This is to certify that the

thesis entitled

IDENTIFICATION OF PTA-1 ON CANINE PLATELETS
USING A HUMAN MONOCLONAL ANTIBODY, LEO-A1

presented by

MARY NINA DIPINTO, VMD

has been accepted towards fulfillment
of the requirements for

MASTERS degree in SCIENCE

Thomas O. Bell

Major professor

Date NOVEMBER 17, 1994

IDENTIFICATION OF PTA-1 ON CANINE PLATELETS USING A HUMAN MONOCLONAL ANTIBODY, LEO A-1

By

Mary Nina DiPinto, VMD

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1994

ABSTRACT

IDENTIFICATION OF PTA-1 ON CANINE PLATELETS
USING A HUMAN MONOCLONAL ANTIBODY, LEO A-1

By

Mary Nina DiPinto, VMD

Basset Hound Hereditary Thrombopathy (BHT), an autosomal recessive canine platelet function defect, causes a complete failure of primary platelet aggregation in affected animals. Previous studies have demonstrated a markedly decreased phosphorylation of a 65 kDa protein on SDS-PAGE in BHT platelets. This protein may be represented by PTA-1, a 65 kDa surface glycoprotein of human platelets and T-lymphocytes, which is important in their activation. A human monoclonal antibody, Leo A-1, was utilized to identify PTA-1 on canine platelets, and to evaluate canine platelet responses. Western blotting revealed that Leo A-1 bound to BHT and control canine platelets at a higher molecular weight than in man under non-reducing conditions only. In radioligand binding studies, Leo A-1 demonstrated lower affinity for canine platelets than human. Leo A-1 did not induce platelet aggregation in normal or affected dogs.

ACKNOWLEDGMENTS

I wish to express my appreciation and thanks to the members of my graduate committee, Drs. Thomas G. Bell, Douglas W. Estry, Kenneth Schwartz, and Vilma Yuzbasiyan-Gurkan, for their encouragement, guidance, and support. Many thanks also to Dr. Edward C. Mather, Associate Dean for Research and Graduate Studies, without whose help this project would never have been completed.

I would like to especially acknowledge the endless trust, loyalty, love, and cooperation of Agatha, Bertha, Buster, Button, Guinivere, Kewpie, Lacey, Lily, Max, Pippin, Sparrow, and Squire. Without them, of course, there would have been no project.

Special thanks must also go to Jim for everything.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1,4,5 IP_3	inositol 1,4,5-triphosphate
ADP	adenosine diphosphate
BCIP	5-bromo-4-chloro-3-indoloyl phosphate
BHT	Basset hound hereditary thrombopathy
BSA	bovine serum albumin
Ca^{2+}	ionized calcium
cAMP	cyclic AMP
CPM	counts per minute
DAG	diacylglycerol
DEA	diethanolamine
GFP	gel-filtered platelets
H_2O_2	hydrogen peroxide
HB 43	monoclonal antibody to human IgG
HPLC	high performance liquid chromatography
HTA	Hepes-Tyrodes-Albumin
IgG	immunoglobulin G
IV 3	monoclonal antibody to human F_c receptors
K_d	dissociation constant
kDa	kilodalton

LEO-A1	monoclonal antibody to PTA-1
M	molar
mA	milliamperes
MoAb	monoclonal antibody
NBT	nitro blue tetrazolium
ng	nanograms
PAF	platelet activating factor
PBS	phosphate buffered saline
PGE_1	prostaglandin E_1
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
PMSF	phenyl methyl sulfonyl flouride
PRP	platelet-rich plasma
PTA-1	platelet-thymocyte antigen-1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TxA_2	thromboxane A_2
U	units
μg	micrograms
vWF	von Willebrand's factor
TxB_2	thromboxane B_2

CHAPTER 1

INTRODUCTION

This thesis project arose as a sequel to a study of an inherited, severe hemorrhagic diathesis in purebred Basset hounds that has been ongoing at three universities since the late 1970s. The support for extended study of this and other platelet disorders in humans and other mammalian species was in response to the major contributions that such studies have made to the current understanding of platelet biochemistry and physiology. Support for the initial investigation of Basset Hound Hereditary Thrombopathy (BHT) arose because of the superficial similarity of this disease to a specific inherited human platelet disorder. It has since been determined that BHT is not a model for any known human platelet disorder but represents a unique and as yet undefined platelet defect. Because a functional disturbance of a 65 kDa protein had been identified in BHT, the initial purpose of this project was to determine if a protein analogous to the human 65 kDa platelet-thymocyte antigen (PTA-1) is present and functional in the canine. Further, it is anticipated that the project may serve to refine what is known of canine platelet function and may be of value in increasing our understanding of the BHT defect.

Hemostasis is a complex interaction between platelets, soluble coagulation factors, and the vascular endothelium. Exposure of the subendothelium following vessel injury initiates the ***platelet response***, which culminates in the formation of the primary hemostatic plug. The ***platelet response*** is a sequence of precisely coordinated events in which platelets adhere to the exposed subendothelium of the injured vessel, change shape, spread, begin to aggregate on the adherent platelet layer, and secrete the contents of their cytoplasmic granules. The concurrent production and release of arachidonic acid metabolites is also important in the recruitment of additional platelets into the forming coagulum. During activation, alterations in the platelet membrane provide phospholipid surfaces that enhance the activation of a number of soluble coagulation factors, amplifying the coagulation cascade, and initiating the conversion of soluble circulating fibrinogen to an insoluble fibrin monomer. Polymerization of fibrin then stabilizes the platelet aggregate, resulting in secondary hemostasis[1-5a].

Adhesion of platelets to subendothelial components such as collagen and glycosaminoglycans is directly dependent upon the presence of platelet surface receptors for adhesive proteins such as von Willebrand's factor (vWF), fibrinogen, collagen, and fibronectin[6]. The major function of the surface glycoprotein Ib-IX is to bind vWF in the subendothelium and it is essential for the initial adhesion of platelets and the formation of the platelet monolayer covering the site of injury. Even though non-activated circulating platelets do not bind soluble vWF in plasma, they will adhere to vWF once the latter protein

becomes reconformed on the endothelial surface[12]. However, all other platelet adhesion molecules only become functional receptors in response to platelet agonist-mediated activation[6]. This may occur in response to soluble agonists that are present in the surrounding milieu, such as ADP and thrombin, or agonists that are exposed during vessel injury, such as collagen. The interaction of these extracellular "agonists" with their specific platelet surface receptors through the signal transduction process initiates platelet activation, exposure and induction of receptors for adhesion molecules, and recruitment of additional platelets to the site of injury. An important example of receptor induction is demonstrated by the fibrinogen receptor, glycoprotein IIb-IIIa (GP IIb-IIIa). This receptor is composed of two surface glycoproteins and is a member of the integrin supergene family. These proteins exist as a complex on the platelet surface, and at 50,000 copies per platelet, are the most abundant platelet receptors[7]. Platelet activation leads to conformational changes in this protein complex that permit the binding of fibrinogen[8-11].

Fibrinogen-receptor binding is calcium dependent, and is essential for platelet aggregation. Once bound to its receptor, fibrinogen is thought to cross-link adjacent platelets by virtue of its dimeric structure. GP IIb-IIIa possesses many functional domains, and has the ability to bind other adhesion molecules besides fibrinogen, such as vitronectin, fibronectin, and von Willebrand's factor(vWF)[12].

Primary platelet aggregation, which consists of adhesion, shape change, and fibrinogen binding, is a reversible phenomenon. If the initiating stimulus is

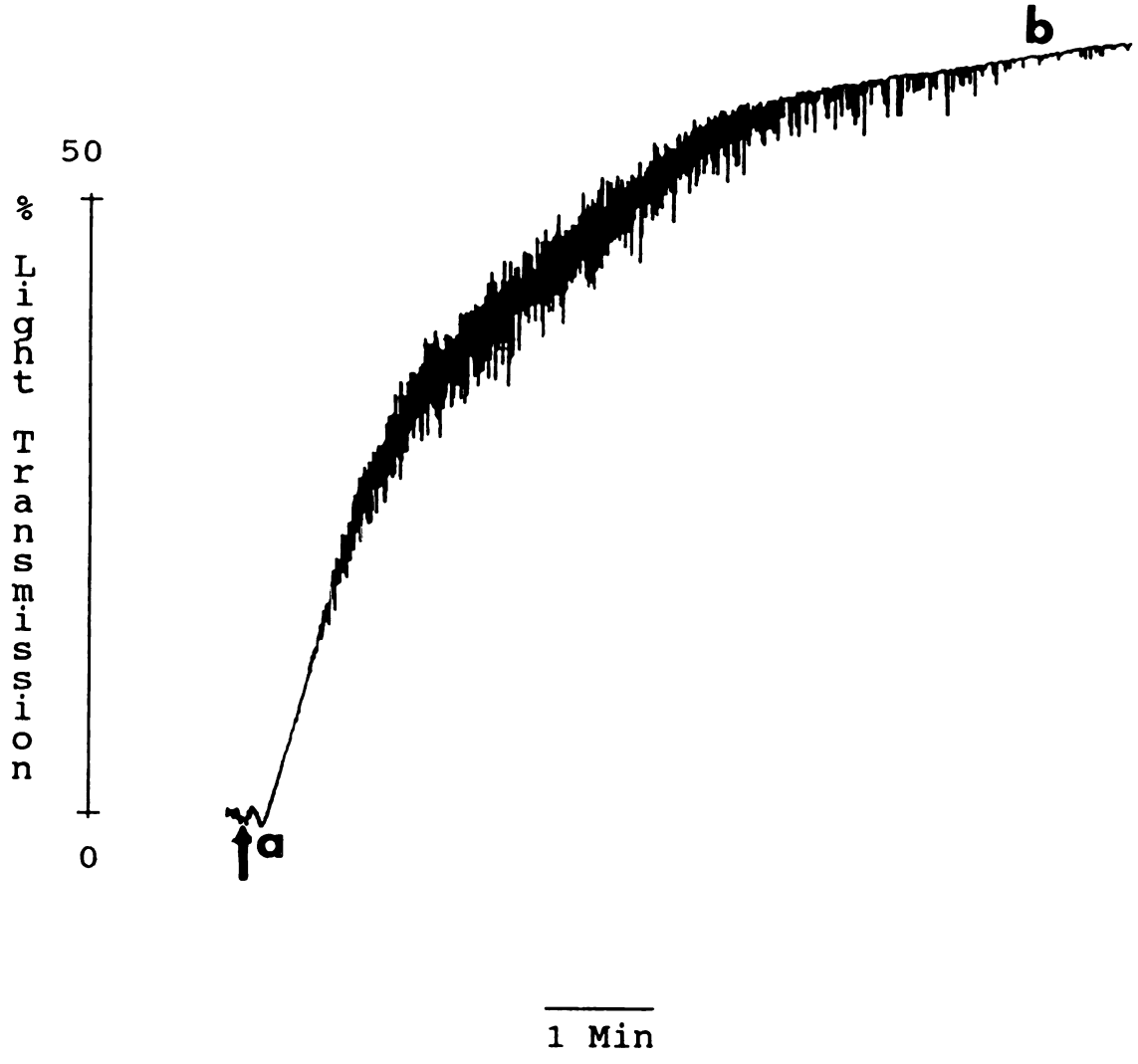
of sufficient strength, primary aggregation will proceed to an irreversible secondary phase, which is accompanied by, and is dependent on, the platelet release response[4,13]. Platelet release involves both the secretion of alpha and dense granule contents and the production/release of arachidonic acid from membrane phospholipids. The contents of platelet alpha granules include coagulation factors (fibrinogen, factor V, factor VII), growth factors, mediators (thrombospondin, fibronectin, platelet factor 4), and vWF. Dense granules contain primarily ions (calcium and magnesium), adenine nucleotides (ADP and ATP), pyrophosphates, and serotonin[4,18,19]. Thus, the release response ensures that high concentrations of agonists, calcium, and adhesive proteins become localized on the surface of the forming platelet plug. Platelet agonists are classified as "weak", "intermediate", or "strong" based upon their ability to provoke irreversible secondary aggregation and the release response[13-15].

The optical aggregometer is the method most frequently used to study platelet aggregation in vitro. This instrument measures changes in light transmission when platelet agonists are added to stirred platelet suspensions, and the results are displayed on a strip chart recorder[20,21]. Platelets respond to most agonists by an initial shape change converting the resting discoid morphology to a spiny sphere. This conformational alteration produces a small but detectable change in light transmission. Phorbol esters and epinephrine are agonists that do not induce shape change prior to inducing aggregation. As aggregation proceeds with the formation of the platelet clump, light transmission through the suspension increases to a plateau (see Figure 1). Primary

aggregation is reversible, and not associated with platelet secretion, so a plateau phase may not be attained. However, if the agonist is strong, or present in high enough concentration, the release reaction will occur, resulting in irreversible secondary aggregation. Figure 1 outlines a typical curve generated during platelet aggregation stimulated by ADP, and measured by an aggregometer.

The ***platelet response*** is ultimately determined by the balance between excitatory and inhibitory signals mediated by cell surface receptors. The signal transduction process, initiated by the binding of an agonist to its receptor, is mediated by transducer molecules known as G proteins. G proteins act to transmit the signal across the plasma membrane and regulate the function of specific effector mechanisms such as enzymes or ion channels[6,26,17]. Although platelets respond to a wide variety of stimuli, they possess only a finite number of effector mechanisms by which these cellular reactions can be mediated. These effector pathways determine cellular responses by controlling the production of intracellular second messengers, which then modulate the structure, activity, or phosphorylation of intracellular proteins[4,6,13,16,17]. The signal transduction process is tightly regulated and contains many backup systems that permit communication between different parts of the platelet activation sequence. This arrangement provides opportunities for modulation (i.e., amplification or inhibition) of the signal at any step.

