FELINE PLATELETS IN HEALTH AND DISEASE

AND AN ASSESSMENT OF A NEW ANTICOAGULANT TO MINIMISE PSEUDOTHROMBOCYTOPENIA AND PSEUDOLEUKOCYTOSIS

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ABSTRACT

Platelets are complex blood cells whose primary function is to participate in haemostasis, aggregating at the site of injury to obstruct blood flow out of the vessel. They are non-nucleated cells, derived by division of multinucleated bone marrow stem cells called megakaryocytes. Production of platelets is increased when the total circulating platelet mass declines, through the action of thrombopoietin and other stimulatory cytokines. Platelets contain a complex cytoskeleton and contractile protein network which enables shape change upon stimulation, active extrusion of the contents of storage granules and clot retraction. Platelets synthesise a number of factors which influence other platelets, the coagulation cascade, leukocytes and blood vessels. These are stored in granules and are extruded upon stimulation along a complex series of channels to reach the exterior. Platelet adhesion and aggregation begins with the binding of proteins to specific glycoprotein receptors in the platelet plasma membrane. Collagen, exposed at the site of damage to blood vessels is an important agonist in vivo, but other agonists are numerous and include substances released from activated platelets themselves, such as adenosine diphosphate (ADP) and serotonin, circulating substances such as adrenalin and vasopressin, products of the coagulation cascade such as thrombin, physical factors such as shear stress and stirring, and many foreign substances. The signal derived from the agonist binding is transferred across the plasma membrane to trigger a series of biochemical events which result in platelet shape change, fibrinogen binding, attachment of platelets in aggregates and secretion of granule contents, which recruit other platelets, and culminates with platelets losing their individual integrity to form a platelet mass. Platelet activity is constrained by the physical nature of the blood vessel wall and substances secreted by platelets and endothelial cells, as well as the rapid inactivation of free agonists.

Platelet enumeration is a routine part of a full blood count. Both manual methods, using a haemocytometer, and automated methods are available. Estimations of platelet number can be obtained by counting platelets seen in several microscope fields of the blood smear. Automated methods are more accurate than manual methods because of the larger number of cells counted. However, overlap of feline red cell and platelet size results in inaccuracies when aperture-impedance analysers are used. The presence of platelet aggregates in samples produces inaccuracies by all methods, because individual platelets within aggregates cannot be differentiated.

Anticoagulants are designed to inhibit blood coagulation and preserve cell morphology for haematological analysis. EDTA is the most frequently used anticoagulant; however, it causes platelet shape change and swelling and does not completely prevent activation or aggregation. In some individual human blood samples pseudothrombocytopenia and concurrent pseudoleukocytosis is seen when EDTA anticoagulant is used and this has been documented in other species. Other anticoagulants may also produce this effect. Citrate is not routinely used for anticoagulation for haematological analysis because of its dilutional effect.

Because of the central role of platelets in haemostasis, changes in platelet number or function can have significant consequences. Immune-mediated thrombocytopenia is a rare disease in the cat in contrast to other species. More commonly, feline thrombocytopenia accompanies other diseases such as neoplasia, gastrointestinal disease and virus infection and may be a component of disseminated intravascular coagulation; however, resultant clinical signs of bleeding are not often seen. A variety of drugs and diseases may affect platelet function including renal disease, liver disease, dysproteinaemias, infectious diseases and disseminated intravascular coagulation. Hereditary platelet function disorders recognised in cats include Chediak-Higashi syndrome and von Willebrand's disease. Platelet neoplasia and thrombocythaemia is rare in cats; however thrombocytosis is not and may reflect physiological release from splenic and non-splenic pools or a reactive component of diseases, resulting in accelerated haematopoiesis, inflammatory diseases, some types of neoplasia or following splenectomy.

Difficulties in accurately enumerating feline platelets in the laboratory are well known; however, studies of the frequency of errors are lacking. A retrospective study was undertaken to examine the prevalence of low automated platelet counts compared with low blood smear-estimated platelet counts in feline blood samples over a twelve month period in the University of Glasgow Veterinary Haematology Laboratory. Platelet counts by means of an impedance counter (Minos® Vet, Abx Hematologie) were less than 200 x 10^9/l in 71% of samples and in 12% were less than 50 x 10^9/l. However based on estimation of platelet numbers from smears, only 3.1% of samples had platelet counts less than 200 x 10^9/l, with 2.5% being less than 50 x 10^9/l. Four cats with blood smear-estimated thrombocytopenia had clinical signs of a bleeding disorder. Diseases associated with thrombocytopenia included neoplasia, cytotoxic chemotherapy and infectious diseases. There was no evidence that delay due to mailing of samples was associated with lower automated platelet counts than analysis on the day of sampling. The common occurrence of apparent thrombocytopenia in automated platelet counting was attributed to a combination of platelet aggregation, and the impedance method of cell type differentiation by size. It was concluded that vigilance and careful examination of blood smears is required to identify the few cats with true thrombocytopenia.

A further, prospective, study was then performed to determine if the use of an anticoagulant which inhibits platelet activity in human blood samples would reduce the difficulties in feline platelet enumeration associated with platelet aggregation. Blood samples from 51 cats were divided into aliquots and anticoagulated with EDTA, a citrate based anticoagulant containing the platelet inhibitors theophylline, adenosine and dipyridamole (CTAD) and, in 12 cases, citrate solution. Use of CTAD resulted in significantly higher ($P \leq 0.001$) platelet counts yielded by an impedance counter (Minos® Vet, Abx Hematologie), hemocytometer and smear estimation, than were obtained with EDTA. Subjective assessment of the degree of platelet aggregation on smears was significantly reduced ($P < 0.001$) in CTAD. Although often similar, automated and smear estimates of platelet counts were significantly higher ($P < 0.05$), and the degree of platelet aggregation

significantly lower ($P < 0.05$) in CTAD than in citrate. This suggests that the platelet inhibitory activity of the CTAD, leading to reductions in platelet aggregation, was responsible for this effect. Automated total white cell counts in CTAD anticoagulated samples were significantly lower ($P < 0.001$) than automated counts in EDTA anticoagulated samples, and were similar to manual white cell counts in EDTA anticoagulated samples. Changes in both platelet and white cell counts in CTAD were clinically relevant. Mean platelet volume (MPV) and mean red cell volume (MCV) were significantly lower ($P < 0.05$) in CTAD than in EDTA. Although a statistically significant increase in red cell counts was seen with the use of CTAD compared with EDTA, the difference was not clinically relevant. No effect was seen on cell morphology or staining characteristics. It was concluded that the use of CTAD anticoagulant appears to offer advantages over either EDTA or citrate for feline haematology in avoiding pseudothrombocytopenia and pseudoleukocytosis.

Although CTAD had advantages over the use of EDTA for feline haematological analysis, a disadvantage is that it is a liquid and introduces a dilutional error. In addition, species differences in the response of platelets to antagonists, mean that it may not represent the optimal combination of platelet inhibitors for use in the cat. Alternatives such as a dry anticoagulant containing pyridoxal-5'-phosphate, which has shown promise in samples from human patients, may warrant investigation for use in feline samples. The use of specific techniques to evaluate the degree of platelet activation and aggregation may have advantages over the techniques used in this study in assessing the efficacy of a new anticoagulant for feline haematology. However, many of these techniques suffer from being technically difficult, requiring specialised equipment, requiring large volumes of blood for analysis or requiring sample handling which may itself induce activation or aggregation. The measurement of a new platelet parameter, mean platelet component concentration (MPC) by particular haematology analysers gives a useful assessment of platelet activation in unaggregated platelets, but is only suitable for use with samples collected in EDTA-based anticoagulants.

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PREFACE

Because platelets are such a reactive cell, their study is fraught with technical difficulty. Nevertheless the study of platelet physiology and pathology has great importance in human medicine because of the common occurrence of vascular disease and thrombosis. Despite a great deal of research on platelets, many aspects of platelet function and the pathophysiology of platelet disease remain unknown. Aspects of platelet function and disease specific to many species are less well studied than in man. Platelet studies in the cat are complicated by the propensity for aggregation which is a feature of this species.

This study was prompted by the difficulties experienced in commercial haematology laboratories, in gaining an accurate platelet count in the cat, when sampling and sample handling were performed under routine clinical conditions using commonly available haematology equipment. Though this difficulty has been noted before, recommended methods to circumvent the problem, such as manual haemocytometer platelet counting, are time consuming for the commercial laboratory. It was also felt that these methods may not be entirely accurate in themselves.

The aims of this study are: to review the literature regarding platelet physiology and platelet disorders in the cat; to document the frequency of occurrence of inaccurate platelet counts in the commercial laboratories using standard procedures; and to investigate the usefulness of an alternative anticoagulant in preventing these difficulties.

The work was performed generous support of the Division of Small Animal Clinical Studies and the University of Glasgow Veterinary Haematology Laboratory, University of Glasgow Veterinary School, Bearsden Road, Glasgow, Scotland G61 1QH, and HemaTechnologies Ltd, Unit 13D/8 Anniesland Village Business Park, Netherton Road, Glasgow, Scotland G13 1EU. The work formed part of a Clinical Scholarship in Small Animal Medicine at the University of Glasgow Veterinary School.

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for statistics; to Professor Max Murray for his enthusiasm and special encouragement in the early stages of the project, without which it may not have gone ahead; and to Professor David Bennett for his encouragement and practical support of the project.

AUTHOR'S DECLARATION

This thesis represents the work of the author except for technical assistance. The literature review and study plan were performed by the author. All data collection and analysis was performed by the author from the laboratory records. All smear estimations of platelet counts and aggregation grade scoring were performed by the author. The anticoagulant tubes for the study comprising Chapter 5 were all prepared by the author.

The author acknowledges the practical assistance of Ronnie Baron and Kenny Williamson, technical staff of the University of Glasgow Veterinary Haematology Laboratory, for performance of full blood counts, morphological assessment of smears and manual platelet and white cell counts.

The author also acknowledges the assistance of the clinical staff of the University of Glasgow Veterinary Hospital who assisted the author in collecting feline blood samples for the study comprising Chapter 5.

Chapters 4 and 5 represent papers that have been accepted for publication in Veterinary Clinical Pathology in 2001.

1. Platelet Anatomy and Physiology

1.1. Overview of the Function of Platelets

Platelets are small non-nucleated cells which circulate in the peripheral blood and have an important role in haemostasis, acting in concert with vascular mechanisms and circulating coagulation factors. They accumulate at the site of vascular injury, binding to each other and to the injured endothelium to form a plug which restricts haemorrhage. Platelet adhesion to damaged endothelium is followed by aggregation with other platelets. Aggregation culminates in platelets losing their individual integrity and becoming enmeshed in fibrin which strengthens and stabilises the haemostatic plug against the force of reperfusion. Factors released from platelets contribute to the vasogenic response to haemorrhage (Kuter, 1991).

Activation of platelets occurs by exposure to a variety of stimulatory substances such as collagen and is amplified by release of factors from activated platelets themselves, endothelial cells, white blood cells and by products of the coagulation cascade. Exposure of binding sites for coagulation proteins during activation of the platelet leads to the formation of procoagulant complexes which accelerate the formation of thrombin.

Control of platelet activity is achieved by endothelial and circulating inhibitory factors and physical properties. Factors produced by endothelial cells which prevent adherence of platelets to normal endothelium and to each other, include prostacyclin (PGI_2), endothelium-derived relaxing factor (EDRF), thrombomodulin and ADPase (Ware and Heistad, 1993). The negative surface charge of both platelets and endothelial cells results in mutual repulsion (Green and Thomas, 1995).

Platelets have additional functions unrelated to haemostasis. It has long been hypothesised that they promote the integrity of the vascular endothelium. Although there are numerous contradictory studies, the evidence suggests that the interactions of platelets and endothelial cells help to maintain the continuity and nonthrombogenicity of the vascular endothelium, but the mechanism remains poorly defined (Bithell, 1993a). Release of factors such as prostaglandins, collagenase, elastase, histamine, serotonin and chemotactins can initiate or contribute to the inflammatory response, however platelets have also been found to have some anti-inflammatory activity (Jain, 1986a). Infliction of endothelial damage coupled with secretion by activated platelets of an endothelial growth factor which stimulates smooth muscle and fibroblast proliferation, is thought to play a role in the development of atherosclerosis in humans (Jain, 1986a).

Platelets also have the ability to phagocytose bacteria, bind endotoxin and they are thought to secrete β-lysin, a bactericidal serum protein (Jain, 1986a). Aggregation of bacteria with platelets

may aid in their clearance from the circulation by the mononuclear phagocytic system (Jain, 1986a). However, detrimental consequences such as disseminated intravascular coagulation (DIC), formation of septic emboli, occlusion of blood vessels, vasculitis and haemorrhage due to thrombocytopenia may result from platelet-bacterial interactions during sepsis (Jain, 1986a). Aggregation of platelets around tumour cells may play a role in tumour metastasis, preventing rapid clearance of tumour cells from the circulation and allowing adherence and penetration of the vascular endothelium (Jain, 1993).

1.2. THROMBOPOIESIS

Platelets are the final cell in a lineage that begins with a self-perpetuating progenitor cell and continues through megakaryoblasts, promegakaryocytes and megakaryocytes (Jain, 1986a) (Figure 1.1). Megakaryocytopoiesis differs from erythropoiesis and granulocytopoiesis in that it involves successive divisions of nuclear material without nuclear or cytoplasmic division (termed endomitosis or nuclear endoreduplication), resulting in precursor cells with a polyploid nuclear DNA content of 4N, 8N, 16N, 32N and sometimes 64N (Jain, 1986a). Cytoplasmic maturation occurs after completion of nuclear division (Bithell, 1993b). There is an increase in cytoplasmic volume (Jain, 1986a), formation of dense and alpha granules and production of platelet specific products such as platelet factor-4 (Kuter, 1991), von Willebrand factor (vWf), thrombospondin, β-thromboglobulin and the vWf receptor protein GPIb (Reagan and Rebar, 1995). Some substances such as fibrinogen are thought to be endocytosed into the cell (Reagan and Rebar, 1995). An extensive demarcation membrane system forms by deep invagination of the plasma membrane (Kuter, 1991) which has luminal openings to the cell surface (Jain, 1986a) and delineates the developing platelets (Burkitt *et al.*, 1993). The external surface of the demarcation membrane system becomes coated with glycoprotein which will form the glycocalyx of the mature platelet (Jain, 1986a).

The mechanism of platelet production from the megakaryocyte has been the subject of some controversy. Currently it is generally accepted that individual platelets are derived by the formation of "proplatelets" and their subsequent fragmentation at sites of demarcation (Jain, 1986a). This process has been observed both *in vivo* and *in vitro* (Choi *et al.*, 1996). Megakaryocytes lying in the subendothelial region of marrow sinusoids extend cytoplasmic projections called proplatelets into the sinusoid (Jain, 1986a), by rupturing the endothelial cell body (Bithell, 1993b). These proplatelet processes then extend into venous sinuses and begin to segment along the demarcation membranes, which then fragment and enter the circulation (Bithell, 1993b). This process is assisted by the platelet and megakaryocyte contractile proteins (Jain, 1986a). Large pieces of megakaryocyte and proplatelet processes may be found in the circulation as well as occasional megakaryocytes (Bithell, 1993b). The main site of thrombopoiesis is the bone marrow, however some megakaryocytes can be found in extramedullary locations such as lungs, spleen, liver, kidneys and heart (Jain, 1986a).

A mature megakaryocyte produces 2000-8000 platelets in a period of 3-12 hours, with the number being proportional to the cytoplasmic volume and ploidy value of the megakaryocyte (Jain, 1986a). Stimulation of thrombopoiesis causes release of platelets from megakaryocytes within 8 hours in humans, and is accompanied by an increase in the average ploidy of the megakaryocytes in the bone marrow within 2 days, and an increase in megakaryocyte numbers within 3-5 days (Kuter, 1991). A cohort of larger platelets enters the circulation increasing mean platelet size temporarily, with mean platelet size returning to normal before maximal changes in precursor megakaryocytes

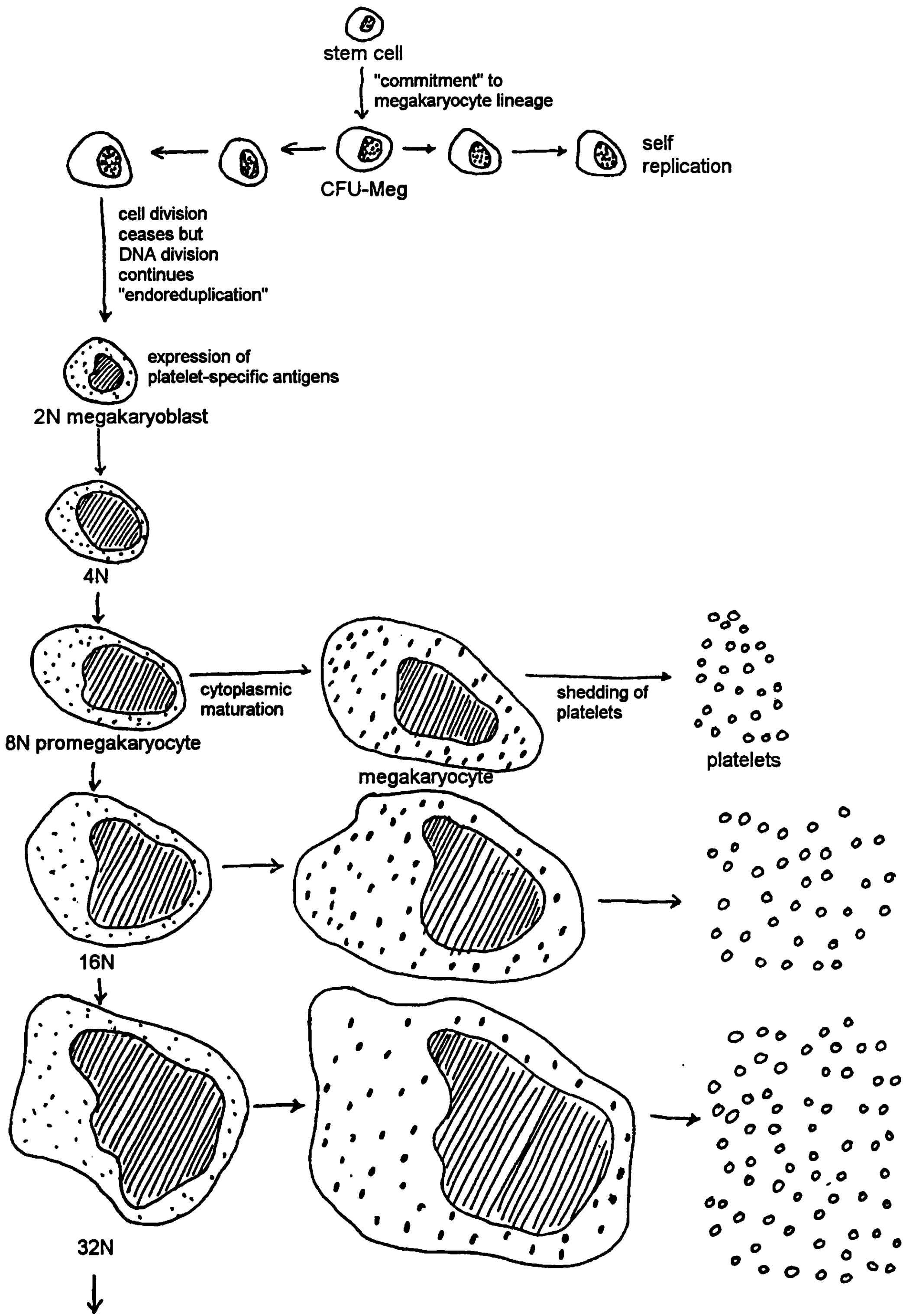

Figure 1.1 *Stages of thrombopoiesis showing progression from stem cell to megakaryocyte and platelet.*

(Bithell, 1993b). Inhibition of thrombopoiesis is accompanied by a decrease in ploidy of megakaryocytes in the bone marrow (Kuter, 1991).

