation in this study.

It has been shown that neutrophils adhere to endothelial cells by CD11b/CD18 dependent mechanisms before there is maximal recruitment of new copies from the cytosol to the surface and binding actually reverses before maximal quantitative upregulation (Zimmerman et al. 1992). This study reinforces that quantitative methods are not sufficient for function, in that although a likely increase in CD18 density occurred in some cells, there were other cells which were shown to down-regulate with LPS-stimulation.

Monoclonal antibody #25 showed no significant alteration of labeling percentage with an increase in mean channel number fluorescence intensity. However, it was not possible to confirm the hypothesis of increased density of β-integrin-like surface antigen up-regulation on neutrophils stimulated with LPS, because the peak channel number fluorescence intensity actually decreased marginally. Significance could not be ascribed to fluorescence intensity data. The MAb #25 was initially thought to be another marker for CD18 (Tumas et al. 1994) as the molecular weight matched, however, it has only been found on equine myeloid

cells, not lymphocytes, which differs markedly from other species, in which CD18 is also expressed constitutively on lymphocytes (Davis W.C.* - personal communication; Beatty et al. 1984).

Another putative reason for the occurrence of unexpected down-regulation (i.e. 60.3 and #38) or no alteration (i.e. #25) in percentage labeling after LPS-stimulation was that the cell population was not pure. Endotoxin has markedly different effects on other cell populations (e.g. monocytes), causing elaboration of cytokines (e.g. IL1, TNF, which increase binding via activation of neutrophil CD11/CD18). However, this is not relevant in this situation, as the laboratory's isolation techniques produced > 99 percent pure neutrophils, which appeared subjectively to be activated after LPS-stimulation. Percentage labelling (indicating CD11/CD18) actually decreased. Conversely, Bochsler et al. (1990) reported purity > 95%, so their cell suspension may have contained monocytes able to elaborate cytokines and cause activation of neutrophils with subsequent alterations in CD11/CD18 expression, not due to LPS. However, negative control results in both studies make this unlikely.

* Davis W. C., Washington State University - personal communication.

CHAPTER 6

Contributions to Knowledge and Future Directions

1) In this study, almost constitutive expression of CD18 surface antigen was demonstrated on non-stimulated equine neutrophils, by using two MAbs (i.e. 60.3 and #38). In another study, equine neutrophils were only studied using MAb 60.3. The use of MAb #38 helps ratify results.

2) The β-integrin-like surface antigen was also highly expressed on non-stimulated equine neutrophils with a labeling percentage of 87.67%, using MAb #25.

3) The ranked labeling percentage data revealed significant differences between the three MAbs. The actual surface antigen labeled by MAb #25 is not known, except that it is of molecular weight 95 kd (Tumas et al. 1994) and is not present on equine lymphocytes (Davis W.C.* - personal communication). Data in this study appeared to confirm a different degree of labeling with MAb #25 in comparison to MAbs 60.3 and #38, which supports data from Washington State University (Davis W.C. - personal communication) proposing that MAb #25 does not label CD18. Peak and mean channel data also support this.

4) Most non-stimulated equine neutrophils have at least one CD18 and β-integrin-like antigen on their surface. Aggregation studies should be performed to confirm that these are in a quiescent state and are not causing active adhesion.

5) Autofluorescence of negative control neutrophils occurs, but the use of FITC-GAM $F(ab')_2$ minimizes non-selective fluorescence and is a major improvement in MAb-FACS systems. This secondary MAb should be used on a routine basis.

6) Lipopolysaccharide - stimulation of equine neutrophils induced changes in the labeling percentage and peak/mean channel numbers that supported quantitative up-regulation of individual neutrophils, with an increase in CD18 (and possibly β-integrin-like antigen) density. However, it is possible that qualitative alterations in CD18 antigen surface expression also took place as significantly less neutrophils were actually labeled after stimulation with LPS. Conversely, MAb #25 labeling of LPS-stimulated neutrophils tended to decrease, but this was not significant. More studies of neutrophil function should be preformed to elucidate the relative importance of both quantitative and qualitative up-regulation in the horse.

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* Davis W. C., Washington State University - personal communication.

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VITA

Catherine Jane (Kate) Savage was born on May 26, 1965, in Melbourne, Australia. Kate graduated from the University of Melbourne School of Veterinary Science with her BVSc in 1987. After this she completed a year in large animal private practice in rural Victoria, Australia. In September, 1988, she returned to the University of Melbourne, School of Veterinary Science to commence a PhD under the supervision of Professor L.B. Jeffcot. The PhD thesis was entitled "The Influence of Nutrition on Skeletal Growth and Induction of Osteochondrosis (Dyschondroplasia) in Horses." Here, she pursued the mechanisms of osteochondrosis and also alterations in bone remodelling with varying nutrition. She won the Barrenger Overseas Scholarship in 1990 and completed a portion of her research in Aarhus, Denmark and Uppsala, Sweden.

In January, 1992, Kate arrived in Blacksburg, Virginia, to commence a large animal medicine residency at the Virginia-Maryland Regional College of Veterinary Medicine. Kate's large animal medicine residency was completed on June 30, 1994, under the direction of Dr. W.K. Scarratt. Her research for her Master of Science degree has been supervised by Dr. M.V. Crisman. Kate's interests include water-skiing, skiing, roller-blading, tennis, golf, and horseback riding. She is engaged to Charles Kuntz and resides with Giles (the legendary, rock-gathering black Labrador), Holly the Puffkini, and Leo.