Figure 1. Typical aggregation curve for normal canine platelet-rich plasma stimulated by 10 μM ADP. The arrow indicates the point of agonist addition, (a) shape change, (b) maximal aggregation.


b
50
% Light Transmission
0
a
1 Min

Several enzyme systems play a pivotal role in platelet signal transduction: 1) **phospholipase C** (PLC), which mediates the hydrolysis of membrane inositol phospholipids, resulting in generation of the second messengers **inositol 1,4,5 triphosphate {(1,4,5) IP3}, and sn-1,2-diacylglycerol (1,2 DAG)**; 2) **protein kinase C** (PKC), which phosphorylates various intracellular proteins; 3) **phospholipase A2**, which cleaves arachidonic acid from membrane phospholipids; and 4) **adenylate cyclase,** which controls the generation of **cyclic AMP** (cAMP), an important inhibitory second messenger[4,13,17].

Physiologic platelet agonists such as thrombin, platelet activating factor (PAF), and thromboxane A_2 (TxA_2) transmit their signal across the platelet membrane via the receptor-mediated hydrolysis of membrane phosphoinositides by phospholipase C (PLC). This generates the second messengers 1,2 DAG and 1,4,5 IP3, which activate protein kinase C (PKC) and increase cytosolic calcium concentrations[13]. These events are associated with the phosphorylation of the 47 kDa substrate of PKC and the 20 kDa myosin light chain. The 47 kDa protein has been purified[22], and its cDNA sequenced[22a]. This protein is called "pleckstrin", and it may represent the IP3 5'-monoesterase that is phosphorylated and activated by PKC[24], although its function is still controversial[22a,23,24]. Platelets may also be activated by non-physiologic agonists such as phorbol myristate acetate (PMA), a tumor-promoting phorbol ester. PMA intercalates into the lipid bilayer of the platelet plasma membrane, and directly activates PKC by virtue of its structural similarity to 1,2 DAG[13]. Free arachidonic acid cleaved from membrane phospholipids is metabolized to TxA_2

and other eicosanoids, which function as intra- and inter-cellular messengers. Arachidonic acid and its cyclooxygenase metabolites are of major importance in normal platelet function. Weak physiologic agonists such as ADP, epinephrine, and low concentrations of PAF and thrombin require arachidonic acid to be liberated and metabolized to TxA_2 for irreversible aggregation and dense granule secretion to occur[13].

An interesting class of platelet agonists is represented by antiplatelet IgG monoclonal antibodies (MoAb), autoantibodies, and alloantibodies, all of which are capable of inducing platelet aggregation and secretion. These immunoglobulins can bind via their F_{ab} components to specific platelet glycoproteins or to proteins that are adsorbed to the platelet surface[43]. Of this group, the most is known about monoclonal antibodies, which have been extensively used to identify, quantify, and isolate platelet membrane glycoproteins[44-47]. The majority of platelet-activating MoAb are of the IgG1 subclass[49]. It has been shown that these MoAb also require binding of the antibody to the platelet F_c receptor to initiate platelet aggregation and release[43].

Platelets express approximately 1500 F_c receptors of a single class ($F_{c\gamma}RII$) on the plasma membrane[50]. MoAb can initiate platelet activation following F_c receptor occupation on adjacent platelets (termed interplatelet activation), or, through antibody binding to the F_{ab} and F_c receptors on the same platelet (intraplatelet activation)[43]. These mechanisms of antibody-mediated platelet activation are diagrammed below. There is also evidence to suggest that intraplatelet F_c receptor cross-linking may also be an important element in

MoAb activation of platelets[49]. Platelet activation by MoAb binding appears to be mediated by G protein activation of PLC and the generation of 1,2 DAG[49].

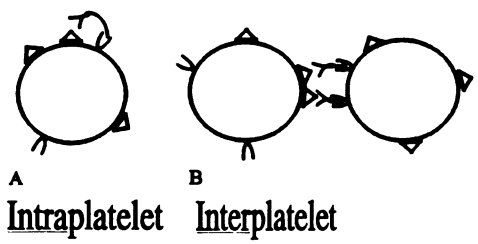


Figure 2. Proposed mechanisms of antibody-mediated platelet activation. (▲) platelet surface antigen, (U) platelet F_c receptor, (Y) antibody molecule[43].

Basset hound hereditary thrombopathy

The study of inherited disorders of platelet function has contributed a great deal to the current understanding of platelet biochemistry and physiology. Identification of absent or dysfunctional glycoproteins, enzymes, and other structures has helped to clarify their role in normal platelet function.

An inherited, severe hemorrhagic diathesis in purebred Basset hounds was first described in 1979[25]. Animals affected with the disorder exhibit clinical signs of platelet dysfunction such as mucosal bleeding, petechiation, easy bruising, and excessive hemorrhage associated with estrus cycles and the loss of deciduous teeth. Pedigree analysis of 92 related animals[26], as well as breeding studies conducted in our own colony strongly support an autosomal recessive mode of inheritance. To date, no similar disorder has been described in man or other animal species.

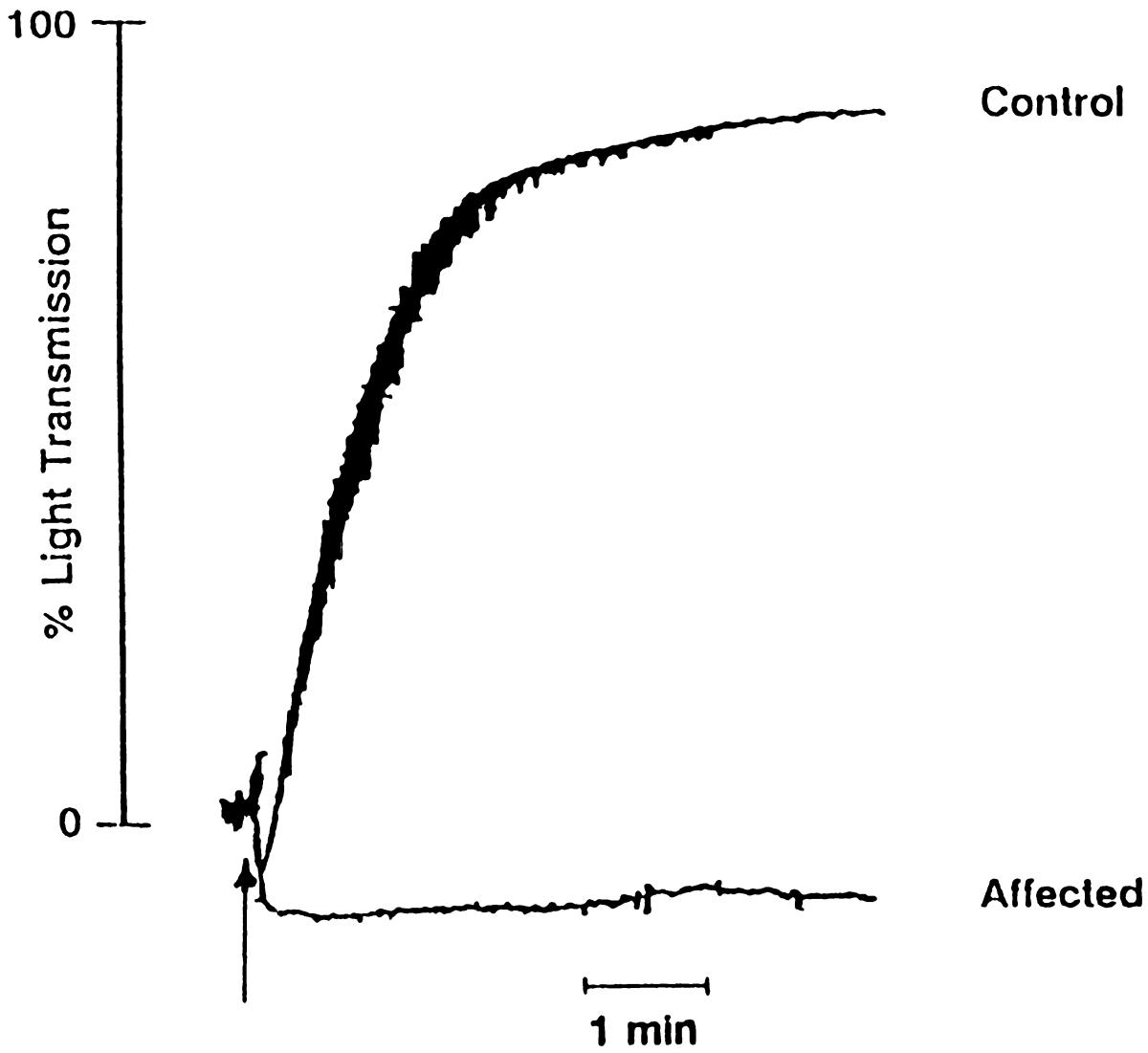
The initial investigation of BHT centered around its potential as an animal model for Glanzmann's thrombasthenia, a hereditary disease of human platelets in which the membrane GP IIb-IIIa complex is either absent or dysfunctional[27]. As the fibrinogen receptor, GP IIb-IIIa is essential for platelet-to-platelet adhesion during aggregation[28]. Affected dogs share many clinical features with Glanzmann's patients, such as prolonged bleeding times, normal coagulation profiles and von Willebrand's factor levels, normal platelet numbers and morphology, as well as the failure of primary platelet aggregation in response to ADP or collagen. It was found, however, that affected BHT platelets have normal GP IIb-IIIA content[29], and bind I-125 or gold-labelled fibrinogen to the same extent as control platelets when stimulated by ADP[30,31]. Also, unlike Glanzmann's patients, affected Basset hounds demonstrate normal clot retraction. Therefore, it was apparent that BHT is not a model for Glanzmann's thrombasthenia, but represents a unique platelet defect involving an as yet undefined post-fibrinogen binding event(s).

The function of the different platelet activation pathways was investigated in BHT by measuring aggregation and ATP dense granule secretion in response to a variety of physiological and non-physiological agonists. These included ADP, PAF, thrombin, collagen, the thromboxane-mimetic U46619 plus epinephrine, PMA and the calcium ionophore A-23187[32]. Although affected platelets changed shape in response to all physiologic agonists tested, they did not aggregate unless activated by PMA or high concentrations of thrombin, both of which are able to bypass phospholipase C and the production of the

arachidonic acid metabolite TxA_2[13]. The method by which PMA-mediated activation of protein kinase C (PKC) induces aggregation is currently unknown. Figure 3 illustrates typical aggregation curves for control and BHT-affected platelet-rich plasma following stimulation by ADP. Dense granule ATP secretion from affected platelets occurred in response to some, but not all, agonists, and the release response was not linked to the ability to aggregate. ATP secretion in the affected platelets was also inhibited by aspirin to a greater degree than in controls, suggesting an increased dependence on arachidonate metabolites[32].

In BHT, the evaluation of TxA_2 production, when measured as the inactive metabolite TxB_2, revealed that affected platelets produce significantly more TxB_2 than controls in response to those agonists that induce secretion, but decreased TxB_2 in response to those agonists that do not cause secretion. These studies confirm the hypothesis that dense granule release of ATP is strongly linked to TXA_2 production in affected platelets. Although the TxA_2 production pathway appears to be present and functional in BHT platelets, it does not appear to be properly regulated[32]. In addition, dense granule secretion occurs earlier and more rapidly in affected platelets[66].

Figure 3. Typical aggregation curves for canine control and BHT-affected platelet-rich plasma stimulated by 10 μM ADP.


100
0
% Light Transmission
Control
Affected
1 min

It was hypothesized that the BHT defect might involve a dysfunction in the signal transduction system and/or the generation of second messengers within the platelet. Due to the importance of cAMP as an inhibitory second messenger, Boudreaux et al. examined both the concentrations of cAMP and the activity of the cAMP phosphodiesterase in control and affected platelets. It was found that affected platelets had increased resting levels of cAMP[33]. It was concluded that affected platelets have functionally intact regulatory G proteins that regulate cAMP production. cAMP phosphodiesterase, which catalyzes cAMP removal and breakdown, is also functional. In BHT, however, the control of this enzyme appears to be impaired[34]. Although concentrations of cAMP are slightly but significantly increased in affected BHT platelets, it is highly unlikely that this alteration is responsible for the failure of primary aggregation, particularly since all other post-activational events, including phosphorylation of the 20 and 47 kDa proteins, occur normally. One hypothesis for this elevation is that it is merely a reflection of an underlying primary control defect within the affected platelet. It would be useful to determine if the derangements in arachidonate metabolism seen in affected platelets also produce increased concentrations of those prostanoids (i.e., PGE_1/PGE_2) that act to increase cAMP.

Studies in our laboratory examined signal transduction more closely in control and affected platelets through the measurement of a number of end products: 1) resting and post-stimulation cytosolic free ionized calcium concentrations[35], 2) cytosolic pH changes in response to agonist stimulation[35],

3) PLC activity through the generation of the intracellular second messenger 1,2 DAG[35], and 4) resting and post-stimulation phosphorylation of the 20 and 47 kDa proteins in 32-P-labelled platelets[32].

The activity of many intracellular effector systems is regulated by cytosolic free ionized calcium concentrations. Calcium also affects the organization of platelet cytoskeletal proteins[13,36]. The measurement of resting and post-stimulation cytosolic calcium concentrations in fura-2 or aequorin-loaded platelets indicated that calcium fluxes across the plasma membrane are normal in affected platelets. However, affected platelets released less calcium from internal stores than controls[35]. Despite this, the amount of calcium released from internal stores in affected platelets was adequate to support calcium-dependent processes such as shape change and the activation of the myosin light chain kinase, in the absence of external calcium[32].

Cytosolic alkalinization frequently accompanies platelet activation, and appears to potentiate phospholipase A_2 activation[37,38], release of internal calcium, and cytoskeletal organization[39]. Regulation of cytosolic pH is dependent upon the Na^+/H^+ antiport, which is closely associated with the 64 kDa alpha$_2$-adrenergic receptor in the platelet plasma membrane[40]. Measurement of cytosolic pH indicated that the Na^+/H^+ antiport is able to generate adequate alkalinity during activation to support phospholipase A_2 activity in affected platelets[35].