The early stages of megakaryocytopoiesis are stimulated by several cytokines that also stimulate other haemopoietic lineages including: interleukin-3 (IL-3); granulocyte-macrophage (GM)-CSF; c-kit ligand (KL, also called stem cell factor and steel factor) (Kaushansky, 1997). A theoretical late-acting lineage-specific hormone for platelet production was named thrombopoietin in the 1950's (Kaushansky, 1997). A range of physiological properties was predicted based on experimental findings which showed that plasma from thrombocytopenic animals increased thrombopoiesis *in vitro* and *in vivo* and plasma from thrombocytotic animals inhibited thrombopoiesis (Kuter, 1991). These properties are listed in Table 1.1.

- Plasma concentration inversely proportional to platelet mass or numbers
- Causes differentiation of megakaryocyte lineage
- Has no effect on megakaryocyte progenitor cell proliferation
- Increases megakaryocyte size, ploidy and expression of membrane glycoproteins
- Specific to platelet lineage

Table 1.1 *The predicted properties of thrombopoietin (Kaushansky, 1997)*

Recently in the course of murine leukaemia research a protooncogene (c-mpl) was identified which was found to be a member of the haemopoietic cytokine receptor family. This receptor was found in megakaryocytes and their precursors and neoplastic cell lines exhibiting features of megakaryocytes. Purification and study of the receptor ligand (termed Mpl ligand (ML)) has shown it to have nearly all of the predicted properties of thrombopoietin, but also some unexpected properties (Table 1.2) (Kaushansky, 1997). It is likely to be the primary regulator of platelet production (Boudreaux, 1996).

- ML plasma concentration is inversely proportional to platelet counts in humans (with some exceptions)
- Administration of ML to animals increases platelet counts for the duration of treatment
- Increases megakaryocyte size, ploidy and expression of membrane glycoproteins
- ML-induced megakaryocytes have been shown to produce functional platelets
- Induces proliferation of megakaryocyte progenitor cells
- Supports the proliferation of many haemopoietic progenitor cells in combination with other cytokines

Table 1.2 *Properties of Mpl Ligand (ML) (Kaushansky, 1997)*

The site of thrombopoietin formation remains unknown. There is some evidence that the kidney is a source of thrombopoietin or its precursors (Jain, 1986a; Bithell, 1993b). However the expression of thrombopoietin mRNA by several different types of endothelial cells and fibroblasts suggests that the distribution of production may be widespread (Boudreaux, 1996). Platelet mass may be more important than platelet number in regulating thrombopoiesis (Bithell, 1993b; Reagan and Rebar,

1995) however the evidence is inconsistent and the "detector" mechanisms for recognition of platelet mass or number remain undefined (Bithell, 1993b). It has been proposed that thrombopoietin production is constant but its uptake by platelets or megakaryocytes in proportion to their total mass results in inversely proportional circulating concentrations (Boudreaux, 1996). Another theory involves regulation of gene expression by a negative feedback mechanism provided by either platelets or megakaryocytes (Boudreaux, 1996). There is also some evidence to suggest the presence of a thrombopoietic inhibitory factor derived from the spleen (Jain, 1986a; Bithell, 1993b).

About 30-40% of the total platelet mass in humans, dogs and rabbits is sequestered in the spleen and can be rapidly mobilised with adrenalin administration (Jain, 1986a). The splenic pool contains a high proportion of large young platelets (Jain, 1986a; Bithell, 1993b). This splenic pool is not present in all species (for example ponies). Another rapidly mobilisable non-splenic pool of sequestered platelets is released during exercise in dogs and rabbits but does not contain a high proportion of large platelets (Jain, 1986a; Bithell, 1993b). The location of this pool is not yet known, although it is hypothesised to be the lung, and its functional importance is questioned (Bithell, 1993b).

Circulating platelet lifespan ranges from 5 to 10 days in most species (Jain, 1986a), but is much shorter, with a mean of 31 hours in the cat (Reagan and Rebar, 1995). Platelets are cleared by the mononuclear phagocytic system, principally in the spleen (Bithell, 1993b), with liver, heart and bone marrow being additional important sites of clearance in humans and dogs (Jain, 1986a). Whether platelets are removed in an age dependent or random manner remains controversial (Bithell, 1993b). Difficulties in labelling techniques, such as impairment of platelet function or preferential labelling of large platelets, have delayed a definitive answer, and conflicting results to date may also reflect species differences (Bithell, 1993b). The currently prevailing view is that in man, senescence predominates, with random destruction being more important in populations of older platelets (Bithell, 1993b).

Another unresolved issue is the origin of platelet size heterogeneity. Although some studies have shown that large platelets are the youngest platelets and that platelets become smaller and less active with age, other evidence of cohorts of different sized platelets which remain the same size throughout their lifespan, is contradictory (Bithell, 1993b). Existing evidence implies that larger, older megakaryocytes release small platelets and smaller, younger megakaryocytes release large platelets (Bithell, 1993b). This may account for the finding of increased proportions of large platelets in conditions of increased thrombopoiesis (Bithell, 1993b).

1.3. PLATELET STRUCTURE

Feline platelets are larger than those of other species, with a mean volume of 11.0-18.1 fL (Weiser and Kociba, 1984), compared with platelet volumes in the dog, pig and human of 7.6-8.3 fL and in the ox, horse, sheep, rat, guinea pig and mouse of 3.2-5.4 fL (Jain, 1986a). Within an individual animal larger platelets have a greater number of granules, a greater capacity for protein synthesis and a greater amount of many secretory products than smaller platelets and hence are thought to be functionally more active (Jain, 1986a; Bithell, 1993b). Whether the larger size of feline platelets contributes to their propensity to aggregate *in vitro* and for the predisposition to thrombosis in cats is not clear.

Platelet numbers appear to be inversely proportional to platelet size within healthy human individuals, but the relationship is nonlinear (Bessman *et al.*, 1981). The change in platelet size is greatest in the lower range of platelet counts (Bessman *et al.*, 1981). However despite having significantly higher MPVs, a group of 10 Cavalier King Charles Spaniels did not have significantly different manual platelet counts to dogs of other breeds in one study (although automated counts were significantly different) (Brown *et al.*, 1994). Nakeff and Ingram (1970) presented evidence that the relationship between MPV and platelet count is maintained between species, showing that mean platelet volume decreased as the mean platelet count for the species increased, in humans, dogs, rats and mice (see Table 1.3). However, comparison of published reference ranges for size and volume of platelets for a range of species does not appear to reflect this (Table 1.3). Variations in techniques used to determine platelet indices and the dependency of mean platelet volume on platelet number within individuals in a species complicate interpretation of this data.

On stained blood smears feline platelets appear as small pale blue discoid cells with a central cluster of purple granules (Jain, 1986a). They may range in size up to the size of red blood cells (Jain, 1993) and may be clumped together ranging from small groups to large amorphous masses.

Platelets are non-nucleated cells with a complex ultrastructure that can be divided into four functional zones (Jain, 1986a) (Figure 1.2). The peripheral zone consists of the plasma membrane and an outer glycocalyx. The plasma membrane has a phospholipid bilayer structure in common with other cells (Tuffin, 1991). It is punctuated with orifices which are the openings of the open canalicular system (Bithell, 1993b). On the exterior of the plasma membrane is a the thick filamentous glycocalyx, rich in mucopolysaccharides, and containing several types of glycoprotein receptor which play an important role in platelet adhesion, aggregation and signaling (Jain, 1986a; Tuffin, 1991). The plasma membrane differs from that of other cells in that the number of membrane proteins exposed to the external surface is twice as great as the number facing inwards (Burkitt *et al.*, 1993).

Species	Platelet count [‡] (x 10^3/μL)	Mean platelet volume (fL)
mouse	160-410[*] >1000[†] 1302 ± 44[§]	3.3[*] 3.2-5.4[†] 4.02 ± 0.13[§]
rabbit	250-270[*]	3.3[*]
sheep	300-600[*] 250-750[†]	4.4[*] 3.2-5.4[†]
guinea pig	250-850[*]	4.5[*] 3.2-5.4[†]
rat	500-1300[*] 847 ± 36[§]	4.7[*] 3.2-5.4[†] 5.37 ± 0.09[§]
ox	100-800[*] 100-800[†]	5.0[*] 3.2-5.4[†]
horse	120-360[*] 100-350[†]	5.1[*] 3.2-5.4[†]
human	200-400[*] 198 ± 14[§]	6.7[*] 7.6-8.3[†] 7.74 ± 0.22[§]
pig	350-700[*] 100-900[†]	6.9[*] 7.6-8.3[†]
dog	200-900[*] 200-500[†] 380 ± 47[§]	7.5[*] 7.6-8.3[†] 7.17 ± 0.33[§]
cat	300-700[*] 300-800[†] 296-850[#]	12.0[*] 15.1[†] 11.0-18.1[#]

Table 1.3 *Comparison of platelet number and volume of various mammalian species as published by [*] (Poole, 1996), [†] (Jain, 1993) and [§] (Nakeff and Ingram, 1970), and [#] (Weiser and Kociba, 1984).*

[‡] range or mean ± sd

The sol-gel zone consists of the platelet cytoplasm which contains a well developed microfilament and microtubule cytoskeleton. There is a circumferential band of microtubules located around the cell periphery and the cytoplasm is rich in contractile proteins, mainly actin, and other filamentous elements (Tuffin, 1991; Burkitt *et al.*, 1993). These structural proteins help maintain platelet shape, but also enable shape change, pseudopod formation and clot retraction (Jain, 1986a) and probably have a role in extrusion of granule contents (Burkitt *et al.*, 1993).

The organelle zone comprises the other platelet organelles. Platelets retain the enzyme systems for anaerobic and aerobic respiration and contain a few mitochondria and glycogen granules (Burkitt *et al.*, 1993). In some platelets Golgi elements and ribosomes are seen (Jain, 1986a; Burkitt *et al.*, 1993). However, the most conspicuous organelles, occupying about 20% of platelet volume, are the four types of electron dense granules (Burkitt *et al.*, 1993). Alpha granules vary in size and contain several polypeptides such as platelet factor 4; β-thromboglobulin; coagulation factors fibrinogen, factor V and factor VIII/vWf; platelet-derived growth factor; fibronectin; thrombospondin; and other growth factors (Burkitt *et al.*, 1993). Dense bodies or dense granules are very electron dense (Burkitt *et al.*, 1993) and contain adenosine triphosphate (ATP) and diphosphate (ADP), Ca^{2+} and serotonin (McNicol *et al.*, 1993). Lysosomes containing hydrolytic enzymes (McNicol *et al.*, 1993) and peroxisomes containing catalase are few in number (McNicol *et al.*, 1993).

Lastly, platelets contain two membrane systems. The plasma membrane-derived open canalicular system is an intercommunicating system of channels which open on the platelet surface (Jain, 1986a) and are associated with the cytoskeleton (Burkitt *et al.*, 1993). They provide a passage for secretion and internalization of substances (Jain, 1986a). The dense tubular system is found just under the circumferential band of microtubules and contains a platelet specific peroxidase (Burkitt *et al.*, 1993). It is the site of prostaglandin synthesis and calcium sequestration within the platelet (Jain, 1986a).

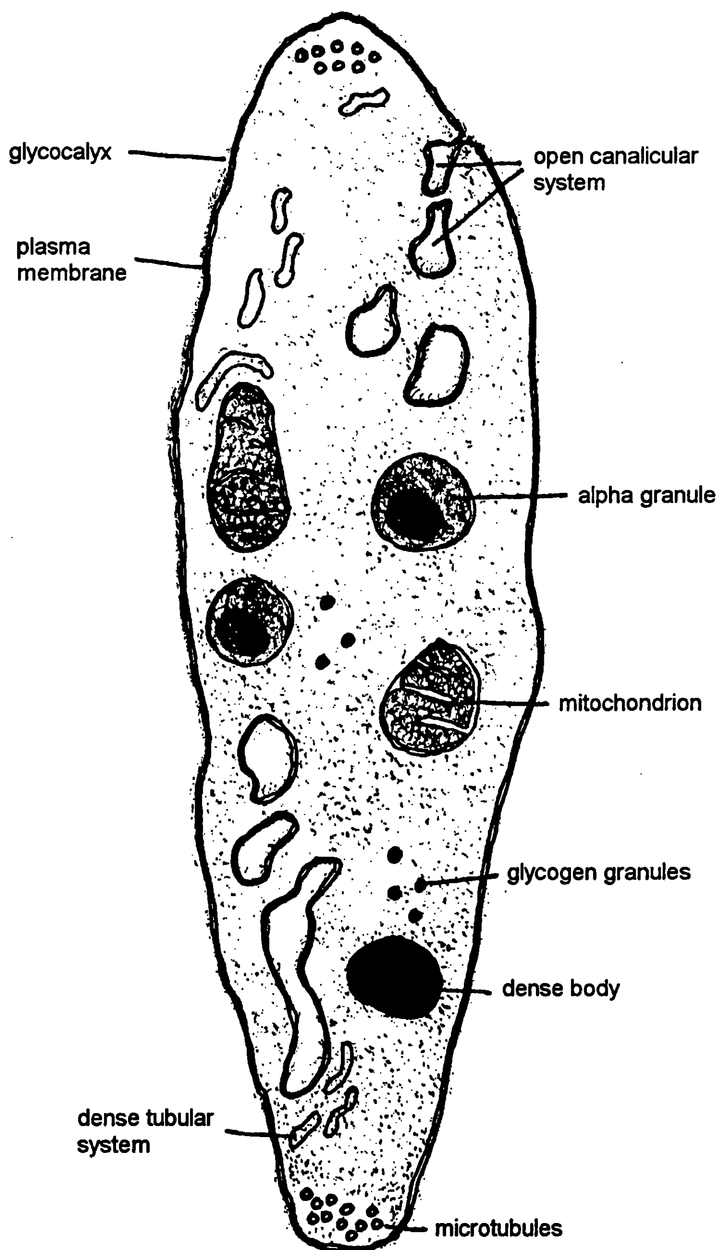

Figure 1.2 *Ultrastructure of platelets as seen on electron micrographs (drawing modified by author based on (Bithell, 1993b)*

1.3.1. Platelet membrane adhesive proteins

Platelet adhesion and aggregation begin with the binding of adhesive proteins to glycoprotein receptors in the platelet plasma membrane-glycocalyx complex. Many of these receptors belong to the integrin family: heterodimeric molecules composed of alpha and beta subunits which are found on virtually all cells. Unique combinations of subunits lead to specificity of the receptor for particular ligands. The nomenclature of integrins has recently changed to reflect their subunit structure (McNicol *et al.*, 1993; Lefkovits *et al.*, 1995). Previously, naming of platelet membrane adhesive proteins was based on the electrophoretic bands formed by glycoproteins of differing molecular weight, with sub-bands being recognised as improved gel systems allowed further discrimination (Plow and Ginsberg, 1991). Whilst the integrin group make up some of these glycoproteins, two non-integrin platelet adhesive glycoproteins are glycoprotein Ib and glycoprotein IV (Lefkovits *et al.*, 1995). There are species differences in the properties and relative abundance of each glycoprotein band (McNicol *et al.*, 1993). Feline platelets lack the glycoprotein I band (Nurden *et al.*, 1977).

The major platelet adhesive proteins are as follows:

- $\alpha_{IIb}\beta_3$ integrin (glycoprotein IIb/IIIa) contains the fibrinogen binding sites critical to platelet aggregation but also has a secondary role in platelet adhesion (Lefkovits *et al.*, 1995). There is more than one binding site for different parts of the fibrinogen molecule. When platelets are activated, a conformational change occurs in the $\alpha_{IIb}\beta_3$ complex to permit fibrinogen binding at these sites. Once fibrinogen binds, additional changes including receptor clustering occur to permit and promote platelet aggregation (McNicol *et al.*, 1993). This receptor also binds fibronectin, vWf, and vitronectin (Lefkovits *et al.*, 1995).
- glycoprotein Ib (nonintegrin) is the principal receptor involved with initial contact between platelets and the vessel wall (Lefkovits *et al.*, 1995). It exists in a complex with glycoprotein IX and glycoprotein V on the platelet surface and binds vWf (Lefkovits *et al.*, 1995). Its cytoplasmic domain is linked to the actin cytoskeleton and this association may be important in maintenance of cell shape and vWf binding (McNicol *et al.*, 1993). The glycoprotein Ibβ subunit is phosphorylated following exposure to agents that increase intracellular concentrations of cyclic adenosine monophosphate (cAMP) and may be responsible for keeping platelets in a resting state (McNicol *et al.*, 1993).
- $\alpha_2\beta_1$ integrin (glycoprotein Ia/IIa) is a collagen receptor which mediates platelet-collagen adhesion (Lefkovits *et al.*, 1995).This appears to be the principal receptor for collagen (Lefkovits *et al.*, 1995).
- $\alpha_v\beta_3$ integrin is a receptor for vitronectin, fibrinogen, fibronectin and vWf which mediates platelet adhesion (Lefkovits *et al.*, 1995).
- $\alpha_5\beta_1$ integrin (glycoprotein Ic/IIa) is a fibronectin receptor which mediates platelet adhesion (Lefkovits *et al.*, 1995). It is also called $\alpha_2\beta_5$ by some authors (McNicol *et al.*, 1993).