PLC-mediated hydrolysis of membrane inositol phospholipids generates two important second messengers in the platelet signal transduction pathway:

1,2 DAG and (1,4,5)IP3. PLC is activated through agonist-specific receptors which are coupled to the enzyme via G proteins[13]. The activity and integrity of this enzyme system were indirectly examined by monitoring the production of 1,2 DAG in affected and control platelets in response to agonist stimulation. Affected and control dogs produced similar quantities of 1,2 DAG, indicating that PLC activity is not impaired in affected platelets[35].

Stimulation of platelets by agonists results in the phosphorylation of a number of proteins. Following electrophoresis and autoradiography of human platelets, agonist-induced phosphorylation is prominent in two protein bands. One is the 20 kDa myosin light chain, which is phosphorylated following activation of its calcium-dependent kinase[41]. The other band is the 47 kDa pleckstrin, the main substrate of PKC[13]. Phosphorylation of the 20 and 47 kDa proteins in response to a range of agonists was similar in control and affected ^{32}-P labelled canine platelets[32]. Phosphorylation of the 20 kDa band correlated with shape change, but phosphorylation of neither the 20 nor the 47 kDa protein correlated with the ability to aggregate. However, a consistent finding on autoradiographs was markedly decreased phosphorylation of a 65 kDa band in affected platelets compared with controls. This difference was evident both before and after agonist stimulation. Minimal time-dependent phosphorylation of this 65 kDa band was observed only in response to those agonists capable of inducing aggregation in affected platelets: PMA or thrombin[32].

In the previous study, it was not possible to determine if this 65 kDa area was a single band, or several bands that migrated closely together.

Subsequent work that I performed in our laboratory utilizing gradient and lower %T resolving gels with more sensitive silver/copper stain techniques has generated data that supports the conclusion that this 65 kDa band migrates as, and is representative of, a single band. The decreased intensity of this band on autoradiography may represent either a missing protein or the inability to phosphorylate an existing band. This phenomenon of reduced phosphorylation raises several possibilities that could explain the results observed: 1) that this 65 kDa protein is defective or dysfunctional and cannot be phosphorylated normally, 2) the protein is present, but in reduced amounts, 3) the protein kinase responsible for phosphorylation of the 65 kDa protein is defective or dysfunctional, or 4) that the protein kinase is present in reduced concentrations. When reduced SDS-PAGE gels stained with Coomassie blue from affected and control dogs were examined both visually and densitometrically, no difference in the 65 kDa band could be appreciated between the two groups. These data tend to favor the possibility that either the 65 kDa protein or its specific protein kinase are defective or dysfunctional, rather than present in reduced amounts. If the latter were true, one would have expected to detect a densitometric difference in the band or bands in the Coomassie-stained gels.

Currently, the identity, cellular location, and function of the protein in this 65 kDa band remains unknown, and its relationship to the BHT defect is a mystery. Several proteins of similar size have been identified on human platelets. One is the $alpha_2$-adrenergic receptor, a 64 kDa glycoprotein which is associated with the Na^+/H^+ antiport[40]. It is unknown if phosphorylation of

this protein occurs, or if phosphorylation is important in the regulation of alpha$_2$-adrenergic receptor activity. With the very low copy numbers present on human platelets (only 200 copies/platelet[42]), it is unlikely to be detected and observed by SDS-PAGE.

A likely candidate is Platelet Thymocyte Antigen (PTA-1), which shares many features with the poorly phosphorylated 65 kDa protein seen in platelets from affected Basset hounds. A 65 kDa membrane glycoprotein, PTA-1 was first identified on T-lymphocytes in the mid-1980s using monoclonal antibody, Leo A-1. Initially thought to be a T cell lineage-specific antigen, studies indicated that this protein was important in T cell activation[51]. However, subsequent investigation revealed that PTA-1 was also expressed on the surface of human platelets and their megakaryocytic precursors. In addition, an intracytoplasmic pool of PTA-1 was demonstrated to be present in membrane-bound vacuolar structures and the canalicular system in a fashion similar to that described for GP IIb-IIIa[52].

Biochemical characterization of PTA-1 shows that the identical protein is present on T lymphocytes and platelets (J. Scott, unpublished data). PTA-1 is a 65 kDa molecule that is composed of a 35-40 kDa protein backbone, with the remainder consisting of heavily sialated N-linked carbohydrate moieties. Partial sequencing of the amino terminus reveals that PTA-1 exhibits no identity with any other known platelet glycoprotein. Scatchard analysis of competitive binding assay data from human platelets indicated that there are approximately 1200 Leo A-1 binding sites per platelet[52]. This is a low copy number, and is

comparable to that seen with the platelet F_c receptor, which is also involved in certain types of antibody-mediated platelet activation[43].

Leo A-1 monoclonal antibody is a powerful platelet agonist, capable of stimulating both platelet aggregation and secretion. The binding of Leo A-1 induces irreversible platelet aggregation that is not preceded by shape change[53]. The aggregation response is distinct, and divided into two phases: an initial "slow" phase, which occurs independent of granule secretion and does not require fibrinogen; and a subsequent "fast" phase, during which the release reaction occurs[53]. This latter phase most resembles the ***platelet response*** seen with the secretion-dependent weak physiologic agonists. In dose-response evaluations of Leo A-1 induced aggregation, the time to onset of aggregation was inversely proportional to the concentration of antibody used. At very low antibody concentrations (i.e., < 0.3 μg/ml), this lag phase was as long as 5 minutes[52].

Leo A-1, like other platelet activating MoAb, appears to require antibody interaction with the platelet Ig F_c receptor for the aggregation and release response to occur[53]. Previous studies suggest that platelet activation induced by the binding of Leo A-1 may be mediated by protein kinase C, rather than indirectly via a cyclooxygenase-dependent pathway or by the release of dense granule ADP, as agents that interfere with the activation of protein kinase C inhibit Leo A-1 induced platelet aggregation and secretion[52]. Leo A-1 aggregation curves are typical of those observed with other platelet activating MoAbs, and closely resemble those generated when platelets are stimulated

with phorbol ester. As previously stated, phorbol esters activate protein kinase C directly and irreversibly, unlike MoAb which act via G protein stimulation of PLC to activate PKC. Aggregation responses induced by phorbol esters are characterized by a lack of shape change, a slow aggregation response, and delayed granule secretion[54]. Both phorbol and Leo A-1 stimulate the phosphorylation of a 47 kDa protein of unknown function that is a substrate of protein kinase C[55,56]. In addition, PTA-1 antigen is itself phosphorylated in response to the binding of Leo A-1 as well as phorbol. This response is inhibited by blockers of protein kinase C, further strengthening the case for the importance of this enzyme as a mediator of Leo A-1 platelet activation.

Summary

In summary, three metabolic derangements following activation have been identified in affected platelets: 1) altered arachidonate metabolism with overproduction of TxA_2; 2) early, rapid dense body secretion, which is linked to 1); and 3) increased concentrations of cAMP. **These derangements are most likely secondary manifestations of an underlying primary control defect because none of these alterations would result in complete failure of primary aggregation.**

The 65-67 kDa protein, which is poorly phosphorylated in platelets from dogs affected with BHT, may play an important role in the pathogenesis of the defect. This protein exhibits physical and functional similarity to human platelet-thymocyte antigen (PTA-1). The purpose of the studies outlined in this

thesis was to determine if a protein analogous to the PTA-1 antigen in man was present in the dog. If this were the case further investigations may determine if PTA-1 represents the dysfunctional mechanism in BHT. This potential relationship was investigated by determining if the monoclonal antibody developed against human PTA-1, Leo A-1, exhibits functional and immunological cross-reactivity with canine platelet proteins. This was accomplished by:

1) functional studies investigating platelet aggregation responses to Leo A-1 monoclonal antibody in control and BHT affected Basset hounds;

2) the use of Western blotting techniques and radioligand binding analysis to determine if cross-reactivity existed between Leo A-1 and platelet proteins in control and BHT affected dogs, and if the specificity of any cross-reactivity was associated with the 65-67 kDa area;

3) if successful cross-reactivity was observed in the 65-67 kDa area, Leo A-1 would be utilized to immunoprecipitate the protein for further characterization in control and BHT affected dogs.

The success in accomplishing these experimental procedures was dependent upon demonstration of the cross-reactivity of the human monoclonal antibody Leo A-1 with a canine platelet protein. Although cross-reactivity

between canine platelet proteins and monoclonal antibodies to analogous human proteins has been demonstrated[29], it could not be assumed that: 1) the dog possesses an analogue to PTA-1, or 2) that even if such an analogue exists, it will bind Leo A-1 antibody. The experimental design was developed to first address these two issues.

CHAPTER 2

MATERIALS AND METHODS

Experimental subjects

Experimental subjects consisted of Basset hounds from a colony established at Michigan State University for the study of Basset Hound Hereditary Thrombopathy (BHT). This colony is composed of 8 dogs, ranging in age from 3 to 7 years, of known genotype for the disorder. In the colony there is only a single known normal Basset hound; other healthy dogs of various breeds were recruited as a source of additional normal canine platelets. All canine subjects were receiving no medication (including heartworm preventative) at the time of blood collection. Human platelet proteins were utilized for comparison, and to link the study to established identification procedures for PTA-1.

Part I. Identification of platelet-thymocyte antigen (PTA-1) on canine platelets

Experimental design

Preliminary experiments in this section were designed to identify PTA-1 in canine platelet preparations by immunodetection utilizing Western blotting. The Western blotting technique enables the detection of specific proteins in

complex mixtures following separation by gel electrophoresis. Platelet preparations from a total of 3 affected Basset hounds, 3 normal dogs (1 Basset and 2 non-Bassets), and a human being were used in the immunoblotting studies with human Leo A-1 monoclonal antibody. Each immunoblot compared human, normal dog, and BHT platelets under either reducing or non-reducing conditions. Non-specific binding of the primary and secondary antibody was evaluated in separate immunoblots utilizing HB 43, a murine monoclonal antibody to human IgG, as a negative control.

Studies were then performed using ^{125}I-labelled Leo A-1 to evaluate antibody binding to human, BHT, and normal dog platelets. Dose-response experiments with radiolabelled antibody were designed to evaluate binding affinity and to potentially estimate the number of Leo A-1 binding sites on canine platelets in comparison to human platelets. If Leo A-1 cross-reacted with canine platelets, the next step was to immunoprecipitate PTA-1 and the canine analogue from ^{125}I-labelled platelet preparations using Leo A-1 for accurate molecular weight determinations in man and dog.

Monoclonal antibodies

Leo A-1 monoclonal antibody was the generous gift of Dr. Judith Scott, University of Newcastle, New South Wales, Australia. This antibody, characterized as an $IgG1_{k,}$ was developed by immunizing mice to intact, stimulated human T-lymphocytes[51]. Leo A-1 was precipitated from ascites fluid

and purified by HPLC to a concentration of 1.025 mg/ml in PBS as described by Scott[52].

HB 43 (murine anti-human IgG), and IV3 (murine anti-human Fc) monoclonal antibodies were the generous gift of Dr. Kenneth Schwartz.

Preparation of platelet-rich plasma

For all studies, canine whole blood was collected by atraumatic jugular venipuncture into a plastic syringe containing 3.2% trisodium citrate at a ratio of 9 ml of blood to 1 ml of trisodium citrate. Samples from human subjects were obtained by phlebotomy from the median cubital vein into a plastic syringe containing 3.8% trisodium citrate in the same ratio as for canine whole blood. The citrated blood was then transferred to polypropylene plastic tubes, and the platelet-rich plasma obtained by repeated centrifugations at 1324 x g for 60 seconds. Platelet-rich plasma (PRP) was removed after each centrifugation. A manual platelet count was then performed on the PRP using a Unopette and a Neubauer hemocytometer. All platelet preparations were used within four hours of collection.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Preparation of platelet suspensions

To minimize platelet activation during the subsequent centrifugation steps, prostaglandin E_1 (PGE_1) was added to PRP at a final concentration of 1 μM. The platelets were allowed to rest at room temperature for 5 minutes, and

then sedimented by centrifugation at 730 x g for 15 minutes, washed twice, and the platelet pellet resuspended in the original plasma volume of Buffer #1 containing 120 mM NaCl, 13 mM trisodium citrate, and 30 mM glucose, pH 7.0. Between washes, the platelets were resuspended in 5 ml of Buffer #1 and a manual platelet count performed. Following the second wash, the platelet pellet was resuspended to a final count of 9 x 10^8/ml in Buffer #3 containing 138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, and 1.0 mM EDTA, pH 7.4. An aliquot of this suspension was solubilized by an equal volume of 4% w/v SDS, and the protein content determined by Markwell protein assay. The remaining platelet suspension was solubilized by the addition of an equal volume of a 2x concentrated solubilization buffer containing 4% w/v SDS, 10% w/v 2-mercaptoethanol, 20% w/v glycerol, 0.004% w/v pyronin-Y as the dye front marker, and 125 mM TRIS HCl, pH 6.8, and incubated at 100°C for 3 minutes. For non-reduced samples, distilled water was substituted for 2-mercaptoethanol in the solubilization buffer. Aliquoted samples were frozen at -70°C until used.

SDS-PAGE

Samples were electrophoresed according to the method of Laemmli[57] through a 1.5 mm thick 3% T acrylamide stacking gel, and a 5-10% T acrylamide linear gradient resolving gel, using a Biorad Protean II vertical slab gel electrophoresis apparatus (Biorad, Richmond, CA). The linear gradient gel was used to achieve optimal separation of the proteins in the 65 kDa area.