- $\alpha_6\beta_1$ integrin is the laminin receptor and may be important for platelet adhesion (Lefkovits *et al.*, 1995). It is also called $\alpha_2\beta_6$ in some references (McNicol *et al.*, 1993).
- glycoprotein IV (nonintegrin) in a receptor for collagen and thrombospondin (Lefkovits *et al.*, 1995).

1.3.2. Platelet factors

Substances involved with coagulation that are associated with platelets are designated platelet factors (PF) 1 to 10; however, all except PF-2, PF-3 and PF-4 are rarely used terms.

- PF-1 is plasma coagulation factor V (Bithell, 1993b).
- PF-2 is fibrinogen activating factor. This is a globulin unique to platelets which may have a proteolytic action on fibrinogen but its physiologic role is still undetermined. (Bithell, 1993b). *In vitro*, it inhibits antithrombin III, induces aggregation and accelerates the rate of thrombin-fibrinogen reaction (Bithell, 1993b).
- PF-3. This thermostable lipoprotein is probably the platelet plasma membrane in an activated or available state (Bithell, 1993b). PF-3 activity is also present in phospholipid derived from erythrocytes, leukocytes and most tissues (Bithell, 1993b; Bithell, 1993c). When coagulation is activated by the extrinsic pathway PF-3 activity is provided by tissue factor (Bithell, 1993c). PF-3 serves to bind and orient coagulation factors to form active complexes. The plasma membrane of activated platelets provides the optimum charge mosaic of phospholipids for this purpose explaining the greater coagulant activity of platelet membranes compared to platelet substitutes (Bithell, 1993b; Bithell, 1993c). PF-3 is required for activation of factor X and the formation of prothrombinase (Bithell, 1993b). Formation of a complex of calcium, PF-3, factor VIII' and factor IXa results in formation of an enzyme capable of activation of factor X (Bithell, 1993c). A complex formed from PF-3, factor V or factor V', factor Xa and calcium is termed prothrombinase and leads to the conversion of prothrombin to active thrombin (Bithell, 1993c). Prothrombinase involves PF-3 that is made available following platelet aggregation and release (Bithell, 1993b).
- PF-4. This glycoprotein is stored in α-granules and only traces are found in plasma (Bithell, 1993b). PF-4 combines with and inactivates the anticoagulant heparin (Bithell, 1993b). It also facilitates platelet aggregation (Bithell, 1993b).
- PF-5 refers to platelet fibrinogen (Bithell, 1993b).
- PF-6 refers to platelet-associated plasmin inhibitor (Bithell, 1993b).
- PF-7 is cothromboplastin (Bithell, 1993b).
- PF-8 is antithromboplastin (Bithell, 1993b).

- PF-9 is accelerator globulin stabilising factor (Bithell, 1993b).

The nature and physiological importance of platelet factors 7, 8 and 9 have yet to be elucidated (Bithell, 1993b).

- PF-10 is serotonin and its functions include vasoconstriction and enhancement of platelet aggregation (Jain, 1986a)

1.3.3. Other platelet proteins

β-thromboglobulin is a low affinity antiheparin protein very close in structure to PF-4 and may be derived from the same precursor molecule (Bithell, 1993b). It is stored in α-granules and upon release binds to endothelial cell membranes, where it inhibits prostacyclin formation (Bithell, 1993b). It may be involved in the pathogenesis of thrombosis (Bithell, 1993b).

Platelet derived growth factor (PDGF) is an important serum mitogenic factor, stimulating growth of endothelial cells, smooth muscle cells, glial cells and fibroblasts, and is also chemotactic for fibroblasts (Bithell, 1993b).

Thrombospondin. This glycoprotein is also known as thrombin-sensitive protein, the endogenous "lectin" of platelets, and glycoprotein G (Bithell, 1993b). It is stored in α-granules and secreted by platelets but also produced by endothelial cells, monocytes and macrophages, smooth muscle cells and fibroblasts (Bithell, 1993b). It binds to the surface glycolipids of activated platelets and is apparently involved with platelet aggregation, fibrin formation and platelet induced haemagglutination (Bithell, 1993b).

1.4. PLATELET ADHESION

Attachment of platelets to nonplatelet surfaces is the first step in blood coagulation (Bithell, 1993a). Platelets do not adhere to endothelial cells themselves. The main site of attachment is collagen fibres deep in the subendothelial layers, but platelets may also adhere to elastic fibres and other non-collagenous substances in interactions that require free calcium (Bithell, 1993a). Platelet adhesion to collagen proceeds in the absence of free calcium and involves specific receptors on the platelet and the collagen, vWf and possibly other "adhesive" proteins (Bithell, 1993a). Physical factors such as rate of blood flow, velocity gradient and red cell concentration also appear to affect platelet adhesion (Bithell, 1993a). In blood vessels such as capillaries in which there is a high shear rate, platelet adhesion increases as PCV rises through a wide range, presumably because increasing numbers of red cells deflect platelets laterally, resulting in increased vessel wall contact (Moore, 1991). The effect of increasing PCV is not as marked in large veins (Moore, 1991).

von Willebrand's factor is a large multimeric molecule composed of many dimeric protomers (Bithell, 1993c). It circulates as a complex with factor VIII, in which the larger vWf acts as a carrier molecule for factor VIII (Bithell, 1993c). It is produced by endothelial cells and megakaryocytes and is present in plasma, on endothelial surfaces, on platelet membranes, and secreted from storage granules by activated platelets (Bithell, 1993c). In cats, a higher percentage (25%) of vWf is expressed in platelets than in dogs (Waters *et al.*, 1989). Large vWf multimers are stored in endothelial cells (Weibel-Palade bodies) and secreted under appropriate stimulation (Bithell, 1993c). Adrenalin, vasopressin, exercise and venous occlusion can all stimulate vWf release (Moore, 1991). Circulating vWf is heterogeneous. Platelet vWf consists of larger multimers than plasma vWf. It may be cleaved by circulating calcium activated proteases (Bithell, 1993c).

There is more than one platelet receptor for vWf and it has been shown that dimeric vWf first binds to platelet membrane glycoprotein Ib and subsequently to glycoprotein IIb-IIIa on the activated platelets (Bithell, 1993a). vWf secreted by platelets binds preferentially to the glycoprotein IIb-IIIa receptor (Bithell, 1993a). Collagen adhesion may preferentially involve larger multimers (Bithell, 1993a).

Fibronectin is another adhesive protein which is synthesised by endothelial cells and present in plasma, connective tissue and platelet granules (Bithell, 1993a). Activated platelets have receptors for fibronectin, which may act as a collagen receptor when bound to platelets and may assist in vWf mediated adhesion but is unlikely to be a major factor in platelet adhesion (Bithell, 1993a).

Platelets may also adhere indirectly to the vascular wall by adhering to leukocytes bound to the endothelium (Ware and Heistad, 1993).

1.5. PLATELET ACTIVATION

Platelet activation begins with the interaction of agonists with platelet receptors [#]. The signal derived from attachment of the agonist is then transferred across the plasma membrane to trigger a series of biochemical changes within the platelet via G proteins on the internal membrane surface (McNicol *et al.*, 1993). These biochemical changes lead to a change in cell shape with extension of projections called pseudopods, binding of fibrinogen to the platelet surface, attachment of platelets in aggregates and secretion of platelet granule constituents (McNicol *et al.*, 1993). At the same time as platelets are activated to form a platelet plug, rearrangement of membrane phospholipids and microvesicle sprouting from the platelet surface promotes blood coagulation (McNicol *et al.*, 1993). Following secretion of granule contents (the release reaction) the platelets begin to lose their individual integrity (Gentry and Downie, 1993). A fibrin mesh is formed which stabilises the platelet plug, first around the periphery of the platelet aggregates and then throughout the platelet plug (Gentry and Downie, 1993; Bithell, 1993a) (Figure 1.3).

1.5.1. Agonist interaction with membrane receptors

A variety of agents have been shown to activate platelets including collagen, thrombin, vWf, fibrinogen, adrenalin, vasopressin, ADP, thromboxane A_2 , PAF and serotonin (Brass, 1991; McNicol *et al.*, 1993). The response to various agonists differs, for example collagen and thrombin initiate granule secretion (and are therefore known as strong agonists) whereas ADP and adrenalin are weak agonists (Brass, 1991). Species differences exist in the responses to various agonists (Table 1.4); however, all animal platelets respond to thrombin and collagen by undergoing shape change, irreversible aggregation and release of dense granule contents (Poole, 1996). Feline platelets differ from those of other domesticated species in that serotonin is a strong platelet agonist (Poole, 1996) and low concentrations of ADP can act as a strong agonist (MacMillan and Sim, 1970). Specific receptors have been isolated for many of these agonists (McNicol *et al.*, 1993). The extracytosolic aspect of these transmembrane proteins forms the agonist binding site. Agents such as adenosine, prostacyclin (PGI_2) and PGD_2, can inhibit platelet activation by acting on specific receptors (McNicol *et al.*, 1993).

The cytosolic aspect of the transmembrane receptor proteins is linked to guanine nucleotide-binding proteins (G proteins). There are two broad categories of G proteins: high molecular weight heterotrimeric G proteins and low molecular weight G proteins (McNicol *et al.*, 1993). The family of heterotrimeric G proteins play a regulatory role in the signal transduction pathways of many cell

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[#] Platelets subjected to elevated levels of fluid shear stress will aggregate in the absence of exogenous agonists. The mechanism involves binding of vWf to glycoprotein Ib which leads to opening of plasma membrane Ca channels, resulting in an influx of extracellular Ca. (Chow *et al.* 1992)

types, mediating the interaction of the receptor to the intracellular effector pathway. Individual G proteins either stimulate or inhibit the effector (Brass, 1991). The recently recognised low molecular weight G proteins also appear to have a role in signal transduction in platelets and some may be involved in secretion of α-granule contents (McNicol *et al.*, 1993).

Species	PAF	AA	serotonin	ADP	ATP	adrenalin
Human	Ir	Ir	R	Ir	Ant	Var
Mouse	X	Ir		Ir		P
Rabbit	Ir	Ir	R	R	Ant	P
Dog		Var	Ir/R	Ir	Ant	Var (Ir, P)
Cat		Ir	Ir	Ir		Ir, P
Sheep			P	Ir		X
Cattle	Ir	SC	P	Ir		X
Horse	Ir	R	R	Ir		X

Table 1.4 *Effect of common agonists on platelets of different species (Poole, 1996).*

Ir = irreversible aggregation, R = reversible aggregation, Ant = antagonist of ADP response, Var = variable response, X = no effect, P = potentiatory action only, SC = shape change only

1.5.2. Activation of cytosolic second messenger pathways

At least three effector molecules are present in the platelet cytoplasm: phospholipase C (phosphoinositide pathway), phospholipase A_2 (eicosanoid or arachidonate pathway) and adenyl cyclase (cAMP pathway) (Brass, 1991).

1.5.2.1. Activation of phospholipase C

Phospholipase C activation by G proteins occurs when agonists such as thrombin, serotonin, vasopressin, PAF and thromboxane A_2 bind to transmembrane receptors (McNicol *et al.*, 1993). Several types of this enzyme have been identified (McNicol *et al.*, 1993). Phospholipase C cleaves phosphatidylinositol biphosphate to form inositol 1,4,5-triphosphate and diglyceride (McNicol *et al.*, 1993).

phosphatidylinositol biphosphate $\xrightarrow{\text{phospholipase C}}$ inositol 1,4,5-triphosphate + diglyceride

Inositol 1,4,5-triphosphate interacts with a receptor on the dense tubular system to release calcium causing a rise in cytosolic calcium concentration (McNicol *et al.*, 1993).

Diglyceride activates protein kinase C (Brass, 1991). Protein kinase C transfers a phosphate group from ATP to a number of platelet proteins leading to pseudopod formation, granule membrane fusion and platelet aggregation (associated with fibrinogen binding) (McNicol *et al.*, 1993). Protein kinase C has also been implicated in platelet secretion (McNicol *et al.*, 1993).

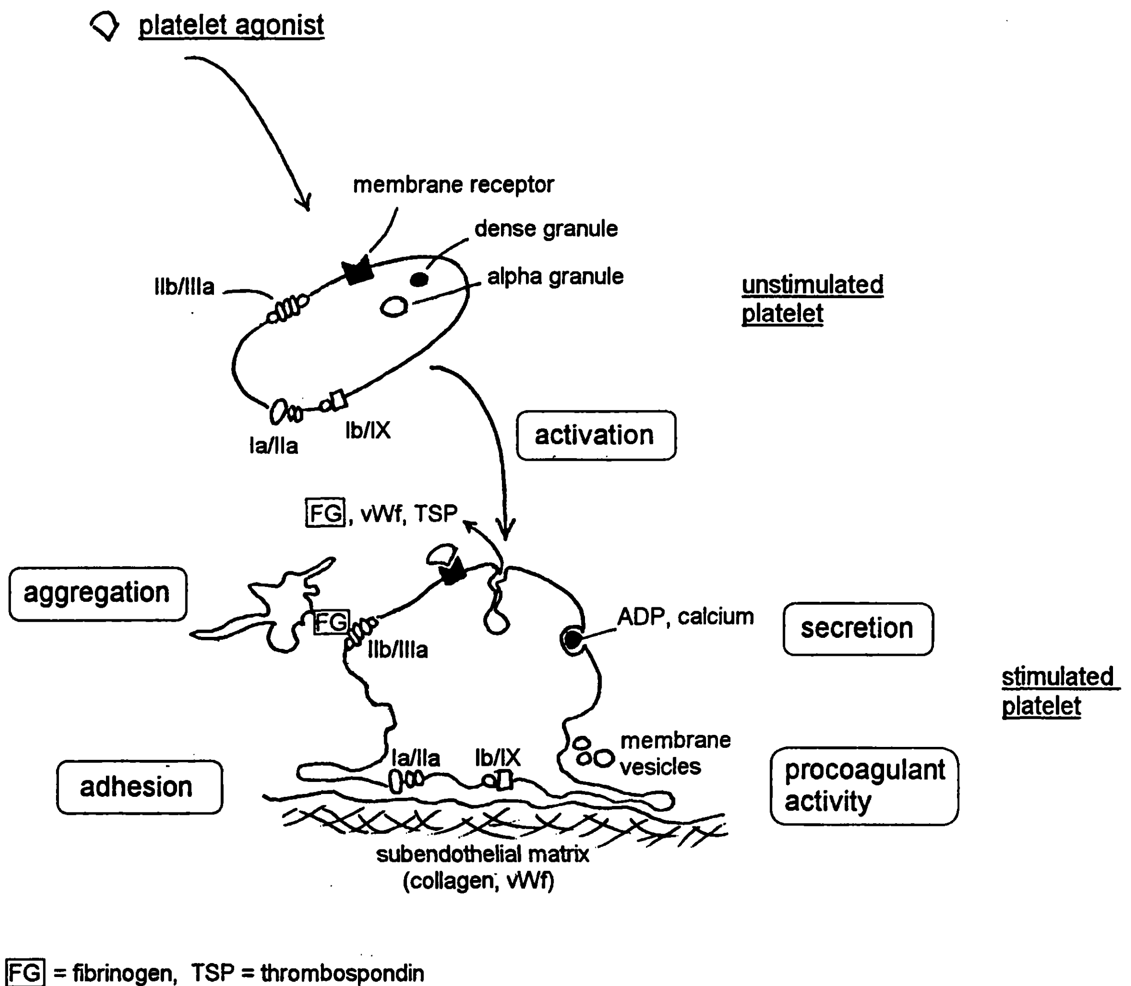
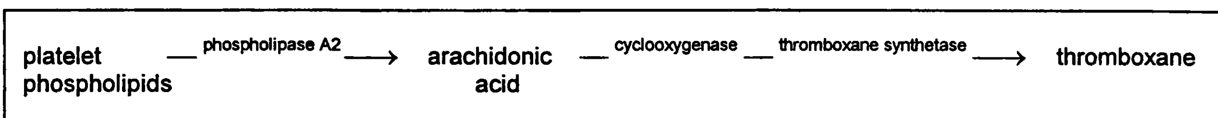


Figure 1.3 *Diagram of platelet activation (Howard and Hamilton, 1997).*

1.5.2.2. Activation of phospholipase A_2

The eicosanoid or arachidonate pathway leads to the synthesis of thromboxane A_2 (TXA_2). Since thromboxane is a platelet agonist this provides a positive feedback loop enabling full platelet activation by agonists such as collagen and adrenalin (McNicol *et al.*, 1993). The arachidonate pathway is well developed in feline, rabbit and human platelets but is rudimentary in equine and bovine platelets (Poole, 1996).



Phospholipase A_2 is activated in the presence of increased cytosolic calcium concentrations (Brass, 1991) but the mechanisms of regulation of its activity are still not fully understood (McNicol *et al.*, 1993). The intracellular availability of arachidonic acid also regulates TXA_2 production (McNicol *et al.*, 1993) and this may be modified by diet in the cat for whom it is an essential fatty acid.

1.5.2.3. Adenyl cyclase

Receptors for adenosine, PGI_2 and PGD_2 are linked via a G protein to the activation of adenyl cyclase (Scrutton and Athayde, 1991; Bithell, 1993a). This enzyme catalyses the synthesis of cAMP, an inhibitor of platelet function, from ATP (Bithell, 1993a). At least two phosphodiesterases in the cytosol degrade cAMP to AMP and it is the balance of this enzyme activity which determines the concentration of cAMP which in the cytosol (Bithell, 1993a). Thus phosphodiesterase inhibitors, such as theophylline and dipyridamole, also lead to increased cAMP concentrations (Bithell, 1993a). Inhibitors of adenyl cyclase include adrenalin, serotonin, thrombin, collagen and PAF (Bithell, 1993a).