Lanes were loaded with 100 μg of protein or 10 μl of either high molecular weight standard or biotinylated high molecular weight standard (Biorad, Richmond, CA). Each gel run contained sample lanes with platelet preparations from a human, normal dog, and BHT-affected dog. The protein standards were prepared according to the manufacturer's instructions by dissolving them in a reducing buffer containing pyronin-Y as a dye front marker. Pyronin-Y is used because it will readily transfer to nitrocellulose during electroblotting, while the more commonly used bromphenol blue will not. The gels were run overnight at 9.5 mA/gel (950 V).

Western blotting

Electroblotting of the SDS-PAGE gels was performed according to the method described by Kyshe-Andersen[58,59], using a Jansen semi-dry blotting apparatus. The gel was allowed to equilibrate in a solution of 25 mM TRIS prior to blotting. A section of nitrocellulose membrane cut to the same dimensions as the gel was soaked briefly in distilled water and then laid gently on top of the moist gel. Eighteen pieces of Whatman #1 filter paper were also cut to the exact size as the gel. Three layers were soaked in anode solution #2 (25 mM TRIS, 20% v/v methanol, pH 10.4) and placed gently on top of the nitrocellulose. This was followed by six layers of filter paper soaked in anode solution #1 (0.3 M Tris, 20% v/v methanol, pH 10.4). The gel was then carefully removed from the glass plate, and laid filter paper side down on the anodic plate of the apparatus. Nine layers of filter paper soaked in cathode

solution (40 mM 6-amino-n-hexanoic acid in 25 mM TRIS, pH 9.4) were added to the top of the gel. The lid, containing the cathodic plate, was placed on the stacked assembly and connected to the power supply (LKB). The transfer was carried out at a constant current of 8 mA/cm^2 of gel at room temperature for 1 hour. Following completion of the blot-transfer, the portion of the membrane corresponding to the non-biotinylated molecular weight standard was cut away and stained with a total protein stain containing India ink to evaluate the efficacy of transfer.

Protein detection on the remainder of the nitrocellulose membrane was performed using a commercial kit for mouse antibodies (Amersham, Chicago, IL). All steps were carried out at room temperature on a shaker plate. The blot was first incubated with reconstituted dried milk for one hour to prevent non-specific binding of the antibodies or detection reagents to the nitrocellulose. The blot was then incubated for one hour with the primary mouse antibody (Leo A-1), washed, and incubated with a biotinylated anti-mouse antibody (2° antibody), also for one hour. This preparation was subsequently washed, and incubated with a detection solution containing a streptavidin alkaline phosphatase conjugate for 20 minutes. This was followed by a final wash, and the addition of a mixture of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoloyl phosphate (BCIP). NBT and BCIP were used to develop the color and visualize the protein band or bands of interest that may have bound the primary antibody.

Molecular weights of proteins of interest on the blot were determined by constructing a standard curve from the standards of known molecular weight. Using semi-log paper, weights of the known proteins were plotted on the log axis versus the migration index for each protein. The migration index can be calculated as follows:

$$\text{Migration index} = \frac{\text{distance of protein migration (cm)}}{\text{distance of dye front migration (cm)}}$$

This way, the migration index of the protein or proteins of interest can be calculated, and the corresponding molecular weight read from the standard curve.

Binding studies

Radioiodination of Leo-A1

Radioiodination of Leo A-1 was accomplished according to the method described by Fraker[60]. A 12 x 75 mm glass tube was first coated with 0.6 μg of iodogen (1,3,4,6-tetrachloro-3a, 6a-diphenylglycouril) (Sigma Chemical Company) dissolved in methylene chloride to a final concentration of 0.001 mg/ml. The methylene chloride was then allowed to evaporate. A PBS solution containing approximately 25 μg of Leo A-1 was added to the tube, followed by 0.25 millicuries of carrier-free Na^{++} I^{125} (DuPont, New England Nuclear). The tube was agitated for 10 minutes at room temperature, and the solution was

run over a Sephadex G-25 column to remove the unbound I^{125}. The fraction containing the labeled immunoglobulin was collected in the void volume of the column, aliquoted, and stored frozen at -20°C.

Direct binding analysis

Leo A-1 binding studies were conducted according to the method described by Newman[47] with some modifications. The assays were performed in duplicate at room temperature, utilizing PRP prepared as previously described. For each assay, PRP was obtained from a human subject, a normal Basset hound, and a BHT-affected Basset hound; platelet preparations were used within four hours of collection.

Prostaglandin E_1 (PGE_1 final concentration 1.0 μM) was added to the PRP to minimize platelet activation during the subsequent centrifugation and handling. The platelets were washed twice by centrifugation at 3000 rpm for 10 minutes, followed by aspiration of the supernatant and gentle resuspension of the platelet pellet in the original volume of a buffer containing 3% w/v bovine serum albumin (BSA) in PBS, pH 6.5. A platelet count was performed after the second wash.

In an initial pilot study to evaluate the binding of Leo A-1 to human and canine platelets, 5.0×10^7 platelets/ml were incubated with 50 ng (final concentration 250 ng/ml) of ^{125}I-labelled Leo A-1, HB 43, or IV3 monoclonal antibodies. HB 43 served as a negative control for canine platelets, and IV 3 as a control for low copy number (the number of Fc receptors on the human

platelet surface is approximately the same as PTA-1). The final volume of each tube was adjusted to 200 μl with 3% BSA/PBS. The tubes were capped, mixed gently, and incubated on a shaker plate for 1 hour. After incubation, the solution was layered over a 20% sucrose gradient (20% w/v sucrose in PBS), and spun at 8200 x g for 4 minutes. The tip of each tube was then cut off, and radioactivity quantified by counting for 2 minutes in a gamma counter. The value obtained represented the amount of activity bound to the surface of the platelets. 10 μl (50 ng) of ^{125}I-labelled Leo A-1, HB 43, and IV 3 was also counted in the gamma counter representing total activity (i.e., bound plus free).

In the later studies, 5.0 x 10^8 platelets/ml were incubated with ^{125}I-labelled Leo A-1 in concentrations ranging from 5-200 ng (final concentration 25-1000 ng/ml), and the binding assay carried out as described above. A final study was performed on normal dog platelets with radiolabeled antibody concentrations from 50-600 ng (final concentration 250-3000 ng/ml) at an adjusted platelet count of 2 x 10^7/ml.

A Klotz plot[65] was used to determine if the platelet surface binding sites had been saturated with Leo A-1, to thereby validate a Scatchard analysis of the data to estimate the number of binding sites/platelet. Linear and non-linear regression of the binding data was performed using the EBDA and LIGAND programs modified for microcomputers[61].

Platelet surface labelling and immunoprecipitation

PRP was prepared from platelet samples from a human donor, a normal dog, and a BHT-affected dog, 1 μM PGE_1 added, and the samples washed three times by centrifugation at 3000 rpm for 10 minutes. The platelet pellet was gently resuspended in the original plasma volume of a wash buffer containing 12 mM sodium citrate, 30 mM glucose, and 120 mM NaCl, pH 6.5. A manual platelet count was performed after the second wash as described previously.

1×10^9 platelets were surface-labelled using a lactoperoxidase-catalyzed reaction. Pelleted platelets, after the final wash, were resuspended in 800 μl of wash buffer, and 200 μg lactoperoxidase (1 mg/ml) was added to the suspension. 0.33 millicuries of ^{125}I was added to each tube, followed by five 5 μl aliquots of 3 mM hydrogen peroxide at 10-second intervals accompanied by gentle agitation. The suspension was then diluted to 4 ml final volume with wash buffer plus 1 μM PGE_1, and washed three times as described above. Following the last wash, the platelet pellet was resuspended in 1 ml 3% BSA/PBS with 1 μM added PGE_1, and 500 μl aliquots placed into 1.5 ml Eppendorf tubes. A small quantity (<10 μl) was preserved, and solubilized with an equal volume of 2x SDS-PAGE solubilization buffer to be utilized as a total platelet lysate sample.

5 μg of Leo A-1 or negative control HB43 (antibody concentration of both MoAb = 1 mg/ml) was then added to appropriately labeled tubes, which were incubated on a shaker plate at room temperature for 1 hour. The platelet suspension was then centrifuged at 8200 x g for 4 minutes, and the superna-

tant aspirated and discarded. The pellet was resuspended in 200 μl of lysis buffer (0.1 M PBS, 1% v/v Triton X-100, 1 mM PMSF, 1 mM EDTA, 1% w/v BSA). 20 μg of anti-mouse IgG agarose (200 μg/ml, Sigma Chemical Co.), which had been previously blocked for 1 hour by incubation with a human non-radiolabelled platelet lysate corresponding to 2 x 10^8 platelets/50 μl, and washed three times in lysis buffer, was then added to the platelet lysate in each Eppendorf tube. The lysate/agarose suspension was incubated on a rocker plate at room temperature for 1 hour. Following this second incubation, the labelled platelet lysate/agarose suspension was washed by centrifugation at 8200 x g for 5 seconds, followed by resuspension in lysis buffer. The lysate was washed and resuspended until the radioactivity of the supernatant decreased to a plateau (about 5 washes). The resultant pellets were resuspended in 50 μl of SDS-PAGE sample buffer (non-reduced) containing 4% w/v SDS, 20% v/v glycerol, .0004% w/v bromphenol blue, and 125 mM Tris HCL, pH 6.8 and heated to boiling for 5 minutes to elute PTA-1 from the agarose. This suspension was then centrifuged at 8200 x g for 5 seconds. The supernatant was decanted, and total counts estimated by use of a Geiger counter.

Aliquots of this supernatant were then loaded into individual wells of an SDS-PAGE slab gel (3% T acrylamide stacking gel, 7%T acrylamide resolving gel). The amount loaded was dependent on the cpm of the supernatant. From the small quantity of previously preserved undiluted labeled platelet lysate containing no agarose, 1 x 10^8 cpm was loaded/lane for man and dog. The

SDS-PAGE gel was run at 7.5 mA overnight, and stained with Coomassie blue to illuminate and determine the molecular weight of the protein bands. For detection of immunoprecipitated proteins by autoradiography, the labelled gels were dried onto porous cellophane in a slab gel drier (Biorad, Richmond, CA). The dried gel was then exposed on Kodak X-OMAT AR film at -80°C for 24-36 hours in a cassette with intensifying screens, and the film developed in Kodak GBX developer.

Part II. Platelet function studies with Leo A-1

Experimental design

Once binding studies had determined that PTA-1 or a similar protein was indeed present on canine platelets, the goal of the next series of experiments was to see if the function of this protein was the same in man and dog. As Leo A-1 antibody has been shown to be a potent activator of human platelets, functional studies in this section examined the ability of this monoclonal antibody to aggregate canine platelets.

Aggregation studies were performed on PRP and gel-filtered platelets from three normal dogs (1 Basset, 2 non-Bassets), three affected dogs, and 3 human subjects. As a control to validate the normalcy of platelet responses, 10 μM ADP and 0.2U/ml thrombin were used in PRP and gel-filtered preparations, respectively. Platelet suspensions were then stimulated with serial dilutions of Leo A-1 in PBS with final concentrations in PRP ranging from 0.04-4 μg/ml. In

man, Leo A-1 is reported to induce half-maximal aggregation at a concentration of 1.5 μg/ml[52].

Platelet isolation

Whole blood was collected, PRP prepared, and a manual platelet count performed as described previously in Part I. Following removal of the PRP, the blood was centrifuged at 1324 x g for 13 minutes to separate platelet-poor plasma. The platelet count was then adjusted using the autologous platelet poor plasma to a count of 3 x 10^8 platelets/ml. 0.5 ml of platelet-rich plasma was then aliquoted into aggregometer cuvettes, covered with parafilm, and the platelets allowed to rest for 30 minutes at room temperature. All platelet preparations were used within four hours of collection.

Gel filtration of platelets

In the preparation of gel filtered platelets, whole blood was collected in 3.8% trisodium citrate in all subjects, and the PRP collected as described above. Prostaglandin E_1 was added to a concentration of 1 μM to minimize platelet activation during the subsequent handling steps and gel filtration. The platelets were allowed to rest for 10 minutes at room temperature, and were then pelleted by centrifugation at 800 x g for 15 minutes. The supernatant plasma was discarded, and the pellet gently resuspended in 1-1.5 ml of HEPES-Tyrodes-albumin buffer (HTA) containing 130 mM NaCl, 2.6 mM KCl, 0.42 mM NaH_2PO_4, 5.5 mM glucose, 0.01 mM HEPES, and 3.0 mg/ml BSA, pH 7.2. The

platelet suspension was layered onto a polyethylene column containing Sepharose 4-B (10 ml bed volume) that had been equilibrated at room temperature with HTA. As platelets traversed the void volume of the column, they were detected visually. Two to three ml of gel-filtered platelets were collected, a manual platelet count performed, and the platelet number adjusted to 3 x 10^8/ml with HTA. $CaCl_2$ and $MgCl_2$ were added to the platelet suspension to a final concentration of 1 mM and 0.8 mM, respectively, for canine platelets; and for human platelets, both were added at a concentration of 1 mM. The gel-filtered platelets were aliquoted into aggregometer cuvettes, and allowed to rest at room temperature for 30 minutes.

Platelet aggregation studies

All aggregation studies were performed in a Lumi-aggregometer (Chrono-log Corporation, Havertown, PA) at 37.5°C. Cuvettes containing 0.5 ml aliquots of PRP or gel-filtered platelets were allowed to equilibrate for at least 4 minutes at 37.5°C prior to being placed in the aggregometer. Following placement of the cuvette in the aggregometer, the platelet suspension was allowed to stir at 900 rpm for approximately 30 seconds and 20 μl of agonist (Leo A-1, ADP, or thrombin) was then added. Results were recorded on a dual pen Houston Omniscribe Chart Recorder (Houston Instrument Division of Bausch and Lomb, Inc., Houston, TX). Failure to record an increase in light transmission above baseline when the sample was permitted to stir for at least 15 minutes after the addition of agonist was designated as zero aggregation.

CHAPTER 3

RESULTS

Western blot analysis

To determine if LEO A-1 recognized and bound a platelet protein(s) in man and dog, initial studies were carried out using Western blotting. Reduced and non-reduced platelet protein preparations from a human, normal dog, and a BHT-affected dog were applied to a 7-10%T polyacrylamide gel. The gel was electroblotted onto a nitrocellulose membrane, and the immunocomplex(es) identified using a biotinylated secondary antibody directed against murine IgG. The signal was then amplified in a streptavidin-biotinylated alkaline phosphatase-catalyzed reaction by BCIP and NBT.

As seen in Figure 4a, LEO A-1 reacted strongly with a human protein in a broad band from approximately 56 to 62 kDa under non-reducing conditions. Leo A-1 also reacted with non-reduced platelet proteins in normal and affected dogs. In this case, 2 fainter but crisp bands could be appreciated at approximately 67 and 70 kDa, respectively. Great difficulty was encountered in approximating molecular weights for the bands detected on the Western blots. This resulted from the detection of additional bands in the molecular weight standard lane not corresponding to known compounds. Unfortunately, this

situation was encountered with several batches of biotinylated standards, and was present in all blots performed.

As seen in Figure 4b, reduced blots showed no interaction of LEO A-1 with platelet proteins in man or dog. However, faint doublet bands could be discerned in the 70-80 kDa area in man and dog that raised concern that the doublet bands seen in the non-reduced blots could represent non-specific binding. A non-reduced blot containing a negative and positive control for the primary and secondary antibodies showed a very faint singlet band in the 70 kDa area. Because of these findings, a direct binding study using ^{125}I-labelled LEO A-1 was performed to determine if the bands seen on the Western blots did indeed represent specific binding of the antibody.

Figure 4a. Western blot of non-reduced platelet proteins incubated with Leo A-1 monoclonal antibody. Lane A: BHT-affected dog, Lane B: normal dog, Lane C: human, Lane D: molecular weight standard, Lane 1: molecular weight standard, Lane 2: human platelet proteins. Lanes 1 and 2 are stained with an India ink total protein stain to evaluate efficacy of protein transfer to the nitrocellulose membrane.

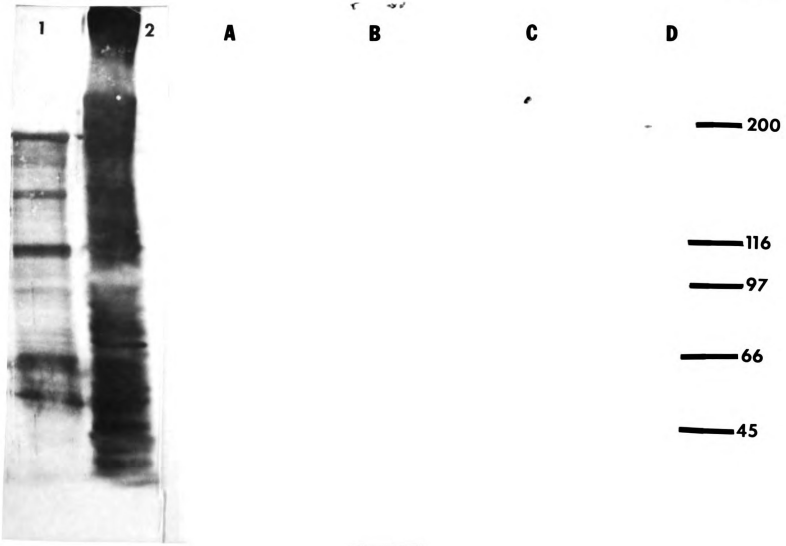
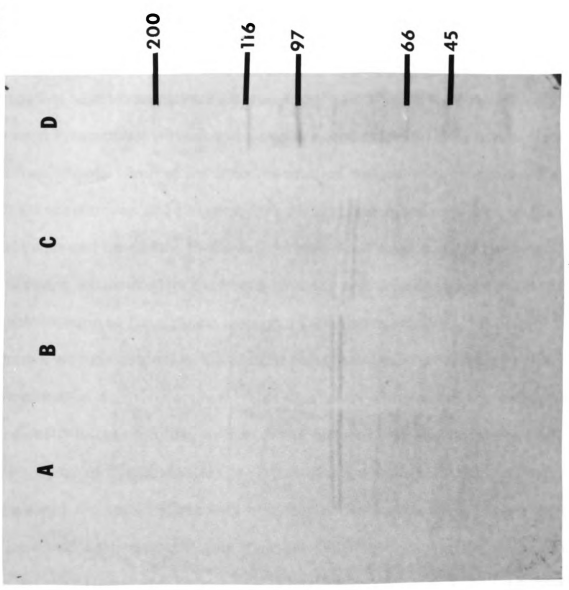

kDa
1
2
A
B
C
D
200
116
97
66
45

Figure 4b. Western blot of reduced platelet proteins incubated with Leo A-1 monoclonal antibody. Lane A: BHT-affected dog, Lane B: normal dog, Lane C: human, Lane D: molecular weight standard.


kDa
200
116
97
66
45
A
B
C
D

Direct binding studies

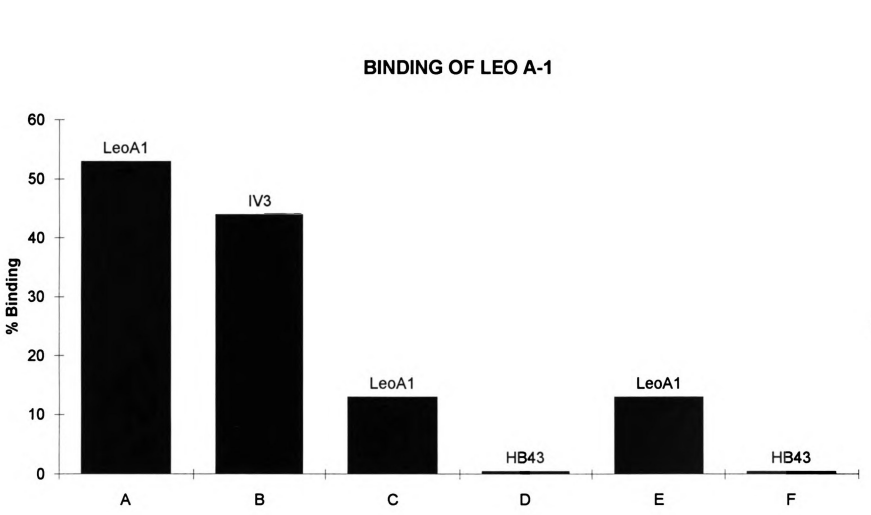
In an initial experiment to evaluate binding of LEO A-1 to the platelet surface, ^{125}I-labelled LEO A-1 was added to a suspension of washed platelets from a human, normal dog, and a BHT-affected dog in a final concentration of 250 ng/ml. The adjusted platelet count of the suspension was 2 x 10^8 platelets/ml. ^{125}I HB 43 (a monoclonal antibody to human IgG) was used as a negative control for canine platelets, and ^{125}I IV 3 (a monoclonal antibody to human F_c receptors) was used as a positive control for low copy number in the human sample. Both of the latter monoclonal antibodies were also used at a final concentration of 250 ng/ml. The platelet suspensions were incubated in an Eppendorf tube with ^{125}I-labelled antibody for 1 hour at room temperature. Following the incubation, the bound antibody was separated from the free by centrifugation of the platelets through a 20% sucrose cushion. The tip of the Eppendorf tube containing the platelet pellet was cut off and its radioactivity measured in a gamma counter. This was taken to represent the amount of radioactivity bound to the surface of the platelet. An equivalent solution of 250 ng/ml of ^{125}I-labelled LEO A-1, HB 43, and IV 3 was also counted to represent the total radioactivity originally added to the tube. The percent binding of each antibody was then calculated by dividing the amount of radioactivity (cpm) of the platelet pellet by the total radioactivity (cpm) for each antibody for canine and human. The data for this initial study are presented in Table 1 and Figure 5. As can be seen, ^{125}I Leo A-1 binds to canine platelets, but to a much lesser degree than to human platelets. This could be the result

of lower affinity of the human monoclonal antibody for the canine protein and/or a lower receptor copy number on the surface of canine platelets compared to human platelets. Leo A-1 binding to human platelets approximates the binding of the positive control, IV 3, whose receptor is present in a comparable copy number to PTA-1.

Table 1. Direct binding of 250 ng/ml ^{125}I Leo A-1 to the platelet surface on man and dog.

Subject	Ab	cpm bound	total cpm	% bound
Human	LEO A-	321205	602402	53
Human	IV 3	192857	438434	44
Normal Dog	LEO A-1	80084	602402	13
Normal Dog	HB 43	279	137973	0.1
BHT	LEO A-1	79295	602402	13
BHT	HB 43	274	137973	0.2

Figure 5. Direct binding of 250 ng/ml ^{125}I Leo A-1, HB 43, or IV 3 to the platelet surface in man and dog. A and B: human platelets, C and D: control canine platelets, E and F: BHT-affected platelets.


BINDING OF LEO A-1
% Binding
60
50
40
30
20
10
0
LeoA1
IV3
LeoA1
HB43
LeoA1
HB43
A
B
C
D
E
F

It can be seen that BHT platelets bound Leo A-1 to the same degree as platelets from the normal dog; therefore, only normal canine platelets were used for further studies because of limited availability of the antibody.

Further experiments using ^{125}I Leo A-1 were performed to determine if the platelet surface receptors could be saturated with antibody, thereby validating a Scatchard analysis of the data to determine the number of Leo A-1 binding sites present on canine platelets. Scott[52] determined that approximately 1500 Leo A-1 binding sites are present on the surface of the human platelet.

^{125}I Leo A-1 was added to suspensions of washed platelets from a human and a normal dog in final concentrations ranging from 25-1000 ng/ml. The platelet count of the suspensions was adjusted to 5 x 10^8 platelets/ml. The data are presented in Tables 2a and b. A Klotz plot[65] was then generated from this data by plotting the ng bound versus the log of the ng free. If the upper inflection point of this sigmoidal plot had been attained, this would indicate that saturation of platelet surface Leo A-1 binding sites had been achieved. As can be seen in the Klotz plots in Figures 6a and b, it could not be determined with confidence that saturation of Leo A-1 binding sites had occurred in man or dog.

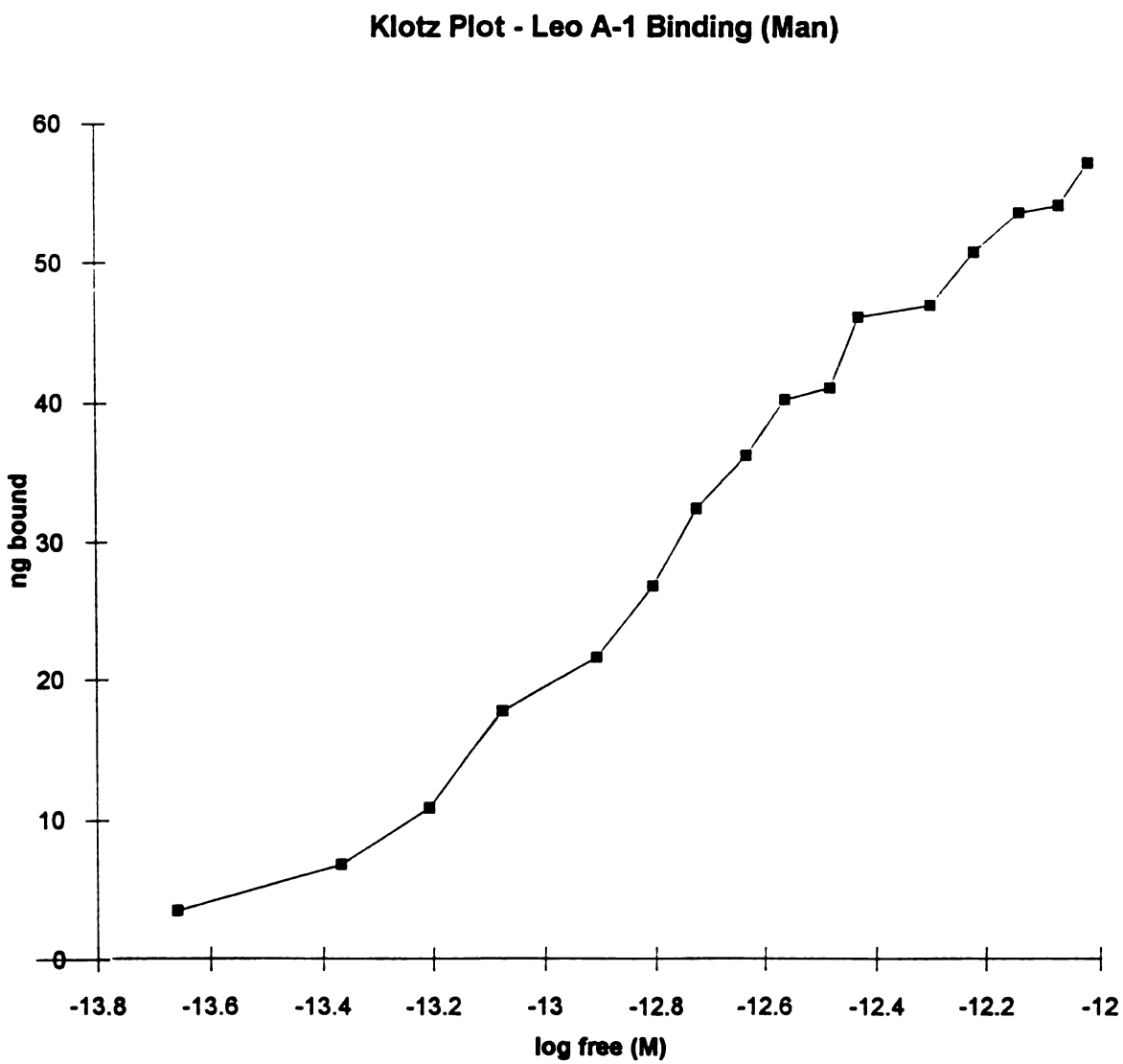
Table 2a. Binding of ^{125}I Leo A-1 to the surface of human platelets. 5 x 10^8 platelets were incubated with concentrations of ^{125}I Leo A-1 ranging from 25 to 1000 ng/ml.