Cyclic AMP inhibits shape change and aggregation but its mechanism of action is unclear (Bithell, 1993a). It may act by maintaining the non-active phosphorylated state of various cytosolic proteins including protein kinases and the enzymes phospholamban and thrombolamban which stimulate the calcium pump in the dense tubules (Bithell, 1993a).

Activation of platelet guanyl cyclase by nitric oxide donors such as endothelium-derived relaxing factor (EDRF) leads to elevations in cGMP and inhibition platelet function (Scrutton and Athayde, 1991; Butt and Walter, 1996). When stimulated by other agonists, however, cGMP appears to have excitatory effects on platelets and the details of cGMP activity and its relation to cAMP activity are still poorly understood (Scrutton and Athayde, 1991).

1.5.3. Increased intracytoplasmic calcium concentration

Calcium concentrations in the cytosol of resting platelets are maintained at a low level by a combination of several mechanisms including the limited permeability of the plasma membrane to calcium and the presence of transmembrane calcium pumps which move calcium from the cytosol into the dense tubular system where it is stored, or out of the cell (Brass, 1991).

Upon activation, cytosolic calcium concentrations rise by influx of calcium from the extracellular fluid and from the dense tubular system (Brass, 1991). Inositol 1,4,5-triphosphate interacts with a receptor on the dense tubular system to cause calcium outflow via a Ca Mg-ATPase pump (Brass, 1991). Influx across the plasma membrane is induced by some agonists such as ADP but the mechanisms are poorly understood at present (Sage, 1996).

The rise in cytosolic calcium is central to platelet activation, leading to shape change, aggregation and secretion of granule contents (Sage, 1996), by activation of calcium-dependent enzymes such as phospholipase A, phospholipase C, myosin light-chain kinase and many others (Brass, 1991).

1.5.4. Shape change

On activation, the shape of platelets changes from discoid to more spherical with long and short cytoplasmic extensions or pseudopods (Tuffin, 1991). Shape change is largely due to reorganisation of the cytoskeletal actin filamentous network (Tuffin, 1991) triggered by activation of protein kinase C and/or a rise in cytoplasmic calcium (McNicol *et al.*, 1993). The proportion of filamentous actin increases through polymerisation of actin monomers by mechanisms which are not yet fully elucidated (Tuffin, 1991). Concurrent myosin phosphorylation by myosin light-chain kinase and protein kinase C and then association with actin provides the contractile unit which is anchored to the plasma membrane by attachment (through actin-binding protein) to the glycoprotein Ib/IX complex (Brass, 1991). Contraction of the actin and myosin cytoskeleton also results in centralisation of granules prior to release of their contents (McNicol *et al.*, 1993).

1.5.5. Platelet fibrinogen binding and aggregation

Aggregation of platelets occurs when fibrinogen bridges the glycoprotein IIb/IIIa receptors on adjacent platelets (Figure 1.4). Although it is clear that the glycoprotein IIb/IIIa $(_{IIb}\beta_3)$ complex on the platelet surface is the binding site for fibrinogen, several aspects of the mechanism are not fully understood. Fibrinogen binding sites are unavailable in unstimulated platelets, but activation results in a calcium dependent conformational change in the glycoprotein IIb/IIIa complex which exposes the fibrinogen binding site (Tuffin, 1991). After binding of fibrinogen, clustering of glycoprotein

IIb/IIIa receptors occurs, and it is proposed that this facilitates anchoring of cytoskeletal proteins to the plasma membrane (Tuffin, 1991). The binding of fibrinogen to human platelets is initially reversible, becoming irreversible upon release of granule constituents (Tuffin, 1991) (Figure 1.4).

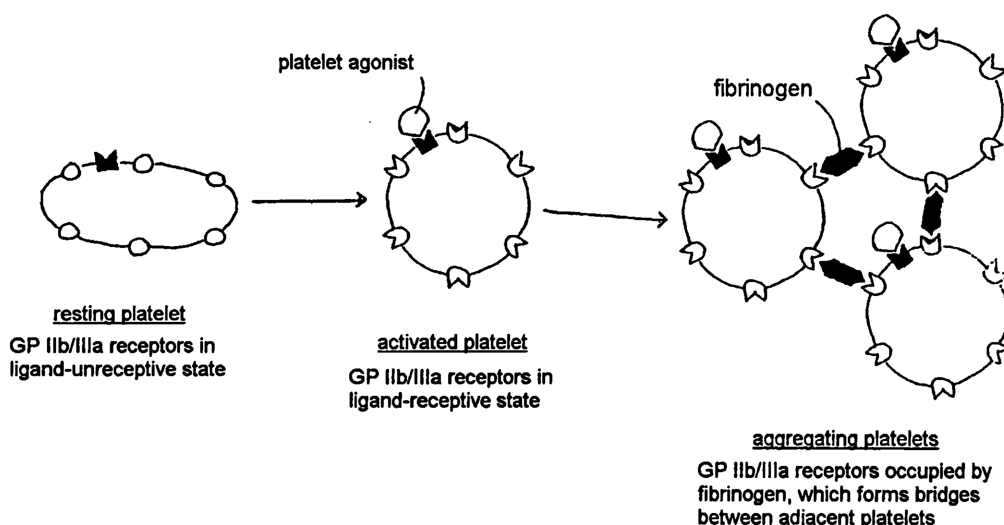


Figure 1.4 *Diagram of platelet-fibrinogen binding to form aggregates (Lefkovits et al., 1995)*

1.5.6. Secretion of platelet granule constituents (release reaction)

Platelet granules are moved to the centre of the platelet by the action of the microtubule and microfilament cytoskeleton (Bithell, 1993a). This process is regulated through calcium, calmodulin and myosin light chain kinase (McNicol *et al.*, 1993). The platelet granule membranes then fuse with the membranes of the open canalicular system resulting in extrusion of their contents to the external environment (Bithell, 1993a). Membrane fusion is driven by a protein kinase C - mediated process which may involve the intracellular synthesis of histamine (McNicol *et al.*, 1993). The contents of dense bodies are secreted first and α-granule contents secreted later, with stronger stimulus. Lysosomal enzyme secretion requires still stronger stimuli (Bithell, 1993a). The release of the aggregatory granule contents recruits other platelets and is required for the formation of large irreversible platelet aggregates and the formation of the insoluble fibrin network (Gentry and Downie, 1993).

1.5.7. Procoagulant response

During activation the platelet surface develops significant procoagulant activity which accelerates the coagulation cascade (Michelson and Shattil, 1996). There is movement of plasma membrane phospholipids from the inner layer to the outer surface of the plasma membrane, shedding of microparticles from the membrane surface and an increase in the number of binding sites for factor Va and factor VIIIa on both the plasma membrane and microparticle surface (Michelson and Shattil, 1996).

1.5.8. Clot retraction

Clot retraction is dependent on the presence of platelets, becoming slow and deficient in severe thrombocytopenia (Bithell, 1993a). Retraction of the fibrin stabilised clot may increase clot strength (Bithell, 1993a), draw wound edges together, and reduce the size of the thrombus to allow blood flow around it (Isenberg and Bainton, 1991) but the physiological significance of this is debated. The effect of clot retraction is greater in fibrin and platelet clots than in whole blood clots, where the presence of red blood cells impedes retraction (Bithell, 1993a). Attachment of platelets to the fibrin mesh occurs at the points where fibrin strands cross (Bithell, 1993a). The mechanism of this attachment is not understood (Bithell, 1993a). Contraction of platelet actin-myosin provides the force for clot retraction and requires thrombin and free calcium (Bithell, 1993a).

1.5.9. Constraints on platelet activation

There are several mechanisms which prevent uncontrolled amplification of aggregatory stimuli. The endothelial cell has an important role in actively degrading aggregatory agonists such as ATP, bradykinin, serotonin and prostaglandins (Bithell, 1993a). The endothelial surface protein thrombomodulin avidly binds excess thrombin rendering it incapable of activating platelets or cleaving fibrinogen (Ware and Heistad, 1993). This interaction leads to the formation of the anticoagulant protein C (Ware and Heistad, 1993). The endothelium also produces heparans which activate antithrombin III (which inactivates thrombin in plasma) (Bithell, 1993a). The presence of heparan anionic groups prevents platelet-endothelial adhesion by promoting the electronegativity of the endothelium, with resulting platelet repulsion (Moore, 1991).

When appropriately stimulated, endothelial cells release prostacyclin (PGI_2) (Bithell, 1993a). Prostacyclin is a vasodilator, potent antagonist of platelet aggregation and also antagonises platelet adhesion (Bithell, 1993a). Its mechanism of action involves stimulation of adenyl cyclase, resulting in increased platelet cAMP concentrations and effects on transmembrane calcium transport

(Moore, 1991). This may lead to inhibition of exposure of fibrinogen binding sites, inhibition of platelet spreading and blocking of the vWf receptor (Bithell, 1993a).

Endothelium-derived relaxing factor (EDRF, or endothelium-derived nitric oxide) is synthesised and released by endothelial cells (Ware and Heistad, 1993). Release of EDRF is stimulated by acetylcholine, histamine, bradykinin, substance P, ADP, ATP and thrombin (Moore, 1991). This nitric oxide stimulates guanyl cyclase in platelets and vascular smooth muscle. Consequent increasing cytosolic GMP results in relaxation of vascular smooth muscle and inhibition of platelet aggregation (Ware and Heistad, 1993), probably by influencing transmembrane calcium transport in a similar manner to cAMP (Moore, 1991). EDRF is inactivated by haemoglobin and hence its effects are likely to be concentrated near the vessel wall (Ware and Heistad, 1993).

Other factors that limit platelet aggregation include rapid inactivation of cyclooxygenase in activated platelets, modulating effects of plasma lipoproteins and 13-hydroxycatadecanoic acid, poorly defined proteins released from leukocytes and endothelium, rapid breakdown of free ADP by plasma adenyl cyclase and hydrogen peroxide production by leukocytes in exudates (Bithell, 1993a). Release of plasminogen-activator inhibitor type-I from activated platelets neutralises the endothelial-derived tissue plasminogen activator (t-PA), which causes platelet aggregation (Ware and Heistad, 1993). High concentrations of ATP, adenosine and serotonin are themselves inhibitory (Bithell, 1993a). In addition, the fact that coagulant enzymes such as thrombin are attached to the surface of activated platelets aids in keeping them within the platelet plug and out of general circulation (Bithell, 1993a).

2. ASSESSMENT OF PLATELET NUMBER

2.1. METHODS OF PLATELET COUNTING

2.1.1. Manual cell counting

2.1.1.1. Haemocytometer

Manual or visual cell counting can be performed using a haemocytometer. This glass counting chamber holds a defined volume of fluid within a marked counting area. Various types are available, the most commonly used are the Neubauer and improved Neubauer. In these the counting area is made up of 9 primary squares each 1 mm x 1 mm which are further subdivided, and the depth of fluid is 0.1 mm. Thus each primary square holds 0.1 μL (Figure 2.1). Phase contrast microscopy enables the platelets to be visualised as refractile bodies. A special thin bottomed (1 mm) counting chamber is best for optimal phase contrast effect (Dacie and Lewis, 1991a). Many veterinary laboratories use brightfield microscopy with field contrast increased by condenser adjustment (Weiser *et al.*, 1989). However the presence of non-platelet particles of a similar size to platelets are difficult to distinguish using this technique, and their presence, such as in leukaemias with high leukocyte counts and leukocyte fragmentation, may mask a concurrent thrombocytopenia (Weiser *et al.*, 1989).

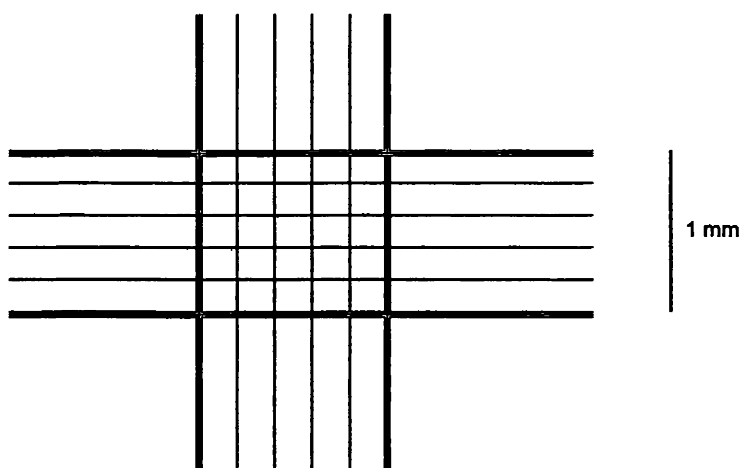


Figure 2.1 *Haemocytometer counting chamber.*

Blood is normally diluted for counting in a haemocytometer to enable cells to be dispersed in a monolayer. Various diluents have been described. Formal-citrate diluent (1% formalin in 32 g/l

trisodium citrate) which is also used for red cell counting, gives incorrectly low platelet counts, because intact red cells obscure platelets (Lewis *et al.*, 1979). The platelet count decreases with increasing red cell count (Lewis *et al.*, 1979). Additionally in cats the large size of platelets makes them more difficult to differentiate from red cells. Ammonium oxalate (1%) lyses red cells, however the resultant debris may be mistaken for platelets (Dacie and Lewis, 1984) and experience and care are needed. Variable degrees of platelet lysis have been associated with its use in bovine blood samples (Maxie, 1977). In cats, the use of this diluent may be associated with increased platelet aggregation (R.J. Barron, pers. com.). Aggregation of platelets may be associated with significant errors as the haemocytometer does not allow large platelet clumps to travel within its 0.1 mm depth and clumps are often seen collecting at the edges of the cover glass. Small platelet clumps may be seen within the counting area but the numbers of platelets within them may be impossible to count accurately.

Although considered by many to be the gold standard for platelet counting, the accuracy of haemocytometer counts is generally less than with automated counts in most species. The coefficient of variation for human platelet counting by the visual method is 8-10% and by automated methods is 3-4% (Dacie and Lewis, 1991a). Inherent errors in manual cell counting arise from the random distribution of cells in the counting chamber and are minimised by counting the cells in a larger area (Dacie and Lewis, 1991a). It is better to count the cells in a second haemocytometer with a second pipetting than to duplicate the count in a single chamber (Dacie and Lewis, 1991a). Technical errors include those arising from poor blood sampling, inadequate sample mixing, inaccurate pipetting, poor filling of haemocytometers and use of equipment of poor accuracy (Dacie and Lewis, 1991a). These can be minimised by careful technique, attention to cleanliness and use of equipment of known accuracy (Dacie and Lewis, 1991a). The use of phase contrast microscopy significantly reduces errors due to the counting of dirt particles as does the use of a commercial prepackaged diluent system (Unopette™, Becton, Dickinson and Company, Franklin Lakes, NJ USA) comprising 1% ammonium oxalate, which presumably contains less particulate matter than solutions prepared in the laboratory (Wertz and Koepke, 1977). However, this commercial system produces greater dilutional errors, resulting in an increased number of falsely low platelet counts (Wertz and Koepke, 1977).

2.1.1.2. Smear estimation

Platelet counts can be estimated from routinely stained smears by counting the number of platelets seen per 100 white cells and multiplying this by the white cell count (per µL) to give the platelet count per µL (Jain, 1986a). Another method involves determining the average number of platelets in 5-10 oil immersion fields in the thin area of the smear (Tvedten, 1994a). A normal platelet count in the cat corresponds to 11-29 platelets per oil immersion field (Weiss, 1984). A third method is to consider each platelet seen per oil immersion field as equivalent to approximately 15 000 platelets

per µL (Tvedten, 1994a). In dogs, smear estimations were found to correlate very highly with cell counts on the Technicon H-1 analyser (Tvedten *et al.*, 1988). However if platelet clumping is present the technique is inaccurate (Tvedten, 1994a). Recently, Tasker *et al.*, (1999) and Tasker and Mackin, (1999) showed that each feline platelet per oil immersion field corresponds to a circulating platelet count of 23.6 x 10^9/L if the field number (FN) of the microscope ocular is 18; 19.1 x 10^9/L if the FN is 20; 15.8 x 10^9/L if the FN is 22; and 10.9 x 10^9/L if the FN is 26.5. The presence of platelet aggregates at the periphery of the smear is taken to indicate adequate platelet numbers for normal haemostasis (Tasker *et al.*, 1999).

2.1.2. Automated cell counting

2.1.2.1. Aperture impedance counters

An aperture impedance method is used in Coulter counters (Beckman Coulter Inc, Fullerton CA, USA), the Sysmex (Sysmex Corporation, Kobe, Japan), the Celloscope (Boule Medical, Stockholm, Sweden), the Minos® Vet (Abx Hematologie, Montpellier, France) and the Cell-Dyn (Abbott Laboratories, Abbott Park, IL, USA) analysers. Blood is diluted in an isotonic electrolyte solution and drawn through an aperture. The magnitude of electrical resistance across the aperture is proportional to the size of the cells passing it and the frequency of changes in resistance indicates the numbers of cells (Tvedten, 1994b). In this type of analyser in which differentiation between platelet and red blood cells is by size alone, the overlap in sizes between normal feline platelets and red cells leads to errors in both counts, as well as to MPV estimates (Zelmanovic and Hetherington, 1998). The magnitude of this error of the platelet count is much greater than for the red blood cells because of the 20-fold difference in relative numbers of each cell type (Mischke *et al.*, 1995). The presence of leukocyte fragments in leukaemias, which may be of a similar size to platelets, results in false high errors in platelet counts, and may mask a concurrent thrombocytopenia (Weiser *et al.*, 1989). Differential leukocyte counting can be achieved by the addition of a high frequency electromagnetic probe (England, 1996).

2.1.2.2. Laser (optical or light scatter) cell counters

Diluted cells pass a focused light source in single file and the resulting scattered light is converted into signals based on the size and internal complexity of cells (Dacie and Lewis, 1991a; Tvedten, 1994b). These analysers include the Technicon H-1 analyser (Bayer Corporation, Tarrytown, NY, USA) and the Ortho ELT analyser (Ortho Diagnostic Systems, Westwood, Massachusetts, USA). Advances such as examining the light scatter from different angles, or measuring light absorption allows the analyser to detect subpopulations of white cells (a differential white cell count) (England, 1996). A platelet histogram is produced allowing detection of platelet size variations (Tvedten,

1994b). Platelet and red cell scattering patterns do not overlap, thereby avoiding the errors produced by impedance analysers (Zelmanovic and Hetherington, 1998). However, since platelet aggregates have a differing light scatter pattern to that of individual platelets, they are not included in the platelet count and hence aggregated blood samples result in inaccurate platelet counts (Zelmanovic and Hetherington, 1998). Analysis of platelet refractive index allows assessment of the state of platelet activation (Zelmanovic and Hetherington, 1998).