ng LEO-A1 added	ng bound	ng free	log free(M)	bound/free
5	3.34	1.66	-13.66	2.01
10	6.59	3.41	-13.37	1.93
20	10.53	9.47	-13.21	1.11
30	17.26	12.74	-13.08	1.35
40	21.11	18.89	-12.91	1.11
50	26.11	23.89	-12.81	1.09
60	31.51	28.49	-12.73	1.10
70	35.25	34.75	-12.64	1.01
80	39.1	40.90	-12.57	0.95
90	39.94	50.06	-12.49	0.79
100	44.97	55.03	-12.44	0.81
120	45.8	74.2	-12.31	0.61
140	49.5	90.5	-12.23	0.54
160	52.3	107.7	-12.15	0.48
180	52.8	127.2	-12.08	0.41
200	55.8	144.2	-12.03	0.38

Table 2b. Binding of ^{125}I Leo A-1 to the surface of normal canine platelets. 5 x 10^8 platelets were incubated with ^{125}I Leo A-1 in concentrations ranging from 25 to 1000 ng/ml.

ng LEO-A1 added	ng bound	ng free	log free(M)	bound/free
5	.264	4.73	-13.51	0.055
10	.409	9.59	-13.20	0.042
20	.960	19.04	-12.91	0.050
30	1.13	28.87	-12.72	0.039
40	2.51	37.49	-12.56	0.059
50	2.59	47.41	-12.51	0.054
60	2.51	57.59	-12.43	0.043
70	3.81	66.19	-12.36	0.057
80	3.53	76.47	-12.30	0.046
90	3.08	86.92	-12.25	0.035
100	4.5	95.5	-12.21	0.047
120	5.64	114.36	-12.13	0.049
140	6.55	133.4	-12.06	0.049
160	7.6	152.4	-12.0	0.049
180	9.2	170.8	-11.95	0.053
200	9.5	190.5	-11.91	0.049

Figure 6a. Klotz plot depicting ^{125}I Leo A-1 binding to the surface of human platelets.


Klotz Plot - Leo A-1 Binding (Man)
60
50
40
30
20
10
0
ng bound
-13.8
-13.6
-13.4
-13.2
-13
-12.8
-12.6
-12.4
-12.2
-12
log free (M)

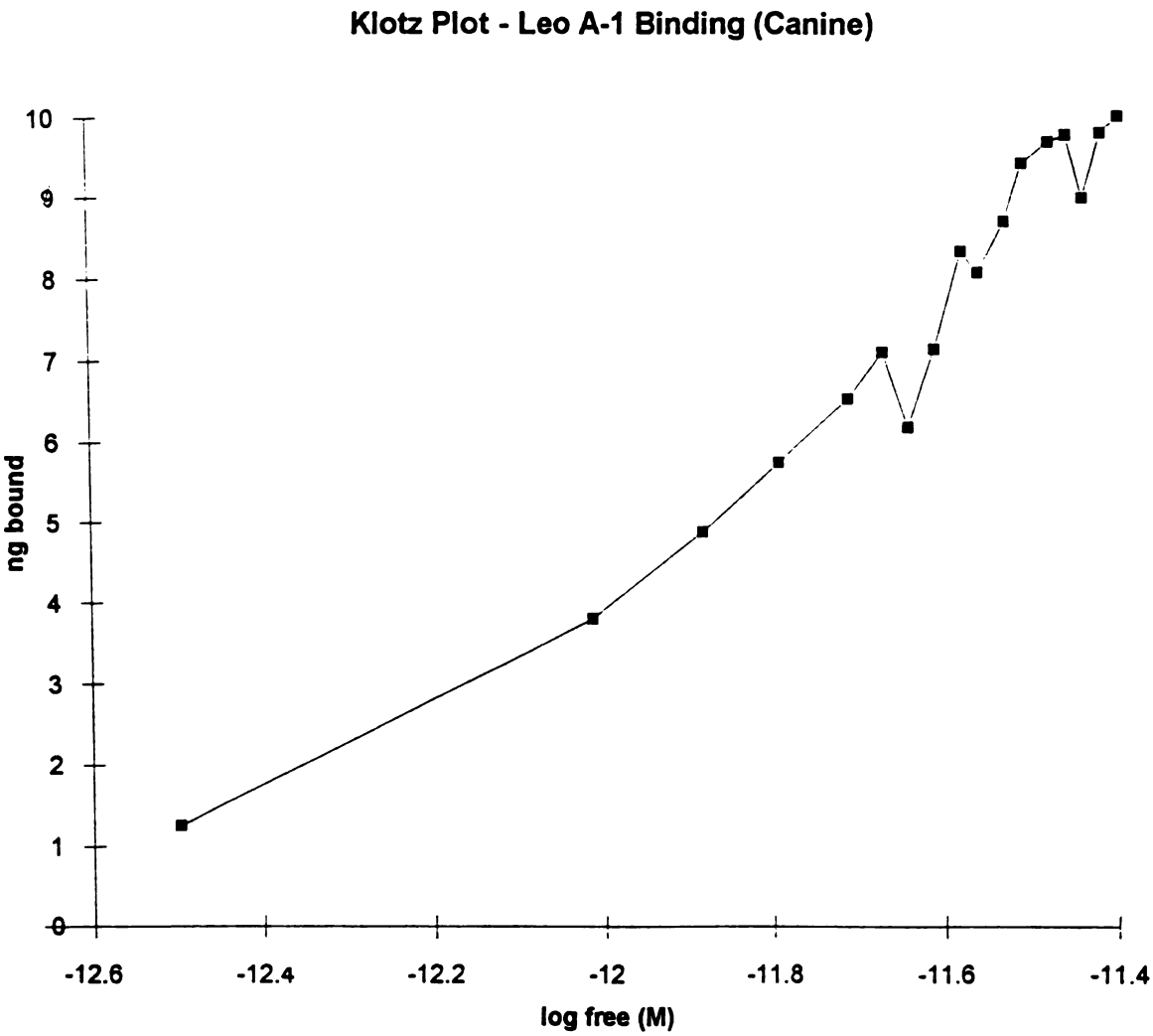
Because the availability of radiolabelled Leo A-1 was limited, a final experiment was performed to attempt to saturate antibody binding sites on normal canine platelets. Washed platelet suspensions from a normal dog were incubated with ^{125}I Leo A-1 in final concentrations ranging from 250 to 3000 ng/ml. The final platelet count of the suspension was adjusted to 2 x 10^7 platelets/ml to favor saturation. The data are presented in Table 3. As can be seen, for large increases in antibody concentration, only very small increases in binding could be appreciated. A Klotz plot of this data (represented in Figure 6b) still did not support saturation of binding sites by Leo A-1. These data strongly suggests that low binding affinity of the human monoclonal Leo A-1 for the canine platelet protein was responsible for the binding data obtained in the experiments.

The data from all of the binding studies were analyzed using two computer programs, LIGAND and EBDA[61]. These programs perform linear and non-linear regression and Scatchard analysis of binding data, and calculate the dissociation constant (K_d) for ligand binding. The K_d value is defined as that concentration of radioligand at which 50% of its receptor sites are occupied. K_d is therefore a measure of binding affinity: the lower the K_d, the higher the affinity. The K_d value obtained for the binding of Leo A-1 to human platelets was 1.38 x 10^{-9} M, and for normal dog platelets 3.78 x 10^{-8} M.

Table 3. Direct binding of ^{125}I Leo A-1 to the surface of normal canine platelets. 2 x 10^7 platelets were incubated with increasing concentrations of ^{125}I Leo A-1 ranging from 250 to 3000 ng/ml.

ng Leo A-1 added	ng bound	ng free	log free (M)	bound/free
50	1.23	48.77	-12.5	0.025
150	3.71	146.29	-12.02	0.025
200	4.77	195.23	-11.89	0.024
250	5.61	244.39	-11.80	0.023
300	6.38	293.62	-11.72	0.022
325	6.94	318.06	-11.68	0.022
350	6.04	343.96	-11.65	0.017
375	6.98	368.02	-11.62	0.018
400	8.15	391.85	-11.59	0.020
425	7.90	417.10	-11.57	0.018
450	8.51	441.49	-11.54	0.019
475	9.22	465.78	-11.52	0.019
500	9.48	490.52	-11.49	0.019
525	9.57	515.43	-11.47	0.018
550	8.8	541.20	-11.45	0.016
575	9.6	565.38	-11.43	0.017
600	9.8	590.20	-11.41	0.016

Figure 6b. Klotz plot depicting ^{125}I Leo A-1 binding to the surface of normal canine platelets.


Klotz Plot - Leo A-1 Binding (Canine)
ng bound
10
9
8
7
6
5
4
3
2
1
0
-12.6
-12.4
-12.2
-12
-11.8
-11.6
-11.4
log free (M)

Using the following formula, it is possible to detemine the percent of receptor sites bound at the maximal concentration of antibody used in each experiment for human and canine platelets:

$$\frac{\text{concentration of radioligand } (10^{-9}\ M)}{K_d + \text{concentration of radioligand } (10^{-9}\ M)} = \%\ \text{receptor sites bound}$$

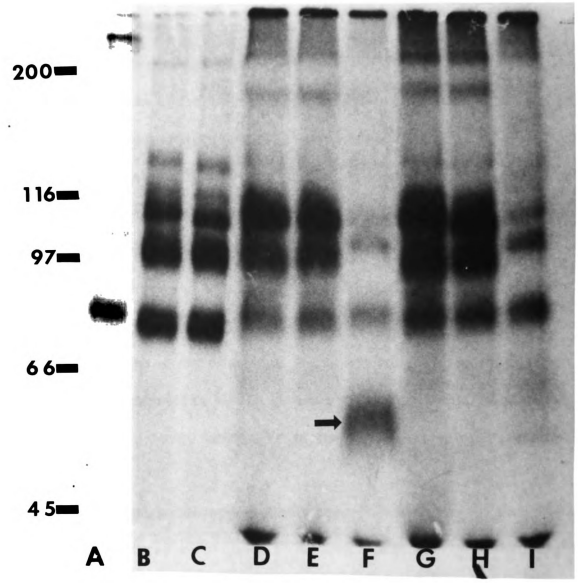
At 1000 ng/ml final concentration of ^{125}I Leo A-1 in human platelets, 79% of the receptor sites were occupied by antibody. In the case of canine platelets, at 3000 ng/ml final concentration of ^{125}I Leo A-1 only 39% of available binding sites were occupied by antibody. Thus, in neither man nor dog had we managed to achieve saturation of binding sites with ^{125}I Leo A-1 antibody. Therefore, the number of binding sites for Leo A-1 on the platelet surface could not be accurately determined using Scatchard analysis. This inability to achieve saturation of canine platelet receptors for Leo A-1 was probably a function of the low binding affinity of the human monoclonal antibody for the canine protein. In the case of human platelets, however, we were merely limited by the inadequate supply of ^{125}I Leo A-1 to complete further experiments that would only serve to confirm previous work[52]. The K_d for Leo A-1 binding to platelet PTA-1 in man was determined by Scott[52] to be 1.2×10^{-9} M, very close to the value of 1.38×10^{-9} M that we obtained in our experiments.

Immunoprecipitation

To more accurately determine the molecular weight of human PTA-1 and the dog platelet protein that bound Leo A-1 for comparison to Western blot results, immunoprecipitation of both proteins was attempted using Leo A-1. 1 x 10^9 platelets from a human, normal dog, and a BHT-affected dog were surface labelled with ^{125}I. The labelled platelets were then incubated with 10 μg/ml final concentration of unlabelled Leo A-1 or HB 43 (used as a negative control for the dog). The labelled platelets were pelleted by centrifugation, and the supernatant containing the excess unbound antibody discarded. The pellet was solubilized in a lysis buffer, added to a solution of anti-mouse IgG agarose, and incubated. The platelet-agarose suspension was washed by centrifugation. The resultant radiolabelled pellet was resuspended in a non-reduced SDS-PAGE sample buffer and boiled to lyse the platelets and elute the bound PTA-1 from the agarose. The suspension was centrifuged, and the supernatant containing the platelet lysate with radiolabelled PTA-1 was electrophoresed on a 7%T SDS-PAGE slab gel. The dried gel was autoradiographed to detect the immunoprecipitated labelled PTA-1. Figure 7 represents the autoradiograph of the immunoprecipitation of PTA-1.

Immunoprecipitation of PTA-1 was only succesful from the human platelet lysate. PTA-1 can be detected as a broad band from approximately 60 to 67.5 kDa, roughly similar to the band visualized on the Western blot, which was immunodetected at approximately 56 to 62 kDa.

Figure 7. Autoradiograph following SDS-PAGE of immunoprecipitated PTA-1 from canine and human surface ^{125}I-labelled platelets with Leo A-1. Lane A: human whole platelet lysate, Lane B: normal canine whole platelet lysate, Lane C: BHT whole platelet lysate, Lane D: normal canine + Leo A-1, Lane E: BHT + Leo A-1, Lane F: human + Leo A-1, Lane G: normal canine + HB 43, Lane H: BHT + HB 43, Lane I: human + HB 43. The arrow represents PTA-1 immunoprecipitated from human platelet proteins.


200
116
97
66
45
A
B
C
D
E
F
G
H
I

Effects of Leo A-1 on platelet aggregation in vitro

The efficacy of Leo A-1 monoclonal antibody as an in vitro platelet agonist was tested in PRP and GFP from humans, normal dogs, and BHT-affected dogs. ADP (final concentration 10 μM) was used as a control agonist for PRP, and thrombin (final concentration 0.2 U/ml) as the control agonist for GFP. Leo A-1 was used in final concentrations ranging from 0.04 to 4.0 ug/ml.