It is now possible to combine both aperture impedance and light scatter technologies in the same machine (England, 1996) (Table 2.1).

	red cell and platelet counting channel	**differential white cell channel**
Cell-Dyn	aperture impedance	light scatter
Coulter STKS	aperture impedance	aperture impedance, light scatter and electromagnetic probe
Sysmex	aperture impedance	aperture impedance and high frequency alternating current technology
Technicon H*3RTX	light scatter	light scatter and absorbance

Table 2.1 *Technology used by some of the modern machines for cell counting*

The accuracy of cell counts performed on these instruments is generally much greater than manual counts because of the much greater numbers of cells counted (Kjeldsberg, 1993). However platelet aggregation can produce a significant error in white cell counts and also in the red cell counts of very anaemic cats (Jain, 1986a). Error codes ("flags") produced by automated cell analysers pinpoint those samples in which results may be unreliable or in which there may be abnormal cells present, necessitating smear evaluation for assessment (England, 1996). However in one study (Schrezenmeier *et al.*, 1995) only 29-77% of falsely low human platelet counts triggered an error code in the Coulter T540 and Coulter STKS machines.

2.1.2.3. Quantitative buffy coat analysers

This system involves separation of cells of various sizes in an expanded buffy coat layer by centrifugation in a specialised microhaematocrit tube (Thrall and Weiser, 1992). A cylindrical plastic float is used to expand the lengths of the buffy coat layers to allow their measurement. QBC (Idexx, New York, NY, USA) is an example of this type of analyser. Measurement of the width of various layers of cells gives a partial white cell differential count as well as a red cell count and platelet count. Indistinct band separation is a major technical problem (Tvedten, 1994b) as is variation of cell sizes in disease states and platelet clumping, which alter the relative density of the cells. In addition, large platelet aggregates are trapped above the float and are thus not included in the platelet-crit measurement (Levine *et al.*, 1986). Examination of a blood film can help distinguish these problems. Good accuracy is obtained for red cell counts and white cell counts, but correlation with reference methods for platelet counts is only fair (Tvedten, 1994b).

2.1.2.4. Centrifugal-based laser analysers

The VS2000 (HemaTechnologies Ltd, Glasgow, UK) is a centrifugal-based laser analyser being developed at Glasgow University. It uses a laser to measure the density of the cloud of platelets at the top of the buffy coat of a spinning microhaematocrit tube. Cloud density is correlated with the platelet count (Adams, 1985). Serial measurements are taken along the length of the platelet layer and the resulting curve used to calculate a platelet count. This analyser also operates as a quantitative buffy coat analyser giving an accurate platelet-crit measurement. The laser is used to locate interfaces between air, plasma, platelets, white blood cells, red blood cells and the haematocrit seal and this allows accurate measurement of the lengths of the various layers without the use of a float to expand the buffy coat layers. Changes in the optical density of platelets occur with activation and aggregation (McNicol, 1996) and may be a source of error with this system. In addition, platelet aggregates may be trapped amongst the red cell layer leading to a falsely low platelet count (R.J. Barron, pers. Com.). However the use of this system appears to provide the best available automated method for measurement of platelet-crit in aggregated feline samples at this time (R.J. Barron, pers. com.).

2.1.2.5. Specific function of the Minos® Vet analyser

The measurement principle of the Minos® Vet (Abx Hematologie, Montpellier, France) is based on the variation of impedance caused by the passage of a particle through a calibrated micro-aperture. The blood sample is diluted with an iso-osmotic current-conductive electrolyte solution and then aspirated through the microaperture. As a cell passes through the aperture, the electrical resistance (impedance) between two electrodes placed either side of the aperture, increases in proportion to

the cell's volume. A histogram is generated for analysis by plotting the number of impulses (cell count) versus pulse height (impedance or cell size). Threshold settings and pulse amplification are used to discriminate cells from noise and debris. Coincident passage of cells through the aperture is corrected by an algorithm incorporated into the analysis software (R.J. Barron, pers. com.).

The Minos is a twin-channel analyser. Red cells and platelets are analysed in one channel and white cells in the other. The leukocyte dilution chamber uses a 100μ-diameter aperture. Addition of a red cell lysing reagent to the leukocyte diluting fluid excludes the red cells from the count. The leukocyte lower threshold is fixed around 30 fL. Simultaneously a portion of the leukocyte lysate is diverted into a haemoglobinometer for measurement of free haemoglobin by the cyanmethaemoglobin method at 540nm. The erythrocyte dilution chamber uses a 50μ-diameter aperture. Platelet count is determined concurrently with the erythrocyte count. The platelet lower threshold is fixed at 2 fL. A fixed upper platelet and lower erythrocyte threshold of 17.5 fL is used for cats and 27 fL for dogs, which in most cases cuts the histogram at the trough between the platelets and red cells . These settings have been determined by the manufacturer. While there is no upper threshold for erythrocytes (other than the 50 μ-diameter of the aperture) to discriminate small leukocytes from erythrocytes, the relative numbers of the two cells means that this is not usually a problem (R.J. Barron, pers. com.).

MCV is derived from analysis of the degree of impedance produced by each particle. The relationship between the degree of impedance (pulse height) and the particle size is determined in the factory by use of stabilised human blood. However impedance is influenced not only by the particle size, but also by the particle shape as it passes through the sensing zone. Red cells deform to become fusiform during flow through the aperture. Cell flexibility can vary with red cell membrane defects such as spherocytosis but also varies between species. Therefore a correction factor for each species must be determined on whole blood and is entered into the machine. It is calculated by comparing the analyser calculated packed cell volume (haematocrit, HCT) with the manually measured packed cell volume (PCV) of 30 samples. The PCV of these samples should be approximately 30% in order to minimise the error caused by trapped plasma. Platelet size calibration is not possible since a packed cell volume for platelets cannot be determined. This limits the accuracy of mean platelet volume (MPV) and "platelet-crit" measurements, although they remain useful as comparative values (R.J. Barron, pers. com.).

2.1.2.6. Preparation of platelet rich plasma

A suspension of platelets in plasma, free from other cells, can be prepared by centrifugation or gravity sedimentation of a whole blood sample and aspiration of the uppermost platelet column. Counting of the obtained platelet rich plasma (PRP) solution can then be performed using any method described above. PRP is frequently used in experiments involving platelet counts and there

is considerable agreement between automated and manual counts performed on PRP. However to avoid erythrocyte contamination only the upper part of the PRP is aspirated. Thus the platelet rich plasma obtained will not truly reflect the total platelet count, tending to be lower, than the whole blood platelet count, however the numerical effect of this may be minimised with some techniques (Zelmanovic and Hetherington, 1998) and it has been stated by others that sufficient accuracy should be obtained for clinical purposes (Weiser and Kociba, 1984). Considerable (though not statistically significant) falls in PRP platelet counts were found with time over 3 hours, with platelet aggregation or adherence to glass offered as explanations (Weiser and Kociba, 1984). Hence the problems produced by aggregation of platelets are not entirely overcome by methods using PRP. The use of PRP may also lead to reporting of a falsely low MPV, as large platelets have a greater buoyant density and concentrate at the lower levels of the PRP (Weiser and Kociba, 1984).

Addition of density gradient media to the sample facilitates separation of PRP from erythrocytes using sedimentation or centrifugation techniques (Greene *et al.*, 1985). Successful application using centrifugation has been reported in a range of species, however, the technique was unsuccessful in feline samples because of the similarity in specific gravity of platelets and red blood cells (Greene *et al.*, 1985).

2.2. CHOICE OF ANTICOAGULANTS

Inhibition of coagulation of the blood sample is required to allow examination of the cellular components of blood.

2.2.1. Ethylenediamine tetra-acetic acid (EDTA)

Salts of EDTA are powerful anticoagulants that work by chelating calcium which is required for clotting (Jain, 1986a). EDTA is generally preferred for examination of blood cell morphology (Jain, 1986a), but is unsuitable for use for coagulation tests (Dacie and Lewis, 1991a). The use of dry salts avoids the dilution effects that occur with solutions. The disodium salt is less soluble than the dipotassium salt which is therefore the better of the two, and is commonly used at a concentration of 0.5-2.0 mg/mL of blood (Jain, 1986a). Commercially produced EDTA tubes ensure rapid solution of the EDTA by coating the container with a thin film of the salt.

Despite some contradictory evidence it is generally agreed that platelet swelling occurs in EDTA. This effect appears to be concentration and time dependent and can also be influenced by storage temperature and pH, and variations in these aspects of technique may explain contradictory findings (Waner *et al.*, 1989). At concentrations of 0.15 mg/mL EDTA, canine platelets become spheroidal and develop long pseudopods, causing an increase in the measured MPV (Handagama *et al.*, 1986). Similar changes in MPV have been noted in human samples (Handagama *et al.*, 1986) and are assumed to occur in feline samples (Zelmanovic and Hetherington, 1998). This change from discoid to spherical shape is potentially advantageous, allowing the application of Mie Scattering Theory (which applies to spherical bodies) for analysis of the light scattering data produced by laser cell counters such as the Bayer (Technicon) H*1 analyser (Zelmanovic and Hetherington, 1998). However changes in MPV with EDTA depend on the analyser used; in contrast to the Coulter counter, a decrease in MPV was noted with both the Minos and Bayer (Technicon) H*1 analysers because of the reduced optical density of swollen platelets (Byrne *et al.*, 1994).

Excessive EDTA can cause shrinkage of red and white blood cells, causing degenerative changes (Dacie and Lewis, 1991a) and can result in significant (5-10%) reductions in the PCV of feline blood at concentrations of 10 mg/mL and greater (Penny *et al.*, 1970). This concentration would be achieved by the addition of only 1 mL of blood to a 5 mL EDTA vacutainer (Penny *et al.*, 1970). Excess EDTA also causes platelet swelling and rupture in human blood samples, producing an artificially high platelet count, as thrombocyte fragments are large enough to be included in the platelet count (Dacie and Lewis, 1991a). Hence the importance of accurate filling of blood tubes and thorough mixing of the sample to obtain the correct final concentration.

2.2.2. Heparin

Heparin is a natural tissue anticoagulant which forms complexes with antithrombin III to neutralise the action of thrombin and activated clotting factors XII, XI, IX and X (Jain, 1986a). These effects render it an unsuitable anticoagulant for use in coagulation studies (Jain, 1986a). Although it has the potential advantage of not altering red blood cell volume in excess concentrations, it is not used for examination of blood cell morphology because it interferes with the action of stains on the blood film (Jain, 1986a), and because of its tendency to induce white cell clumping (Dacie and Lewis, 1991a; Kjeldsberg, 1993). Heparinised platelet rich plasma shows a greater tendency to spontaneous aggregation than citrated plasma (Newhouse and Clark, 1978).

2.2.3. Citrate

Salts of citrate prevent coagulation by chelating calcium (Jain, 1986a). Sodium citrate dihydrate is used as a 3.8% solution, in a 1 to 9 part dilution with blood for the study of platelet morphology and coagulation studies (Jain, 1986a; Narayanan, 1995). The optimal concentration of citrate to prevent platelet aggregation may be higher than this. Aggregatory responses of human platelets to collagen, adrenaline, ADP and serotonin which occurred when a final citrate concentration of 0.38% was used, were inhibited when the citrate concentration was increased to 0.64% (O'Brien *et al.*, 1969). Because it introduces a dilutional effect citrate is not generally used for morphological studies of blood cells (Jain, 1986a). Platelets retain their discoid shape when collected into citrate (Handagama *et al.*, 1986; Zelmanovic and Hetherington, 1998).

Citrate is used in combination with dextrose as acid-citrate-dextrose (ACD) and citrate-phosphate-dextrose (CPD) for long term preservation of whole blood and for transfusion purposes (Dacie and Lewis, 1991a).

2.2.4. Oxalate

Salts of oxalate halt coagulation by precipitating calcium in insoluble crystals (Dacie and Lewis, 1991a). Sodium and potassium oxalates are no longer in common use.

2.2.5. Sodium fluoride

Sodium fluoride is used as to preserve concentrations of glucose for biochemical analysis, alone or in combination with other anticoagulants (Colville, 1992). Significant shrinkage of bovine red blood cells was found in sodium fluoride-potassium oxalate anticoagulant rendering samples unsuitable for haematological analysis (Manston *et al.*, 1974).

2.2.6. Anticoagulant-induced pseudothrombocytopenia

For some time EDTA has been known to cause platelet aggregation *in vitro* in a small percentage of human individuals. The mechanism has been shown to involve the presence of antibody (in most cases IgG) and can be induced by mixing the serum of individuals in which it occurs with unaffected donor platelets in the presence of EDTA (Pegels *et al.*, 1982). The antibody binds to the glycoprotein IIb/IIIa complex and it is thought that this leads to platelet activation by mimicking binding of glycoprotein IIb/IIa ligands such as fibrinogen and fibronectin (Schrezenmeier *et al.*, 1995). Although early work demonstrating that EDTA-induced platelet aggregation was inhibited by addition of calcium, suggested that the mechanism involved calcium chelation by EDTA (Onder *et al.*, 1980), this has been disputed by some authors who have demonstrated anticoagulant induced platelet aggregation in agents which do not cause chelation of calcium (Schrezenmeier *et al.*, 1995).

Anticoagulant-induced pseudothrombocytopenia has now been demonstrated using a variety of anticoagulants such as EDTA, heparin, oxalate and citrate (Schrezenmeier *et al.*, 1995). EDTA causes the greatest aggregatory effect in a time dependent and concentration dependent manner, with ACD (acid-citrate-dextrose) having the least effect (Schrezenmeier *et al.*, 1995). Aggregation of the platelets prevents them from being counted by either automated counters or haemocytometer, resulting in a falsely low platelet count, however aggregates are clearly visible on smears in all cases (Schrezenmeier *et al.*, 1995). Concurrent falsely elevated white cell counts (pseudoleukocytosis) may occur using automated counting because the large platelet aggregates mimic leukocytes in size (Schrezenmeier *et al.*, 1995). At present concurrent pseudothrombocytopenia and pseudoleukocytosis has only been demonstrated with the anticoagulant EDTA and may depend on the type of cell counter used (Schrezenmeier *et al.*, 1995). EDTA-induced pseudothrombocytopenia has been documented in the horse (Hinchcliff *et al.*, 1993; Boudreaux, 1996)) and pig (Ragan, 1972), and is thought to occur in the dog and cat (Hodson and Mackin, 1998). Whether this contributes to the propensity for feline blood samples to aggregate *in vitro* is not clear.

Platelet rosetting around neutrophils (termed platelet satellism) is another *in vitro* phenomenon seen in some human blood samples with EDTA as an anticoagulant held at room temperature. In

this case it is thought that EDTA-dependent antibodies link the platelet glycoprotein IIb/IIIa complex and the neutrophil Fc(gamma) receptor. This process may also result in spurious thrombocytopenia (Shahab and Evans, 1998).

2.3. Effects of Storage on Blood

Storage of human blood at room temperature causes progressive red cell swelling, increased osmotic fragility and prothrombin time and falls in leukocyte and platelet counts which can be striking by 12-18 hours (Dacie and Lewis, 1991a). These changes are more marked with EDTA but also occur with other anticoagulants (Dacie and Lewis, 1991a). At 4°C some of these changes are arrested (Dacie and Lewis, 1991a). In cats storage of blood for 48 hours at 4°C had little effect on the PCV when EDTA concentrations were less than 5 mg/mL (Penny *et al.*, 1970). There was a species difference in the effect on PCV of storage at room temperature, with significant red cell swelling noted in dogs after 24 hours and little effect noted in either feline or equine samples after 72 hours (Penny *et al.*, 1970). Storage of canine blood samples at 4°C resulted in progressive development of pseudopods in platelets over 4 hours, an effect that was amplified when EDTA (at 0.15 mg/mL) was used as the anticoagulant (Handagama *et al.*, 1986). Less change was noted during storage at 37 degrees (Handagama *et al.*, 1986). MPV increases with storage time (Weiser and Kociba, 1984). Laboratory experience is that with storage of samples there is a progressive decrease in platelet count and an increase in haemolysis. While not statistically significant, the progressive decrease reported in feline platelet counts over 3 hours at room temperature (Weiser and Kociba, 1984), supports this observation. It is therefore advisable to make a blood film for evaluation as soon as possible after collection (Dacie and Lewis, 1991a).

The ability of platelets to aggregate is affected by plasma pH (Newhouse and Clark, 1978). The aggregatory response to agonists of human platelets is generally inhibited below a pH of 6.4 and above a pH of 10, with the optimum pH being approximately 8 (Newhouse and Clark, 1978). However this is influenced by the concentration and acid-base properties of the aggregating agent employed (Newhouse and Clark, 1978). pH rises with time in blood samples as carbon dioxide diffuses out of solution. Factors influencing this diffusion include the surface area to volume ratio of the sample, whether the sample is capped and mixing of the sample (Newhouse and Clark, 1978). Changes in aggregatory response induced by pH may be due to changes in calcium concentrations, the platelet membrane, the rapidity of ADP catabolism, net surface charge of platelets or changes in cAMP metabolism (Newhouse and Clark, 1978).

As storage temperature of platelets decreases the tendency for spontaneous aggregation increases (Newhouse and Clark, 1978).

3. FELINE PLATELET DISORDERS

The normal platelet count for the cat appears to be somewhere in the range of 300 to 800 x 10^9/L (Jain, 1993). In a study on platelet counts of healthy cats under ketamine anaesthesia (Weiser and Kociba, 1984) found the mean platelet count to be 460 x 10^9/L (range 263-840 x 10^9/L). Age, breed and sex have little effect on platelet count, with only the very young (less than 3 months old) having lower platelet counts than adults (Jain, 1993). Increases in platelet count and PCV are seen following "fright" (Jain, 1986a; Hammer and Couto, 1994) and is thought to be due to adrenalin related contraction of the splenic pool. While the effect of general anaesthesia on platelet count in cats does not appear to have been evaluated, PCV has been shown to decline, presumably due to relaxation of the spleen and splenic pooling (Frankel and Hawkey, 1980). Thus it is likely that the platelet counts in anaesthetised cats will be consistently less than unanaesthetised cats. The unpredictable degree of adrenalin release when sampling conscious cats is likely to result in a wide range of "normal" platelet counts.

Time of analysis after sampling may affect the platelet count due to *in vitro* platelet clumping. In one study substantial reductions in platelet counts in both whole blood and PRP were found over a 3 hour period after sampling (both changing by approximately 100 x 10^9/L as estimated from the graphs), but these were not statistically significant (Weiser and Kociba, 1984).

Spontaneous bleeding is an uncommon clinical sign in the cat, however abnormal coagulation profiles are found relatively frequently in sick cats (Couto and Hammer, 1994). The most common abnormalities in laboratory findings are those consistent with disseminated intravascular coagulation (DIC) and intrinsic coagulation cascade abnormalities (Couto and Hammer, 1994).

3.1. FELINE THROMBOCYTOPENIA

Thrombocytopenia appears to be a rare cause of clinical disease in the cat (Couto and Hammer, 1994). In a survey of 3300 feline blood samples, thrombocytopenia (platelet count less than 200 x 10^9/L) was observed in 41 (1.2%) cases (Jordan *et al.*, 1993). This compares to an incidence of 5.2% in dogs (Grindem *et al.*, 1991). Only 14 of 41 cats with thrombocytopenia had clinical signs of abnormal haemostasis (Jordan *et al.*, 1993).

When present, the most common cause of feline thrombocytopenia is bone marrow failure, with neoplasia and/or retroviral infections commonly implicated (Couto and Hammer, 1994). Sixteen of 41 cats (39%) with thrombocytopenia had at least one neoplasm, and 13 of these were either leukaemia or lymphosarcoma (Jordan *et al.*, 1993). Malignancy is a common cause of thrombocytopenia in the dog, however the pathogenesis is thought to be multifactorial, involving decreased thrombopoiesis, immune mediated destruction and DIC-related consumption of platelets (Helfand, 1988). Acute leukaemias result in thrombocytopenia in more than 90% of human patients and results from megakaryocyte hypoplasia (Athens and Ward, 1993). Moderate thrombocytopenia can also be seen in human chronic leukaemias and multiple myeloma but is unusual in lymphoma and polycythemia vera prior to cytotoxic therapy (Athens and Ward, 1993). Thrombocytopenia is common in dogs with haemopoietic neoplasia (58% of 41 cases) but also occurred in 20% of 59 cases with solid non-haemolymphatic neoplasia (Madewell *et al.*, 1980). Myelophthisic diseases uncommonly result in thrombocytopenia in human patients (in some cases even resulting in thrombocytosis) and the mechanism by which invasion of the marrow by neoplastic cells can impair thrombopoiesis remains unclear (Bithell, 1993d). Proposed mechanisms include competition between tumour cells and myeloid cells for nutrients, occlusion of marrow blood supply by tumour emboli, lysis of marrow cells by adjacent tumour cells and production of inhibitory factors by tumour cells (Helfand, 1988). In a review of 14 dogs with myelofibrosis, platelet and megakaryocyte counts were generally normal to increased despite the presence of a moderate to marked poorly responsive anaemia in most dogs (Hoff *et al.*, 1991). Increasingly, autoimmune destruction of platelets in human patients with haemopoietic and other neoplasms is being recognised as a contributor to thrombocytopenia (Athens and Ward, 1993). Ineffective thrombopoiesis (in which platelet production is reduced despite normal to increased megakaryocyte numbers) and platelet pooling in an enlarged spleen contribute to thrombocytopenia in some cases (Athens and Ward, 1993).

Of 37 thrombocytopenic cats tested 11 (30%) were infected with feline leukaemia virus (FeLV), 3 of these with concurrent haemic neoplasia, and 2 of 13 cats tested (15%) were infected with feline immunodeficiency virus (FIV) (Jordan *et al.*, 1993). Human thrombocytopenia can be associated with various infectious agents such as rubella, live measles vaccination, influenza, septicaemia, malaria and trypanosomiasis (Bithell, 1993d). Mechanisms of viral infection induced thrombocytopenia involve viral invasion of megakaryocytes and platelets, and viral-antibody immune complex development (Bithell, 1993d). The development of mild thrombocytopenia in cats

infected with FeLV is thought to primarily result from the infection of megakaryocytes (Rojko and Hardy, Jr., 1994). Infection of both megakaryocytes and platelets has been demonstrated in FeLV viraemic cats (Boyce *et al.*, 1986). Platelets may become very large due to expansion of megakaryocyte cytoplasm without concomitant increase in the area of demarcation membrane (Boyce *et al.*, 1986). Defects in platelet function have also been noted, but clinical signs of bleeding are uncommon (Kociba, 1986). Immune mediated platelet destruction may contribute to thrombocytopenia. Circulating immune complexes have been demonstrated in some FeLV infected cats and may react with FeLV antigens expressed by platelets (Boyce *et al.*, 1986). The mechanisms of development of thrombocytopenia and other blood cell abnormalities in FIV infected cats remains unclear and are likely to be multifactorial. Although FIV-related RNA can be found in some megakaryocytes and other bone marrow cells (Pedersen and Barlough, 1991), there does not appear to be a direct effect of the virus on blood cell precursors (Shelton *et al.*, 1991). A serum inhibitory factor (possibly antibody) has been demonstrated (Shelton *et al.*, 1991) and immune mediated thrombocytopenia has been reported (Pedersen and Barlough, 1991). Eight to 10% of FIV-positive cats have thrombocytopenia (Shelton *et al.*, 1991; Hart and Nolte, 1994).

Severe thrombocytopenia in FIP can result from vasculitis and development of DIC (Weiss *et al.*, 1980), and it is thought that an early, direct effect of the virus on platelets may contribute to the tendency to enhanced aggregation and thrombocytopenia (Boudreaux *et al.*, 1990a; Boudreaux *et al.*, 1990b). Human thrombocytopenia may be seen in septicaemia in the absence of DIC and may be due to platelet-bacterial interactions, endotoxaemia, platelet interactions with immune complexes and with damaged vascular surfaces (Bithell, 1993d). Parasitism of platelets, immune processes, intravascular coagulation and hypersplenism may all play a role in parasite-induced thrombocytopenia (Bithell, 1993d). Other infections that have been associated with thrombocytopenia in cats include feline panleukopenia, toxoplasmosis and haemobartonellosis (Jordan *et al.*, 1993), cytauxzoonosis (Hoover *et al.*, 1994), ehrlichiosis (Breitschwerdt, 1995) and histoplasmosis (Wolf and Troy, 1995).

Cardiac diseases were found in 4 (9%) of 41 cats with thrombocytopenia and in 3 of these cases there were clinical signs of haemostatic abnormalities (Jordan *et al.*, 1993). The cause of thrombocytopenia may be related to development of DIC (Bonagura, 1994). The platelets of cats with cardiomyopathy show increased *in vitro* aggregation (Welles *et al.*, 1994a).

DIC represents one of the most common causes of abnormalities of haemostatic profiles in the cat, however it is rarely symptomatic (Couto and Hammer, 1994). DIC was diagnosed in 5 (12%) of 41 cats with thrombocytopenia in one study (Jordan *et al.*, 1993) and in another, 21 (21%) of 106 cats undergoing coagulation testing fulfilled all the criteria for diagnosis of DIC (Couto and Hammer, 1994). Neoplasia, hepatic disease or feline infectious peritonitis are present in the majority of cats with DIC (Couto and Hammer, 1994).

Primary immune mediated thrombocytopenia was diagnosed in only 1 of 41 cats with thrombocytopenia (Jordan *et al.*, 1993) and, in contrast to the dog, represents a rare cause of thrombocytopenia in the cat (Couto and Hammer, 1994). Drug induced thrombocytopenias also appear to be uncommon (Couto and Hammer, 1994).

Haemorrhage does not cause significant reductions in platelet count. In haemorrhage platelets are lost along with other blood cells, however significant thrombocytopenias are not produced because the amount of blood that would need to be lost, would result in the death of the animal (Hodson and Mackin, 1998). The exception is if stored blood is used to replace massive blood loss, in which case dilutional thrombocytopenia occurs (Bithell, 1993d). This can be avoided in humans patients by administration of 1 unit of fresh whole blood for every 5 units of stored whole blood (Bithell, 1993d).

3.2. DISORDERS OF PLATELET FUNCTION

Disorders of platelet function are being increasingly recognised in man and may complicate a wide variety of hereditary and acquired diseases (Bithell, 1993e) (Table 3.1). Often however, these have little clinical significance in the absence of concurrent coagulation abnormalities (Bithell, 1993e). The suspicion of a disturbance of platelet function may arise when bleeding time is prolonged, but platelet counts are normal or only mild to moderately decreased (de Gopegui and Feldman, 1998). Abnormalities of platelet size and shape may also suggest a platelet defect. Confirmation requires specific *in vitro* tests of platelet function and immunoassays (Table 3.2) some of which are not commonly available in commercial veterinary laboratories.

Test	Function evaluated
buccal mucosal bleeding time *	*in vivo* platelet function
glass bead retention test *	platelet adherence
clot retraction in response to thrombin *	qualitative and quantitative integrin $\alpha_{IIb}\beta_3$
fibrin and FDP assay #	inhibitors of platelet aggregation
vWf:Ag assay #	platelet adherence
aggregation in response to agonists	platelet aggregation
nucleotide contents	platelet ADP/ATP contents
cAMP	intracellular cAMP content
thromboxane assay	platelet thromboxane B_2 production
protein phosphorylation	protein kinase C activation
serotonin assay	platelet dense granule secretion
β-thromboglobulin assay	platelet alpha granule secretion, *in vivo* platelet activation
β-glucuronidase assay	platelet lysosomal granule secretion
electron microscopy	platelet morphology
glycoprotein electrophoresis	deficiencies of glycoproteins/integrins

Table 3.2 *Platelet function tests (Clemetson, 1996; McNicol, 1996; de Gopegui and Feldman, 1998)*
** Requires a normal number of platelets (Davenport et al., 1982; Day and Rao, 1986).*
 # Because fibrinogen and vWf are essential for normal platelet function, their quantification should precede other platelet function tests (de Gopegui and Feldman, 1998).

Disturbances of platelet function can be the result of abnormalities in any part of the complex physiology of the platelet and particular diseases have increased the understanding of platelet physiology. In other uncommon and poorly documented platelet function disorders the pathophysiology is still being elucidated. Acquired platelet disorders make up the majority of cases of functional disturbance, with drug therapy being the most common of these (de Gopegui and Feldman, 1998). They are also found secondary to many diseases including renal disease, liver disease, dysproteinaemias, infectious processes, immune mediated disease, and DIC (Table 3.1).

3.2.1. Acquired platelet function disorders

3.2.1.1. Drugs

A wide variety of drugs have been associated with impaired platelet function in man (Table 3.1) and many of these agents are known (or are likely) to have effects on feline platelets. Aspirin inhibits platelet release reaction by irreversibly inactivating platelet cyclooxygenase and blocking thromboxane and prostaglandin synthesis for the life of the platelet (Bithell, 1993e). Other non steroidal anti-inflammatory drugs have differing degrees of reversible or irreversible effect on platelet cyclooxygenase (Bithell, 1993e). Inhibition of platelet phosphodiesterase by dipyridamole, papaverine and methyxanthines inhibits aggregation and release reaction (Bithell, 1993e). Nitric oxide donors stimulate adenyl cyclase and guanyl cyclase with the same effect on the platelet (de Gopegui and Feldman, 1998). Calcium channel blockers interfere with signal transduction within the platelet, inhibiting adhesion, aggregation and release reaction but do not prolong bleeding time (Bithell, 1993e). Other drugs may contribute to platelet dysfunction by stabilising mitochondrial and cell membranes, destabilising phospholipid bilayer structure, coating of platelet membranes and altering surface charge, or by unknown mechanisms (Bithell, 1993e).

3.2.1.2. Uraemia

Platelet dysfunction is a clinically important feature of the bleeding diathesis which accompanies human uraemia (Bithell, 1993e). Platelet function abnormalities include deficiencies of release reaction and aggregation (Bithell, 1993e) and it is likely that these changes are the result of the accumulation of several uraemic metabolites (Bithell, 1993e). Low molecular weight peptides with a common amino acid sequence to adhesive proteins bind membrane integrins and impair aggregation (de Gopegui and Feldman, 1998). Acquired storage pool deficiencies may also contribute to platelet dysfunction in uraemia (Bithell, 1993e). Abnormalities in platelet and endothelial prostaglandin production are also present and are thought to be due to another non-dialyzable factor (Bithell, 1993e). There is an increase in endothelial prostacyclin production with concurrent decreased platelet prostaglandin production leading to impaired aggregation (Bithell, 1993e). Treatment with cryoprecipitate or DDAVP can temporarily control bleeding in uraemic patients but the reason for this effect is unclear (Bithell, 1993e). Although there is some evidence of abnormalities of platelet vWf receptors, plasma vWf concentrations and multimer distribution appear to be normal in uraemia (Bithell, 1993e). Response to oestrogens is also not understood (Bithell, 1993e). Prolonged buccal mucosal bleeding times have been reported in uraemic dogs (Jergens *et al.*, 1987), but no abnormalities in platelet aggregation were found using a whole blood impedance method in uraemic dogs (Forsthye *et al.*, 1989). Hart and Nolte, (1991) found no abnormalities in platelet aggregation in two cats with uraemia.

3.2.1.3. Fibrin degradation products

Accumulation of fibrin degradation products (FDP) can impair platelet aggregation *in vitro*, and concentrations of FDP have been correlated with platelet dysfunction in dogs and severity of bleeding in some human patients with DIC and cirrhosis (Bithell, 1993e). However elevated FDP concentrations do not entirely explain the platelet function defects seen in these disorders (Bithell, 1993e). In DIC, storage pool deficiency and direct effects of plasmin may also be involved (Bithell, 1993e). In cirrhosis, acquired deficiency of glycoprotein I (Bithell, 1993e), and increased nitric oxide production from ammonia metabolism (de Gopegui and Feldman, 1998), may also have a role. Reduced platelet aggregation in response to collagen and arachidonic acid has been demonstrated in dogs with naturally occurring liver disease (Willis *et al.*, 1989).

3.2.1.4. Paraproteinaemias and haemopoietic diseases

Platelet dysfunction may occur as part of the spectrum of abnormalities of haemostasis seen with paraproteinaemias in humans (Bithell, 1993e). It is most commonly seen in association with macroglobulinaemia (Bithell, 1993e). The coating of platelets with abnormal proteins is thought to displace normal proteins and interfere with agonist binding (Bithell, 1993e). In human acute myeloblastic leukaemias and preleukaemias, morphological platelet defects are present because of abnormal thrombopoiesis, resulting in deficient nucleotide storage and release reaction, and accompany thrombocytopenia and DIC to complicate the haemorrhagic diathesis (Bithell, 1993e). Chronic myeloproliferative disorders of several types cause platelet dysfunction in humans (see Table 3.1) (Bithell, 1993e). Mechanisms of platelet dysfunction include acquired storage pool disease, PF-3 deficiency and deficient membrane glycoproteins (Bithell, 1993e).

3.2.1.5. Immune mediated thrombocytopenia

Platelet dysfunction accompanying immune mediated thrombocytopenia has been demonstrated in humans and dogs but its clinical significance has not been determined (Bithell, 1993f; Kristensen *et al.*, 1994). There is evidence to suggest that antibody in immune mediated thrombocytopenia may bind the glycoprotein IIb/IIIa complex (Woods *et al.*, 1984; Bithell, 1993f; Curtis *et al.*, 1994) and, less frequently, other membrane glycoproteins (Chong *et al.*, 1991; Clemetson, 1996) manifesting as platelet dysfunction when there is insufficient quantity to result in clearance by the monocyte macrophage phagocytic system (Bithell, 1993f); (Bithell, 1993e). Reversible platelet activation by antibodies may also result in acquired storage pool deficiency (Bithell, 1993f).

3.2.1.6. Diet

Arachidonic acid is an essential fatty acid in the cat and its deficiency results in a mild impairment of aggregation in response to ADP, collagen and arachidonic acid (Callan and Giger, 1997). Supplementation with n-3 fatty acids may moderately inhibit platelet function by decreasing the amount of available arachidonic acid for thromboxane A_2 synthesis (Bithell, 1993e; Hall, 1996), however no change of platelet function could be demonstrated in one study in cats (Bright *et al.*, 1994).

3.2.1.7. Infections

Feline leukaemia virus infection causes a measurable inhibition of platelet aggregation in response to certain agonists but no clinical significant bleeding tendency has been associated with this (Kociba, 1986). No abnormalities in platelet function were demonstrated in one study of FIV infected cats (Hart and Nolte, 1994). Infectious canine diseases such as ehrlichiosis (Harrus *et al.*, 1996) and parvoviral enteritis (de Gopegui and Feldman, 1998) are associated with platelet function defects in addition to thrombocytopenia.

Enhanced platelet aggregatory responses to *in vitro* agonists has been demonstrated in cats infected with feline infectious peritonitis (FIP) virus, which may be a result of a direct viral effect on platelets (Boudreaux *et al.*, 1990a; Boudreaux *et al.*, 1990b). Platelet hyperreactivity has also been demonstrated in the early stages of canine rickettsial infection (Grindem *et al.*, 1990).

3.2.2. Hereditary platelet function disorders

Few hereditary thrombocytopathies have been recognised in the cat. Two hereditary feline diseases which cause abnormalities in platelet function are Chédiak-Higashi syndrome and von Willebrand's disease.

3.2.2.1. Chédiak-Higashi syndrome (CHS)

Chédiak-Higashi syndrome is an autosomal recessive disorder of Persian cats which occurs in several other species including man (Prieur and Collier, 1981; Baldwin and Cowell, 1997). This multisystem disorder results from abnormal granule fusion, with larger and fewer than normal (and perhaps defective) granules formed in most granule-containing cells through the body (Athens, 1993). The abnormal granules may arise from dilated portions of the Golgi-endoplasmic reticulum-

lysosome apparatus, but exactly how they are formed remains unclear (Athens, 1993). Affected organs include haematopoietic tissues, hair, ocular pigment, skin, adrenal glands, pituitary, gastrointestinal tract, peripheral lymph nodes, kidney, vascular endothelium and fibroblasts (Athens, 1993). A consistent feature in various tissues is that the granule contents are that of the normal cell (Athens, 1993).