The addition of Leo A-1 to either gel-filtered platelets or platelet-rich plasma from human donors resulted in dose-dependent platelet aggregation. As had previously been reported[52], changes in antibody concentration had little effect on the percentage aggregation that was attained. The time to onset of aggregation, however, was inversely related to antibody concentration. Aggregation responses in GFP and PRP were comparable, although GFP were capable of responding to a slightly lower antibody concentration than PRP. One human donor failed to respond to Leo A-1 in any concentration used in either GFP or PRP. In contrast to human platelets, no aggregation was observed in platelets from any canine subject in response to any concentration of Leo A-1 added to PRP or GFP.

Figure 8 illustrates aggregation traces in human GFP in response to increasing concentrations of Leo A-1. Numerical data from canine and human aggregation experiments are presented in Tables 4a and b.

Figure 8. Platelet aggregation induced by Leo A-1 antibody. The aggregation curves illustrated demonstrate the response of gel-filtered human platelets to decreasing concentrations Leo A-1. a: 4 μg/ml, b: 2 μg/ml, c: 1 μg/ml, d: 0.4 μg/ml, e: 0.2 μg/ml. For comparison, aggregation trace f represents the response of normal and BHT-affected canine platelets to 4 μg/ml Leo A-1.

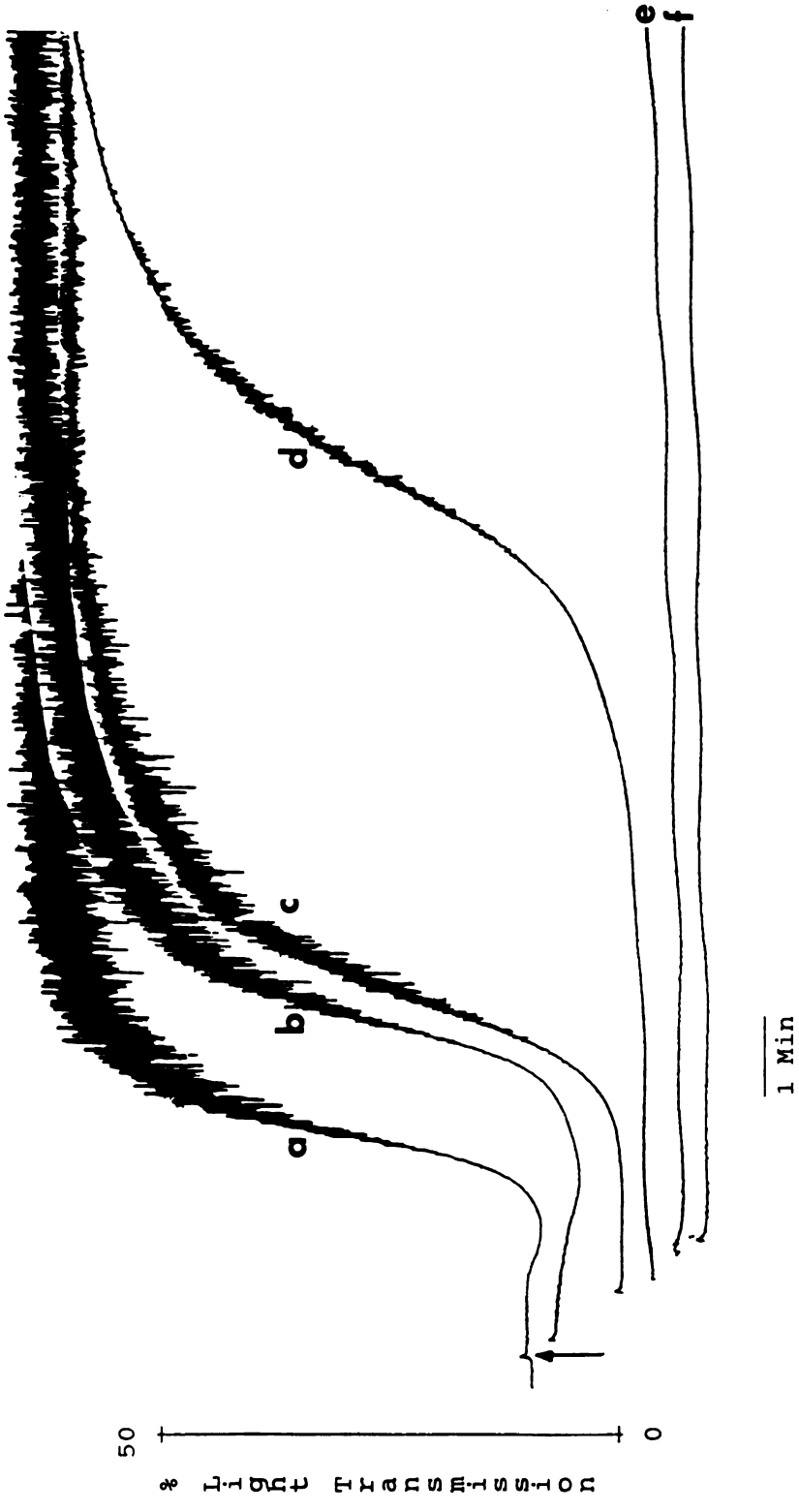

% Light Transmission
50
0
a
b
c
d
e
f
1 Min

Table 4a. Maximum aggregation (%), slope, time to maximal aggregation (min), of human, normal canine, and BHT-affected gel-filtered platelets to increasing concentrations of Leo A-1.

Agonist	SI_m	Time (min)	% Aggregation
HUMAN (n = 3)			
ADP 10 μM	3.55 ± .3	0.3 ± 0.1	60.5 ± 15.5
LEO A-1 .04 μg/ml	0	0	0
LEO A-1 .2 μg/ml	0	0	0
LEO A-1 1 μg/ml	.50 ± .3	11 ± 3	41.9 ± 7.8
LEO A-1 2 μg/ml	1.23 ± .52	5.5 ± 0.5	51.9 ± 2.2
LEO A-1 4 μg/ml	2.4 ± .57	3.5 ± 0.5	60.2 ± 5.2
NORMAL DOG (n = 4)			
ADP 10 μM	2.6 ± .47	.2	49.3 ± 5.9
LEO A-1 .04 μg/ml	0	0	0
LEO A-1 .2 μg/ml	0	0	0
LEO A-1 1 μg/ml	0	0	0
LEO A-1 2 μg/ml	0	0	0
LEO A-1 4 μg/ml	0	0	0
BHT-AFFECTED DOG (n = 4)			
ADP 10 μM	0	0	0
LEO A-1 .04 μg/ml	0	0	0
LEO A-1 .2 μg/ml	0	0	0
LEO A-1 1 μg/ml	0	0	0
LEO A-1 2 μg/ml	0	0	0
LEO A-1 4 μg/ml	0	0	0

Table 4b. Maximum aggregation (%), slope, and time to maximum aggregation (min) of human, normal canine, and BHT-affected platelet-rich plasma to increasing concentrations of Leo A-1.

HUMAN (n = 3)

Agonist	SI_m	Time (min)	% Aggregation
Thrombin .2 U/ml	± 0.3	0.2	69.2 ± 3.2
LEO A-1 .04 μg/ml	0	0	0
LEO A-1 .2 μg/ml	1.17 ± 0.5	10.75 ± 6.2	58.4 ± 10.8
LEO A-1 1 μg/ml	2.29 ± 0.4	6 ± 3	61.0 ± 10
LEO A-1 2 μg/ml	2.8 ± 0.8	3.25 ± 0.75	58.8 ±5.9
LEO A-1 4 μg/ml	4.21 ± 1.2	1.65 ± 0.1	57.4 ± 2.4

NORMAL DOG (n = 4)

Thrombin .2 U/ml	4.8 ± 1.76	0.2	72.9 ± 2.3
LEO A-1 .04 μg/ml	0	0	0
LEO A-1 .2 μg/ml	0	0	0
LEO A-1 1 μg/ml	0	0	0
LEO A-1 2 μg/ml	0	0	0
LEO A-1 4 μg/ml	0	0	0

BHT-AFFECTED (n = 4)

Thrombin .2 U/ml	1.71 ± 0.87	2.2 ± 0.56	73.6 ± 4.1
LEO A-1 .04 μg/ml	0	0	0
LEO A-1 .2 μg/ml	0	0	0
LEO A-1 1 μg/ml	0	0	0
LEO A-1 2 μg/ml	0	0	0
LEO A-1 4 μg/ml	0	0	0

CHAPTER 4

DISCUSSION

The first aim of this thesis project was the identification of PTA-1 in canine platelets by using immunodetection methods with a human monoclonal antibody, Leo A-1. Initial experiments utilized Western blotting as a method to identify PTA-1 in platelet protein preparations following separation by gel electrophoresis. In human non-reduced platelet preparations, PTA-1 was detected with Leo A-1 as a broad band that extended from approximately 56 to 62 kDa, corresponding to previously reported data for non-reduced, purified PTA-1[52]. When human platelet proteins were reduced prior to SDS-PAGE electrophoresis, no antibody binding to PTA-1 was detectable by Western blotting. Scott[52] reported that purified human PTA-1 migrates slightly differently on SDS-PAGE under reducing and non-reducing conditions. Non-reduced PTA-1 migrates at approximately 55-65 kDa, while the reduced protein migrates at approximately 67-70 kDa. This difference in mobility is attributed to the presence of sulfhydryl bonding within the molecule, which is disrupted by the denaturation imposed by reducing conditons. Our findings on non-reduced Western blot preparations correspond to those described after SDS-PAGE by Scott[52], and the inability of Leo A-1 to bind to PTA-1 under reducing

conditions is probably secondary to the loss of sulfhydryl bonding and disruption of the binding site (epitope) when the protein is denatured during preparation. We were able to immunoprecipitate PTA-1 from surface-labelled, non-reduced human platelet preparations using Leo A-1 antibody. Electrophoretic separation revealed a protein of the approximate molecular weight of 60-67.5 kDa, again similar to our findings on Western blot and those reported by Scott[52].

In canine non-reduced platelet preparations, fainter but detectable individual bands were seen at 67 and 70 kDa, respectively. These bands, as in man, could not be appreciated on reduced blots. Subsequent direct binding studies confirmed that Leo A-1 bound to a canine platelet protein(s), but with very low affinity when compared to that seen for human PTA-1. The ability to detect such binding on Western blots was likely due to the high sensitivity of this detection method. The inability to saturate the canine platelet Leo A-1 receptor population with the radiolabelled antibody or to immunoprecipitate the canine protein(s) binding Leo A-1 was probably a direct result of this low binding affinity.

The findings indicative of poor affinity of the human monoclonal Leo A-1 for a canine platelet protein(s) are disappointing, but not surprising. Western blotting and direct binding analyses are supportive of the idea that Leo A-1 recognizes a similar epitope on a canine protein, but it is not possible to definitively conclude that this identified canine protein represents a counterpart to human PTA-1.

The effect of Leo A-1 binding on platelet function was examined using the endpoint of platelet aggregation. While Leo A-1 proved to be a powerful agonist when added to human GFP or PRP (as previously reported[52,53]), canine platelets were completely unresponsive to its effects. One of the human donors used in the Leo A-1 aggregation studies also failed to respond to any concentration of the antibody as an agonist. This has been previously reported in Leo A-1 aggregation studies by Zuzel[53], and presumed to be the result of a relatively well-described polymorphism of the human F_c receptor[62,63] which results in decreased binding affinity of the F_c receptor for its ligand[53,62,63]. Given this data, it could be hypothesized that because Leo A-1 **does** bind to a canine protein(s), the lack of response of canine platelets to Leo A-1 as an agonist may be the result of the following: 1) the low binding affinity of Leo A-1 for a receptor on the canine platelet surface, which may not support aggregation due to lack of bridging of adjacent platelets through the F_c receptor; 2) a polymorphism of the canine platelet F_c receptor resulting in decreased binding affinity for the F_c portion of Leo A-1 in the population studied; 3) a combination of 1) and 2) above, or 4) that canine platelets actually lack the F_c receptors necessary for antibody-mediated platelet activation.

It has been reported that rabbit platelets express no F_c receptors on their surface, although such receptors are present on other cell types[64] in this species. The literature contains no hard experimental evidence one way or the other regarding canine platelets and F_c receptors, or indeed canine platelets and aggregation responses to antibodies. However, following the conclusion of the

experiments for this project, information became available that supports the idea that canine platelets do **not** possess the F_c receptors necessary for antibody-mediated aggregation responses (M. Scott, personal communication). This information would support the fourth hypothesis as a cause of the lack of response of canine platelets to Leo A-1 as an agonist.

In summary, the experimental data outlined in this project have indicated that a human monoclonal antibody-Leo A-1- will bind to a canine platelet protein(s). The molecular weight of the canine protein(s) that is bound is slightly larger than PTA-1. Because of the low binding affinity of the human monoclonal in the canine, it was not possible to immunoprecipitate the protein(s) for further analysis and comparison to PTA-1. It is unknown at this time if this protein(s) represents a canine counterpart to PTA-1 or another protein of unknown identity that contains a similar epitope. Canine platelets, although able to bind Leo A-1, did not respond to this antibody as an agonist, probably because canine platelets lack the F_c receptors necessary to initiate such a response.