In cats, CHS is recognised by the obvious pigmentation abnormality including pale blue-smoke (diluted) coat colour, yellow-green irises and altered fundic pigmentation (Prieur *et al.*, 1979; Baldwin and Cowell, 1997). The finding of abnormal granules in neutrophils, eosinophils and hair shafts of affected cats can be used to confirm the diagnosis (Kramer *et al.*, 1977; Prieur *et al.*, 1979).

Chédiak-Higashi syndrome results in a virtual absence of platelet dense granules and their contents; adenine nucleotides, serotonin and calcium (Meyers *et al.*, 1982). It may be classified as a "storage pool disease" in which there are deficiencies of platelet granule contents leading to deficient platelet responses (Baldwin and Cowell, 1997). Deficiencies in platelet aggregatory response to agonists have been demonstrated (Meyers *et al.*, 1982), and affected cats have bleeding tendencies (Prieur *et al.*, 1979) and prolonged buccal mucosal bleeding times (Parker *et al.*, 1988) but platelet numbers are normal (Prieur *et al.*, 1979; Baldwin and Cowell, 1997). Death from acute haemorrhage may occur (Guilford, 1987).

Increased susceptibility to infection because of abnormal neutrophil function is a feature of the disease in humans and mink (Athens, 1993), and neutrophil dysfunction (Guilford, 1987; Colgan *et al.*, 1992) and neutropenia (Prieur and Collier, 1987) have been documented in affected cats. Affected cats have a shortened lifespan with an increased incidence of neonatal septicaemia, viral and chlamydial respiratory infections (Guilford, 1987). In the late stages of human Chédiak-Higashi syndrome lymphadenopathy, hepatosplenomegaly, neuropathy, anaemia, neutropenia and less often, thrombocytopenia occur, with widespread tissue infiltration with mononuclear cells (Athens, 1993), however this phase has not been reported in animals (Kramer *et al.*, 1977).

3.2.2.2. von Willebrand's disease (vWD)

Deficiency of von Willebrand's factor results in platelet dysfunction and illustrates the importance of this factor in platelet adhesion. von Willebrand's disease is an autosomal group of disorders which may be the most common inherited bleeding disorder of dogs and humans (Dodds, 1991; Bithell, 1993g; de Gopegui and Feldman, 1998). However the disease has only been reported in one cat (French *et al.*, 1987). The disease is characterised by subnormal concentrations of vWf, with a resultant deficiency of platelet adhesion to collagen, prolongation of bleeding time and clinical signs relating to defects in primary haemostasis: petechiation, mucosal bleeding and prolonged bleeding

following surgery. A validated assay for feline vWf:Ag concentration is now available (Callan and Giger, 1997) and some authors suspect this may lead to the diagnosis being more commonly made in cats (Dodds, 1991; Callan and Giger, 1997).

3.2.2.3. Other hereditary platelet function disorders

Recently two cats have been reported with a bleeding disorder characterised by defects in platelet aggregation and storage of adenine nucleotides, resembling a storage pool deficiency (Callan and Giger, 1997; de Gopegui and Feldman, 1998).

Table 3.1 *Disorders of platelet function in man (Bithell, 1993e).*

A: Hereditary disorders

Pure platelet dysfunctions	1. Glanzmann's thrombasthenia 2. Deficient release reactions (including storage pool disease, Hermansky-Pudiak syndrome, cyclooxygenase deficiency, others)
Platelet dysfunctions associated with mild-moderate thrombocytopenia	1. Bernard Soulier syndrome 2. Wiskott-Aldrich syndrome 3. 'Thrombopathic' thrombocytopenia 4. 'Montreal' platelet syndrome 5. Gray platelet syndrome
Platelet dysfunction associated with multisystem disease	1. Hereditary afibrinogenaemia 2. Heritable connective tissue disorders (including Marfan syndrome, osteogenesis imperfecta, Ehrlers-Danlos syndrome, mucopolysaccharidoses)

Table 3.1 continued Disorders of platelet function in man (Bithell, 1993e).

B: Acquired disorders

Drugs: (those shown to impair platelet function at therapeutic concentrations)	1. antiinflammatories including aspirin, phenylbutazone, indomethacin 2. antimicrobials including ampicillin, penicillin, carbenicillin, nitrofurantoin 3. antidepressants including amitryptiline, chlorpromazine, cyproheptadine, phenothiazines, reserpine 4. adrenergic blockers including propanolol, phentolamine 5. miscellaneous drugs including antihistamines, amrinone, daunorubicin, dextrans, dipyridamole, ethanol, furosemide, glicazide, heparin, local and general anaesthetics, methylxanthines, radiographic contrast agents, sulfonylureas, ticlopidine, verapamil and other calcium channel blockers, vitamin E
Diseases of the haematopoietic system	1. paraproteinaemias (including macroglobulinaemia, multiple myeloma) 2. acute myeloblastic leukaemias and preleukaemias 3. chronic myeloproliferative disorders (including myelofibrosis, polycythemia vera, chronic myelocytic leukaemia, thrombocythaemia) 4. miscellaneous haematopoietic diseases (including idiopathic thrombocytopenic purpura, β-thalassaemia, sickle cell anaemia, leukaemic reticuloendotheliosis, SLE, haemophilia A, G6PD * deficiency, infectious mononucleosis)
Disorders associated with increased fibrin degradation products (FDP)	1. DIC 2. fibrinogenolysis 3. liver disease
Uraemia	
Miscellaneous	congenital heart disease, following extracorporeal circulation, hypothermia, microangiopathic haemolytic anaemias, congenital agammaglobulinaemia, essential fatty acid deficiency, post renal transplantation, scurvy, some glycogen storage diseases

* glucose-6- phosphate dehydrogenase

3.3. FELINE THROMBOCYTOSIS AND THROMBOCYTHAEMIA

3.3.1. Physiological thrombocytosis

Physiological thrombocytosis occurs transiently after heavy exercise, parturition and adrenalin administration in humans because of mobilisation of the splenic and non splenic pools (Bithell, 1993h). Adrenalin administration or excitement in cats results in physiologic thrombocytosis (Jain, 1986a; Jain, 1993; Hammer and Couto, 1994).

3.3.2. Reactive thrombocytosis

In reactive or secondary thrombocytosis the platelet count increases because of an increase in megakaryocyte mass and is inversely correlated with the mean megakaryocyte volume (Bithell, 1993h). There is a failure of rising platelet numbers to reduce the stimulus for nuclear endoreduplication in megakaryocyte precursors, but in most cases the factors leading to this process remain obscure (Bithell, 1993h).

Human reactive thrombocytosis occurs in conditions of accelerated erythropoiesis such as following acute haemorrhage, haemolytic anaemias and renal neoplasia, suggesting a link between erythropoietic and thrombopoietic control mechanisms (Bithell, 1993h). Mild thrombocytosis is commonly associated with iron deficiency anaemia in humans (Bithell, 1993h). It has been observed in dogs with iron deficiency (Harvey *et al.*, 1982; Jain, 1986a), and in a cat with iron deficiency anaemia (Fulton *et al.*, 1988). An "overshoot" thrombocytosis may be seen following recovery from thrombocytopenias of various types (Helfand, 1988; Bithell, 1993h). Reactive thrombocytosis is associated with several types of human neoplasia such as Hodgkin's disease, breast carcinoma and lung carcinoma (Bithell, 1993h). Three of 59 dogs (5%) with non-haemolymphatic neoplasia were found to have thrombocytosis (2 had osteosarcoma and one had gingival carcinoma) (Madewell *et al.*, 1980). Reactive thrombocytosis is also seen in a variety of human inflammatory disorders such as rheumatoid arthritis, tuberculosis, osteomyelitis, ulcerative colitis and in cirrhosis (Bithell, 1993h). Platelet count has been found to rise and fall during the course of bacterial infection and recovery in various species (Hawkey *et al.*, 1990). Thrombocytosis may be seen in humans following major surgical procedures other than splenectomy (Davis and Mendez Ross, 1973; Jain, 1986a; Bithell, 1993h). It is common during human pregnancy and the puerperium (Bithell, 1993h) and during the growth phase of many species (Hawkey *et al.*, 1990).

Reactive thrombocytosis is common following splenectomy in humans (Bithell, 1993h). The mechanism is unknown, but the degree of thrombocytosis may be massive, more than can be accounted for by circulation of platelets normally held in the splenic pool (Bithell, 1993h). Platelet counts begin to rise 1 to 10 days following splenectomy to peak in 1-3 weeks (Davis and Mendez Ross, 1973; Bithell, 1993h). No abnormality of platelet function has been demonstrated although

platelet serotonin concentrations are decreased (Bithell, 1993h). In most cases platelet count gradually returns to normal but this process may take years in some cases (Davis and Mendez Ross, 1973; Bithell, 1993h). Post splenectomy thrombocytosis is rarely associated with the thromboembolic or haemorrhagic complications which can complicate primary (essential) thrombocythaemia in man (Bithell, 1993h). Reactive thrombocytosis has been reported in the dog following splenectomy (Jain, 1986a), however no consistent changes in canine platelet count were seen 10 days following splenectomy in another study (Richardson and Brown, 1996). Decreased feline platelet counts following splenectomy have been reported (Jain, 1986a), as have increased platelet counts (Lawrence and Valentine, 1947). The incidence of each in cats following splenectomy has not been reported. This may be because disease of the feline spleen appears to be less common than that of the canine spleen. Canine splenic samples were submitted for histopathological examination approximately 4 times more frequently than feline splenic samples over a similar 4 year period to one laboratory (Spangler and Culbertson, 1992a; Spangler and Culbertson, 1992b). Indications for splenectomy in cats include systemic mastocytosis (Liska *et al.*, 1979) and splenic haemangiosarcoma (Scavelli *et al.*, 1985). Thrombocytosis has been observed postoperatively in both conditions (Helfand, 1988).

In a retrospective study of reactive thrombocytosis in dogs and cats neoplasia was the most frequent underlying disorder (Hammer, 1991). However the use of chemotherapeutic agents may have contributed to the thrombocytosis in approximately half of these cases by causing rebound thrombocytosis after myelosuppression, or by the specific effect of vincristine, commonly used as part of the protocol (Hammer, 1991). When this is considered, gastrointestinal disease, mainly inflammatory in nature, was an important cause of reactive thrombocytosis (Hammer, 1991) (see Table 3.3). However, whether surgical diagnostic procedures could have contributed to the thrombocytosis in these cases is not made clear in the study.

Moderate doses of vincristine are thought to increase platelet count through stimulation of thrombopoiesis (Bithell, 1993h; Mackin *et al.*, 1995), although low doses, which result in a prompt transient thrombocytosis have been proposed to induce megakaryocyte fragmentation (Mackin *et al.*, 1995). Vincristine binds to the platelet microtubule cytoskeleton and may lead to concealment of platelets from the thrombopoietic regulatory system or lead to subtle disruption of platelet precursors inducing release of thrombopoietic factors (Mackin *et al.*, 1995). *

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* The increase in platelet count seen with the use of vincristine in ITP, however, is unlikely to be due to accelerated thrombopoiesis, which is already maximal. It is more likely to be attributable to decreased platelet destruction induced by vincristine. Proposed mechanisms include impairment of the monocyte-macrophage phagocytic system, reduced synthesis of antiplatelet antibodies and decreased antibody-platelet binding (Mackin *et al.* 1995).

gastrointestinal diseases 19% of cases	pancreatitis chronic hepatitis gingivitis/periodontitis colitis inflammatory bowel disease gastric ulceration
neoplasia **in absence of chemotherapy** 13% of cases **neoplasia** **with concurrent chemotherapy** 12% of cases	lymphoma melanoma nasal adenocarcinoma primary CNS tumours mast cell tumour mesothelioma
endocrine diseases 10% of cases	diabetes mellitus hyperadrenocorticism hypothyroidism
other diseases 46% of cases	

__Table 3.3__ The most common causes of reactive thrombocytosis in dogs and cats (Hammer, 1991).

3.3.3. Autonomous thrombocytosis or thrombocythaemia

Autonomous thrombocytosis is called thrombocythaemia (also known as essential, primary, haemorrhagic or idiopathic thrombocythaemia) (Bithell, 1993h). It is an uncommon disorder in humans (Bithell, 1993h), and there is a single report of its occurrence in a cat (Hammer *et al.*, 1990). Thought to be a clonal neoplasm, there is an increase in megakaryocyte number, mass and mean megakaryocyte volume, with subsequent increases in platelet count (Bithell, 1993h). Platelet counts are typically massively increased (greater than 1000 x 10^9/L) (Bithell, 1993h) and in the one cat reported ranged between 1 660 and 3 000 x 10^9/L (Hammer *et al.*, 1990). Striking abnormalities in platelet size, shape and structure are commonly seen in human cases, and MPV is usually increased (Bithell, 1993h). Clinical signs of haemorrhage and thrombosis occur in humans and may reflect intrinsic defects in platelet function and hyperaggregability (Bithell, 1993h). Severe gastrointestinal haemorrhage was a feature in the reported cat, but no evidence of thrombosis was found on necropsy (Hammer *et al.*, 1990). Concurrent anaemia may occur in humans and is thought to be due to iron deficiency of chronic blood loss (Bithell, 1993h). In addition there is leukocytosis and splenomegaly in most cases (Bithell, 1993h).

Other myeloproliferative disorders in humans such as polycythemia vera, chronic myelocytic leukaemia and myelofibrosis can result in thrombocytosis (Bithell, 1993h). Thrombocytosis has been associated with megakaryocytic myelosis and acute megakaryoblastic leukaemia in a cats (Michel *et al.*, 1976; Colbatzky and Hermanns, 1993), and with myelofibrosis in dogs (Hoff *et al.*, 1991). Thrombocytosis does not appear to be a common clinical feature of feline polycythemia vera, though this is an uncommon disease in the cat. Of 32 cats reported with the disease (Reed *et al.*, 1970; Foster and Lothrop, 1988; Swinney *et al.*, 1992; Evans and Caylor, 1995; Hasler and Giger, 1996), only one had a platelet count above the reference range (Swinney *et al.*, 1992).

4. Prevalence of Low Automated Platelet Counts in Cats; Comparison with Prevalence of Thrombocytopenia Based on Blood Smear-Estimation

4.1 Introduction

Thrombocytopenia is uncommon in cats, with a reported prevalence of 1.2% of 3300 cats admitted to a veterinary teaching hospital (Jordan *et al.*, 1993). In only 0.42% (14 cases) were clinical signs of abnormal haemostasis detected (Jordan *et al.*, 1993). However, laboratory results suggesting thrombocytopenia are a common finding when automated cell counters are used. Impedance counters differentiate cells by size alone. However, in cats, red blood cells and platelets overlap in size and hence settings which exclude the red cells from the platelet count, will also exclude a proportion of platelets. An additional problem is caused by *in vitro* aggregation of platelets, which appears to occur readily and frequently in feline blood (Jain, 1986b; Thrall and Weiser, 1992; Zelmanovic and Hetherington, 1998). Aggregation of platelets into large clumps may cause them to be counted as one large cell by impedance cell counters, underestimating the platelet count and falsely increasing the counts of other cell types. Falsely decreased platelet counts occur in laser cell counters as well, because platelet aggregates have a different light scatter pattern than do individual platelets and are not counted as platelets (Zelmanovic and Hetherington, 1998). Aggregation of platelets also interferes with all methods of manual counting.

The frequency of occurrence of low automated platelet counts in feline samples in a diagnostic laboratory setting has not been reported. Laboratory experience at the University of Glasgow Veterinary Haematology Laboratory suggested it was so common, that rarely could an automated platelet count be relied upon. This study was undertaken to retrospectively examine the prevalence of low automated platelet counts compared with low blood smear-estimated platelet counts in feline blood samples over a 12 month period in this laboratory.

4.2 MATERIALS AND METHODS

The records of automated haematology counts for all feline blood samples undergoing full blood count received by the University of Glasgow Veterinary Haematology Laboratory were retrospectively examined for the period from 1/4/97 to 31/3/98. Data were collected from the records, and if necessary, by examination of stored blood films from each sample. When an individual cat was sampled on more than one occasion, the results from the first blood sample were included in the study.

Blood was submitted in EDTA for haematological analysis, using any one of a variety of commercially available EDTA tubes. Samples collected from patients hospitalised at the University of Glasgow Veterinary Hospital (internal samples) were stored at room temperature until analysed within 24 hours (in most cases within 8 hours). Samples also were received from veterinarians elsewhere by first class mail (external samples) and were held at room temperature until analysed on the day of receipt. If clots were seen, or there was grossly inadequate filling of the EDTA tube, such that a disproportionately high concentration of EDTA would be present, this was noted and these samples were excluded from the data in this study.

After thorough mixing of the blood samples on an automated mixer for 10 minutes, a full automated blood count was performed on each sample using an impedance cell counter (Minos® Vet, Abx Hematologie, Montpellier, France) which was maintained and calibrated as recommended by the manufacturer. A full white blood cell differential and smear cytological analysis also was performed on each sample. Thin air dried blood smears made after thorough mixing of the sample were stained with a modified May-Grünwald-Giemsa stain and examined under light microscopy. Platelet numbers were reported to be adequate, either because aggregates were seen or, subjectively, based on the experience of the laboratory technicians. Where the record did not note the results of smear evaluation for platelets, the slides were reexamined by the author for the presence of platelet aggregates. If no aggregates were found, an estimation of platelet count was made by averaging the number of platelets seen in 5 oil fields in the thin monolayer part of the smear. An Olympus BX50 microscope was used with a 100x oil lens, and an ocular with a field number of 22. Mean platelet numbers per oil field were multiplied by a factor of 15.8 to give an approximate count x 10^9/l (Tasker *et al.*, 1999; Tasker and Mackin, 1999).Thrombocytopenia was defined as a platelet count of less than 200 x 10^9/l.

Statistical analyses were performed using Minitab for Windows software (release 10.2, 1994, Minitab Inc., State College, PA). All counts were log transformed and comparisons were made using an unpaired t test. A P value of < 0.05 was considered significant.

4.3 RESULTS

A total of 583 feline blood samples were submitted during this period. Of these the records were incomplete in 14 cases for various reasons including cancellation of the request by the submitting veterinarian. In another 4 samples significant underfilling of the EDTA tube was noted and in 26 samples gross clotting of the sample had occurred, making them unsuitable for further analysis. These samples were excluded from further study leaving a total of 539 samples from 359 cats, comprising 325 internal samples from 227 cats and 214 external samples from 132 cats.