As Leo A-1 does bind, albeit with low affinity, to a canine protein, if a polyclonal antibody were available to PTA-1, it is possible that it may recognize other epitopes on the molecule that exhibit a higher degree of identity between the canine and human protein than the epitope recognized by Leo A-1. This might provide another opportunity to isolate, characterize, and compare the canine to PTA-1. Further investigations could also compare the protein in normal and BHT-affected dogs, and determine if the identified protein(s) may

represent the poorly phosphorylated 65-67 kDa protein seen in BHT. If, however, such an antibody were not available, future work examining the 65-67 kDa area will of necessity involve more cumbersome chemical methodology. Such methods would investigate whether the poorly phosphorylated 65-678 kDa protein band(s) seen in BHT: 1) are the result of a failure of or defective phosphorylation, or an abscence of a substrate protein, or 2) consists of a single protein or more than one protein, one or both of which is not phosphorylated. This could potentially be accomplished through:

a) the use of gradient SDS-PAGE gels to expand the 65-67 kDa area, correlated with phosphorylated bands on autoradiographs.

b) the use of O'Farrell 2-dimensional electrophoresis to identify the isoelectric point of the protein(s) as a prelude to purification.

c) further characterization of the protein(s) through glycoprotein moiety labelling in SDS-PAGE and 2-D gels; and the identification of phosphorylated amino acid residues in SDS-PAGE gels.

d) purification of the protein, which could be accomplished using preparative isofocusing. This may be followed by electroblotting and protein sequencing to compare the sequence to known sequences in the NBRF protein data bank; and the sequences in normal dogs versus BHT-affected animals could then be compared.

LIST OF REFERENCES

LIST OF REFERENCES

1. Leung L, Nachman R. Molecular mechanisms of platelet aggregation. Ann. Rev. Med. **37**:179-186, 1986.

2. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane IIb-IIIa complex during platelet activation. J. Biol. Chem. **260**:1107-1114, 1985.

3. Jain NC. Qualitative and quantitative disorders of platelets. In: Schalms Veterinary Hematology, 4th edition. Lea and Febiger, Phila, PA. pp 431-445, 1986.

4. Steen VM, Holmsen H. Current aspects on human platelet activation and responses. Eur. J. Haematol. **38**:383-399, 1987.

5. Coleman RW, Hirsch J, Marder J Slazman EW. Overview of hemostasis. In: Hemostasis and Thrombosis, 3rd edition. JB Lippencott and Co., Phila, PA. pp 3-19, 1994.

5a. Cotran RS, Kumar V, Robbins SL. Hemodynamic disorders, thrombosis, and shock. In: Pathologic Basis of Disease, 5th edition. WB Saunders, Phila, PA. p 100, 1994.

6. Hawiger J, Brass LF, Salzman EW. Signal transduction and intracellular regulatory processes in platelets. In: Hemostasis and Thrombosis, 3rd edition. JB Lippencott and Co., Phila, PA. p603-628, 1994.

7. Jennings L, Phillips D. Purification of glycoproteins IIb and IIIa from human platelet plasma membranes and characterization of a Ca^{+2}-dependent glycoprotein IIb-IIIa complex. J. Biol. Chem. **257**:10458, 1982.

8. Coller BS. A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb-IIIa complex. J. Clin. Invest. **76**:101-108, 1985.

9. Gogstad Go, Brosstad F, Krutnes M-B, Hagen J, Solume NO. Fibrinogen-binding properties of the human platelet glycoprotein IIb-IIIa complex: a study using crossed immunoelectrophoresis. Blood **60**:663-671, 1982.

10. Nachman RL, Leung LLK. Complex formation of platelet membrane glycoproteins IIb and IIIa with fibrinogen. J. Clin. Invest. **69**:263-269, 1982.

11. Simon MF, Chap H, Douste-Blazy L. Platelet aggregating activity of lysophosphatidic acids is not related to their calcium-dependent ionophore properties. FEBS lett. **166**:115-119, 1984.

12. Charo IF, Kieffer N, Phillips DR. Platelet membrane glycoproteins. In: Hemostasis and Thrombosis, 3rd edition. JB Lippencott and Co., Phila, PA. pp 489-507, 1994.

13. Seiss W. Molecular mechanisms of platelet activation. Physiol. Rev. **69**:58-178, 1989.

14. Charo IF, Feinman RD, Detweiler TC. Interrelations of platelet aggregation and secretion. J. Clin. Invest. **60**:866-873, 1977.

15. Zucker MB, Nachmias VT. Platelet activation. Arteriosclerosis **5**: 2-18, 1985.

16. Brass LF, Hoxie JA, Manning DR. Signalling through G-proteins and G-protein-coupled receptors during platelet activation. Thromb Haemostas. **70**: 217-223, 1993.

17. Kroll MH, Schafer AI. Biochemical mechanisms of platelet activation. Blood **74**: 1181-1195, 1989.

18. Holmsen H. Secretable storage pools in platelets. Ann. Rev. Med. **30**: 119-134, 1979.

19. Holmsen H. Platelet secretion and energy metabolism. In: Hemostasis and Thrombosis, 3rd edition. JB Lippencott and Co., Phila, PA. pp 524-545, 1994.

20. Born GVR. Aggregation of platelets by adenosine diphosphate and it's reversal. Nature **194**: 927-929, 1962.

21. Yardumian DA, Mackie IJ, Machin SJ. Laboratory investigation of platelet function: a review of methodology. J. Clin. Pathol. **39**: 701-712, 1986.

22. Imaoka T, Lynham JA. Purification and characterization of the 47,000 dalton protein phosphorylated during degranulation of human platelets. J. Biol. Chem. **258**: 11404-11414, 1983.

22a. Tyers M, Rachubinski RA, Stewart MI, et al. Molecular cloning and expression of the major PKC substrate of platelets. Nature **333**: 470, 1988.

23. Haolan RJ, Lynham JA. Relationship between the phosphorylation of blood platelet proteins and secretion of platelet granule constituents. Biochem. Biophys. Res. Comm. **77**:714-722, 1977.

24. Connolly TM, Lawing WJ, Majerus PW. Protein kinase C phosphorylates human platelet inositol triphosphate 5'-monoesterase, increasing the phosphatase activity. Cell **46**: 951-958, 1986.

25. Johnstone IB, Lotz TF. An inherited platelet function defect in Basset hounds. Can. Vet. J. **20**: 211-215, 1979.

26. Bell TG, Leader RW, Olsen PM, Padgett GA, Penner JA, Patterson WR. Basset hound hereditary thrombopathy: an autosomally recessively inherited platelet dysfunction with 11 cases in a kindred of 56 dogs. In: One Medicine. Ryder OA, Byrd ML (eds). Springer-Verlag, New York, NY. pp 335-344, 1984.

27. McEver RP. The clinical significance of platelet membrane glycoproteins. Haematol. Oncol. Clinics N. Amer. **4**: 87-105, 1990.

28. Peerschke IEB. The platelet fibrinogen receptor. Sem. Hematol. **22**: 241-259, 1985.

29. Patterson WR, Kunicki TJ, Bell TG. Two dimensional electrophoretic studies of platelets from dogs with Basset houndhereditary thrombopathy: a thrombasthenia-like aggregation defect. Thromb. Res. **42**: 195-203, 1986.

30. Patterson WR, Estry DW, Schwartz KA, Borchert RD, Bell TG. Absent platelet aggregation with normal fibrinogen binding in Basset hound hereditary thrombopathy. Thromb. Haemostas. **62**: 1011-1015, 1989.

31. Estry DW, Mattson JC, Oesterle JR, Patterson WR, Bell TG. Analysis of ADP-induced fibrinogen receptors in dogs with Basset hound hereditary thrombopathy: comparison with normal dog and human platelets. Submitted for publication, 1994.

32. McConnell MF. In: Functional and biochemical abnormalities in Basset hound hereditary thrombopathy. Doctoral dissertation, Michigan State University, 1989.

33. Boudreaux MK, Dodds WJ, Slauson DO, Catalfamo JL. Impaired cAMP metabolism associated with abnormal function of thrombopathic canine platelets. Biochem. Biophys. Res. Comm. **140**: 595-601, 1986.

34. Boudreaux MK, Dodds WJ, Slauson DO, Catalfamo JL. Evidence for regulatory control of canine platelet phosphodiesterase. Biochem. Biophys. Res. Comm. **140**: 589-594, 1986.

35. Thomas JS. In: Basset hound hereditary thrombopathy: measurement of platelet cytosolic calcium, pH, and 1,2 diacylglycerol production. Doctoral Dissertation, Michigan State University, 1991.

36. Fox JEB, Goll DE, Reynolds CC, Phillips DR. Identification of two proteins (actin-binding protein and P235) that are hydrolyzed by an endogenous calcium-dependent protease during platelet activation. J. Biol. Chem. **260**: 1060-1066, 1985.

37. Baron BM, Limbird LE. Human platelet phospholipase-A_2 activity is responsive in vitro to pH and calcium variations which parallel those occurring after platelet activation in vivo. Biochim. Biophys. Acta **971**: 103-111, 1988.

38. Sweatt JD, Connolly TM, Cragol EJ, Limbird LE. Evidence that Na^+/H^+ exchange regulates receptor-mediated Phospholipase A_2 activation in human platelets. J. Biol. Chem. **261**: 8667-8673, 1986.

39. Sweatt JD, Connolly TM, Baron BM, Limbird LE. Involvement of Na^+/H^+ exchange in human platelet activation. In: Progress in Clinical and Biological Research. Vol. 283. Platelet membrane receptors: molecular biology, immunology, biochemistry, and pathology. Jamieson GA (ed). Alan R. Liss, New York, NY. pp 523-557, 1988.

40. Daniel JL, Molish IR, Holmsen H. Myosin phosphorylation in intact platelets. J. Biol. Chem. **256**: 7510-7514, 1981.

42. Kerny R, Scrutton MC. Platelet adenoreceptors. In: The Platelets: physiology and pharmacology, Longenecker GL (ed). Academic Press, New York, NY. p 113, 1985.

43. Horsewood P, Hayward CPM, Warkentin TE, Kelton JG. Investigations of the mechanisms of monoclonal antibody-induced platelet activation. Blood **78**: 1019-1026, 1991.

44. Tetteroo P, Lansdorp P, Leksma O, Kr vonDemBorne A. monoclonal antibodies against human platelet glycoprotein IIIa. Br. J. Haematol. **55**: 509, 1983.

45. McEver R, Baenziger N, Majerus P. Isolation and quantitation of the platelet membrane glycoprotein deficient in thrombasthenia using a monoclonal hybridoma antibody. J. Clin. Invest. **66**: 1311, 1980.

46. McGregor J, Brochier J, Wild F, Follea G, Trzeciak M, James E, et al. Monoclonal antibodies against human platelet membrane glycoproteins IIb-IIIa, different effects on platelet function. Thromb. Res. **38**: 547, 1985.

47. Newman P, Allen R, Kahn R, Kunicki T. Quantitation of Membrane glycoprotein IIIa on intact human platelets using the monoclonal antibody, AP-3. Blood **65**: 227, 1985.

48. Anderson GP, Anderson CL. Signal tranduction by the platelet F_c receptor. Blood **76**: 1165-1172, 1990.

49. Rubinstein E, Urso I, boucheix C, Carroll R. Platelet activation by cross-linking HLA Class I molecules and the F_c receptor. Blood **79**: 2901-2908, 1992.

50. Karas SP, Rosse WF, Kurlander RJ. Characterization of the IgG-F_c receptor on human platelets. Blood **60**: 1277, 1982.

51. Burns GF, Triglia T, Werkmeister JA, Begley CG, Boyd AW. TLiSA1, a human T lineage-specific activation antigen involved in the differentiation of cytotoxic T lymphocytes and anomalous killer cells from their precursors. J. Exp. Med. **161**: 1063-1078, 1985.

52. Scott JL, Dunn SM, Jin B, Hillam AJ, Walton S, et al. Characterization of a novel membrane glycoprotein involved in platelet activation. J. Biol. Chem. **264**: 13475-13482, 1989.

53. Zuzel M, Walton S, Burns GF, Berndt MC, Cawley JC. A monoclonal antibody to a 67 kD cell membrane glycoprotein directly induces persistent platelet aggregation independently of granule secretion. Br. J Haematol. **79**: 1-8, 1991.

54. Zucker MB, Troll W, Belman S. The tumor-promotor phorbol ester (12-O-tetradecanoyl-phorbol-13-acetate), a potent aggregating agent for blood platelets. J. Cell Biol. **60**: 325-336, 1974.

55. Kaibuchi K, Tsuda T, Kikuchi A, Tanimoto T, Takai Y. Enhancement of collagen-induced phosphoinositide turnover by thromboxane A_2 analogue through Ca^{2+} mobilization in human platelets. FEBS Lett. **192**: 104-108, 1985.

56. Castagna M. Phorbol esters as signal transducers and tumor promotors. Biol. of the Cell **59**: 3-14, 1987.

57. Laemmli UK. Cleavage of sructural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685, 1970.

58. Kyhse-Anderson J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Meth. **10**: 203, 1984.

59. Kyhse-Anderson J. Semi-dry electroblotting and transfer using equipment without buffer vessel. In: Handbook of Immunoblotting of Proteins, vol 1. Bjerrum O, Heegaard N (eds). CRC Press, Inc., Boca Raton, FL. p 79, 1988.

60. Fraker P, Speck J. Protein and cell membrane iodonations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril. Biochem. biophys. res. comm. **80**: 849, 1978.

61. McPherson GA. KINETIC, EBDA, LIGAND, LOWRY. A collection of radioligand binding analysis programs. 1985 pp14-97

62. Looney RJ, Anderson CL, Ryan DH, Rosenfeld SI. Structural polymorphism of the human platelet F_c receptor. J. Immunol. **141**: 2680-2683, 1988.

63. Clark MR, Clarkson SB, Ory PA, Stollman N, Goldstein IM. Molecular basis for polymorphism involving F_c receptor II on human monocytes. J. Immunol. **143**: 1731-1734, 1989.

64. Siuha RK, Santos AV, Smith JW, Horsewood P, Andrew M, Kelton JG. Rabbit platelets do not express F_c recptors for IgG. Platelets **3**: 35-39, 1992.

65. Klotz I. Number of receptor sites from Scatchard graphs: facts and fantasies. Science **217**: 1247, 1982.

66. Patterson WR, Padgett GA, Bell TG. Abnormal relaease of storage pool adenine nucleotides from platelets of dogs affected with Basset hound hereditary thrombopathy. Thromb. Res. **37**: 61-71, 1985.