In 256 of 359 cats sampled (71%) the automated platelet counts were indicative of thrombocytopenia (< 200 x 10^9/l) (Figure 4.1). In 43 of 359 cats (12%) platelet counts were severely decreased (< 50 x 10^9/l) and in 7 of 359 cats (1.9%) were < 20 x 10^9/l. Based on evaluation of smears, 11 of 359 cats (3.1%) had platelet counts of < 200 x 10^9/l. Platelet counts were markedly decreased (<50 x 10^9/l) in 9 of 359 cats (2.5%) and in 8 of 359 cats (2.2%) were < 20 x 10^9/l. In all samples with blood smear-estimated thrombocytopenia, the automated platelet count was < 200 x 10^9/l. In only 4 of the 11 samples, was thrombocytopenia noted in the haematology report to the submitting veterinarian.

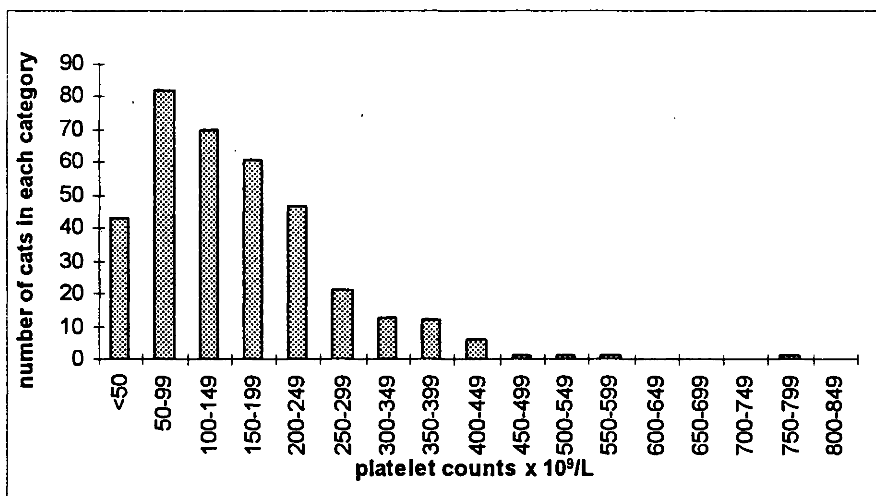


Figure 4.1 Frequency distribution of automated platelet counts from 359 cats during a one year period.

Of the 11 samples with blood smear-estimated thrombocytopenia, 4 cats had histories recorded that suggested a haemostatic defect was present. In all 4 of these cats the platelet count based on both automated count and smear evaluation was < 20 x 10^9/l. One of these 4 cats had pemphigus foliaceus and was being treated with myelosuppressive drugs, one had feline immunodeficiency virus-related disease and in 2 cats an underlying disease was not reported. Of the remaining 7

thrombocytopenic samples, one was from a cat during chemotherapy for lymphosarcoma, one cat had renal neoplasia, one had haemobartonellosis, one had hepatic disease and a positive feline coronaviral titre, and one had intracranial disease of suspected nutritional origin. In two cats no underlying disease was reported.

When external samples were compared with internal samples, there was no significant difference ($P = 0.23$) in the automated platelet counts.

4.4 DISCUSSION

Thrombocytopenia as evidenced by smear examination was present in only 3.1% of cats. Jordan et al. (1993) reported a prevalence of thrombocytopenia of 1.2%. These figures are broadly similar and differences are likely to be due to sampling errors. The study by Jordan et al. (1993) used a much larger sample taken over a 5 year period and, in addition, where haemocytometer counts were not performed, the average of platelet counts in 25 oil fields was used to estimate platelet numbers. This is a much greater number of fields counted than in the present study, and would lessen any inaccuracy resulting from uneven distribution of platelets on the blood smear.

As has been previously reported, neoplasia and infectious diseases are the most common diseases found in cats with thrombocytopenia (Jordan *et al.*, 1993). In some cats, thrombocytopenia may represent a component of disseminated intravascular coagulation (DIC) because 38% of cats undergoing coagulation testing have been found to meet some or all of the diagnostic criteria for DIC (Peterson *et al.*, 1995). No cases of immune mediated thrombocytopenia were diagnosed during this one year survey, in keeping with the low prevalence of this disease in cats (Jordan *et al.*, 1993; Peterson *et al.*, 1995).

Automated platelet counts by use of an impedance counter were low in the majority of cats sampled (71%), whereas the prevalence of thrombocytopenia based on blood smear-estimation was 3.1%. Thus apparent thrombocytopenia represented a significant problem in automated counts with this impedance cell counter. Although the automated counts were low in all cats with blood smear-estimated thrombocytopenia, the frequent occurrence of falsely low automated platelet counts meant that the presence of thrombocytopenia commonly was ignored.

The impedance counting method, in which platelets and red cells are differentiated by size alone, contributes to falsely low automated platelet counts in cats. In the Minos® Vet analyser, red cells and platelets are analysed concurrently in one channel with a 50 μm-diameter aperture. The impedance generated by each particle passing through the sensing zone is plotted against the number of impulses (particles) for analysis. A fixed upper platelet and lower RBC threshold of 17.5 fL for cats has been determined by the manufacturer. In comparison, a threshold of 27 fL is used for dogs. In most cases this threshold cuts the histogram at the trough between the platelets and red cells. However the platelet and red cell histograms are commonly overlapping in cats and this threshold may exclude a proportion of the larger platelets from the platelet count and a proportion of the smaller red cells from the red cell count. Feline platelets are larger than those of other species, with a mean volume of 11.0 to 18.1 fl (Weiser and Kociba, 1984). Mean platelet volume of dogs, pigs and human beings is 7.6-8.3 fl (Jain, 1986c). The magnitude of error in measuring platelet count is much greater than for red cell count because of the difference in relative numbers of platelets and RBCs. Aggregation of platelets increases this effect because the clumped platelets

appear to the cell counter as one larger cell. Although other factors such as red cell microcytosis and schistocytosis will also contribute to this problem, platelet aggregation is a far more frequent occurrence, occurring in at least 50% of feline blood samples analysed during this one year period. Platelet aggregation occurred in 66.6% of blood samples collected from 48 healthy, anaesthetised cats (Zelmanovic and Hetherington, 1998), and in another study, aggregation induced interference was found in 56% of 41 feline blood samples undergoing automated cell counting (Byrne *et al.*, 1994).

The use of optical cell counters in which platelets and red cells are differentiated by their light scattering pattern would be expected to avoid these errors. However the light scattering pattern of a platelet aggregate is not the same as that of a single platelet and hence aggregates are excluded from the platelet count (Zelmanovic and Hetherington, 1998). Manual counting of platelets is also affected by aggregation since individual platelets cannot be counted within aggregates either on smears or in a haemocytometer. The presence of aggregation is likely to lead to uneven distribution of platelets with aggregates accumulating on the edges of smears and counting chambers. Hence aggregation of feline platelets *in vitro* contributes largely to technical difficulties of platelet counting and accurate counting of feline platelets depends on its absence.

Platelets are reactive cells which can be stimulated to aggregate by a variety of factors including substances released from activated platelets themselves, such as adenosine diphosphate (ADP) and serotonin, circulating substances such as adrenalin and vasopressin, extravascular substances such as collagen, products of coagulation cascade such as thrombin, physical factors such as shear stress and stirring, and many foreign substances (Tschopp, 1969; Tuffin, 1991; Scrutton and Athayde, 1991; Brass, 1991; Chow *et al.*, 1992; McNicol *et al.*, 1993). Certain features of feline platelets may result in them being more reactive than those of other species: their larger size (Jain, 1986c), higher concentration of serotonin (Jain, 1986c) and, uniquely among domesticated species, response to serotonin by irreversible platelet aggregation with granule release (Poole, 1996). Irreversible aggregation occurs at lower concentrations of ADP in cat platelets than in other species (MacMillan and Sim, 1970). The small size and imperfectly tractable nature of cats contributes to difficulties in venipuncture which may increase the likelihood of *in vitro* platelet aggregation. Gentle handling of the sample, avoidance of the use of small bore needles for venipuncture and undue negative pressure on the syringe (Poole, 1996), use of siliconised glassware or plastic sample containers (Davenport *et al.*, 1982; McNicol, 1996), and discarding of the first few drops of the sample (McNicol, 1996), have been advocated to reduce aggregation formation. However the problem appears to be unavoidable even in favourable conditions such as under anaesthesia. A simple method to consistently avoid platelet aggregation in cats would be valuable. No significant contribution to platelet aggregation could be attributed to delays and additional handling arising from sample mailing, in this study, with no significant difference ($P > 0.05$) in automated platelet counts between external samples and internal samples.

The findings of this survey demonstrate that while thrombocytopenia is an uncommon occurrence in cats, apparent thrombocytopenia resulting from inability of an impedance counter to accurately quantify platelet counts in feline blood samples is common. This necessitates examination of individual blood smears for the presence of adequate platelet numbers, and vigilance on the part of laboratory workers for the few cats in which thrombocytopenia is actually present. In this study, as is previously reported, thrombocytopenia in cats is most commonly associated with neoplasia, chemotherapy, and infectious diseases.

5. Evaluation of a Citrate Based Anticoagulant with Platelet Inhibitory Activity for Feline Blood Counts

5.1. Introduction

Aggregation of platelets in feline blood samples results in difficulty assessing platelet number by all laboratory means in the majority of feline blood samples (Chapter 4). A citrate based anticoagulant has been developed for use in platelet studies and heparin assays in human patients. CTAD anticoagulant (Diatube-H) contains citrate, theophylline, adenosine and dipyridamole, and is available commercially as 5 ml Vacutainers® (Becton Dickinson, Oxford, UK). The platelet inhibitors in CTAD inhibit platelet activation by maintaining a high intracellular cyclic adenosine monophosphate (cAMP) concentration. Theophylline and dipyridamole inhibit phosphodiesterase degradation of cAMP. Adenosine activates adenyl cyclase, which catalyzes cAMP production from ATP. Dipyridamole prevents uptake of adenosine by red blood cells, and thus a synergistic response is seen when these compounds are combined (Contant *et al.*, 1983; Narayanan, 1995). Cyclic AMP keeps platelets in a resting state by regulating the sequestration of calcium in intracytoplasmic organelles (Vermylen and Deckmyn, 1993).

The use of this combination of platelet inhibitors to prevent platelet activation in vitro has not been reported previously in cats. However, addition of prostaglandin E_1 (PGE_1), an adenyl cyclase activator, to citrate anticoagulant, resulted in significant reduction in feline platelet aggregatory responses to agonists, and reduction in the occurrence of spontaneous platelet aggregation (Welles *et al.*, 1994b). The purpose of this study was to evaluate the use of CTAD in feline haematology, with assessment of its effects on platelet aggregation and any artifacts created in other cells .

5.2. MATERIALS AND METHODS

Aliquots of a commercially available anticoagulant (CTAD) were withdrawn from Vacutainer® collection tubes and used to make up smaller, 1 ml sample tubes. CTAD tubes contain 0.109 M citric acid, 15 mM theophylline, 3.7 mM adenosine, and 0.198 mM dipyridamole with pH adjusted to 5.4. To 1 ml plastic blood collection tubes which contained no anticoagulant, 105 μl of either CTAD or a solution of 32 g/l of trisodium citrate was added. A fill line marked at 1050 μl to give a 9:1 ratio of blood to anticoagulant. Total sample volume of 1050 μl was chosen because a reference mark was already present on the tubes at this point. Tubes were labelled and color coded for ease of identification. Commercially available 1 ml tubes containing potassium EDTA were used.

Blood samples were obtained from cats undergoing blood collection for routine haematology for investigation or monitoring of various diseases at the University of Glasgow Veterinary Hospital. Cats were not selected on the basis of their illness. From each cat, a blood sample collected via jugular venipuncture with a needle and syringe was divided into preprepared CTAD tubes, EDTA and/or citrate tubes. Blood samples were obtained under normal clinical conditions by different operators of varying experience, from cats of differing tractability, and therefore a single clean venipuncture was not always possible. Needle size and syringe size were not standardised. Most samples were obtained without sedation or anaesthesia.

The blood samples from each cat were stored at room temperature until analysis on the same day, usually within 4 hours, at the University of Glasgow Veterinary Haematology Laboratory. Notation was made of inadequate filling of sample tubes or if clots were seen in samples. These samples were excluded from the study. After mechanical mixing for 10 minutes, a blood smear was prepared from each sample, and a full automated blood count was performed which included determination of red blood cell count, haemoglobin concentration, haematocrit, MCV, MCH, MCHC, total white cell count, platelet count, mean platelet volume (MPV), "platelet-crit" and platelet distribution width (PDW). An impedance cell counter (Minos® Vet, Abx Hematologie, Montpellier, France) was used. It was maintained and calibrated as recommended by the manufacturer.

White blood cell differential and smear cytological analysis were performed on each sample by laboratory technical staff. Air dried blood smears, stained with a modified May-Grünwald-Giemsa stain, were examined under light microscopy by the author in a blinded manner for the degree of platelet aggregation. A score was given as follows: aggregates of 30 or more platelets present = 5, aggregates of 20-30 platelets = 4, aggregates of 10-20 platelets = 3, aggregates of 4-8 platelets = 2, few aggregates of 2-4 platelets only = 1, no aggregates found = 0. An estimation of platelet count was made by averaging the number of platelets seen in 10 oil fields in the thin monolayer part of the smear. An Olympus BX50 microscope was used with a 100x oil objective, and an ocular with a field number of 22. Mean platelet numbers per oil field were multiplied by a factor of 15.8 to give an approximate count x 10^9/l (Tasker *et al.*, 1999; Tasker and Mackin, 1999).

Haemocytometer platelet and white blood cell counts were performed on the same day by laboratory technical staff using standard methods. Blood was diluted 1:100 for platelet counting with 1% formalin in 32 g/l trisodium citrate and the number of platelets in five 0.2 x 0.2mm squares were counted. The count was then divided by the volume counted ($0.004 \times 5 \times 10^{-6}$l) and multiplied by the dilution factor (100) to give total platelet count per litre. For white cell counting, blood was diluted 1 in 20 with 2% acetic acid coloured pale violet with gentian violet and the number of white cells in four 1mm^2 squares were counted. The count was then divided by the volume counted ($0.1 \times 4 \times 10^{-6}$l) and multiplied by the dilution factor (20) to give total white cell count per litre. In both techniques, the average count of the two sides of the haemocytometer chamber was recorded.

All counts performed in CTAD or citrate were multiplied by a factor of 1.11 to correct for dilution (MCV, MCH, MCHC, MPV and PDW were not corrected). The difference in values being compared were plotted against their mean. The mean of the difference and the standard deviation of the difference (s_{diff}) were calculated for assessment of agreement between methods (Bland and Altman, 1986). Statistical analyses were performed using Minitab for Windows software (release 10.2, 1994, Minitab Inc., State College, PA). All counts were log transformed and comparisons were made using a Wilcoxon test. A P value of < 0.05 was considered significant.

5.3. RESULTS

Samples were collected from 55 cats but only 51 samples were included in the study. Gross clotting was observed in one citrate sample which was excluded from study but was not observed in any EDTA or CTAD anticoagulated sample. Three other samples were excluded from study because of incorrect filling of blood tubes. In the remaining samples, blood was collected into EDTA and CTAD tubes in 39 cases and into EDTA, CTAD and citrate tubes in 12 cases.

In 36 of 51 (71%) EDTA samples automated platelet counts were less than 200 x 10^9/l. In 16 (31%) samples the platelet count was less than 100 x 10^9/l and in 3 (6%) samples it was less than 50 x 10^9/l. In comparison, in only 12 of 51 (24%) CTAD samples, the automated platelet count was less than 200 x 10^9/l and one was less than 100 x 10^9/l but greater than 50 x 10^9/l. In 6 of 12 (50%) citrated samples the automated platelet count was less than 200 x 10^9/l, in 2 (17%) samples platelet counts were less than 100 x 10^9/l and in one (8%) sample platelet count was less than 50 x 10^9/l. An example of the platelet volume distribution curve produced by the impedance counter is shown in Figure 5.1.

Automated platelet counts were higher in CTAD samples than in their EDTA pairs in 49 of 51 (96%) samples (Figure 5.2). Automated platelet counts were higher in citrate samples than in their EDTA pairs in 10 of 12 (83%) samples. The agreement between automated platelet counts in CTAD samples and their citrate pairs was close for 11 of 12 (92%) samples, however a bias was apparent with the counts being higher in CTAD samples than in their citrate pairs in 11 (92%) of the samples and exactly the same in one of 12.

Platelet counts were significantly higher ($P \leq 0.001$) in samples containing CTAD, than in samples containing EDTA . This relationship persisted whether platelet numbers were assessed by impedance counter, haemocytometer or smear estimation. Mean (± sd) automated platelet count in EDTA was 178 (± 131) x 10^9/l compared with a mean of 316 (± 153) x 10^9/l in CTAD. On examination of smears, platelet aggregation appeared less severe in 45 of 51 (88%) CTAD samples when compared with their corresponding EDTA sample (Figure 5.3), and the difference in aggregation grade of samples between the two anticoagulants was significant ($P < 0.001$). The mean differences ± s_{diff} are tabulated in Table 5.1.

No significant difference ($P \geq 0.05$) was found between citrate and EDTA anticoagulated samples with respect to either automated platelet counts, smear estimates of platelet counts or aggregation grades. For the 12 samples which included a citrated sample, the mean (± sd) automated platelet count in EDTA was 153 (± 79) x 10^9/l compared with a mean of 213 (± 109) x 10^9/l in citrate. Significantly higher automated platelet counts ($P = 0.004$) and smear estimates of platelet counts ($P = 0.045$) were found in CTAD samples than their corresponding citrate pairs, and aggregation grades were significantly lower ($P = 0.014$) in CTAD than in citrate. Platelet aggregation grade was

lower in CTAD samples than in their citrate pairs in 8 of 12 (66%) samples and exactly the same in the remainder (Figure 5.3).

The mean differences (± s_{diff}) of white cell counts are tabulated in Table 5.1. Automated total white cell counts in EDTA were significantly higher (P < 0.001) than those performed in CTAD; however, when white cell counts were performed by haemocytometer, there was no significant difference (P = 0.258) between EDTA and CTAD anticoagulated samples (Figure 5.4). In samples for which all three were available (n = 30), the mean (± sd) automated EDTA white cell count was 17.2 (± 7.1) x 10^9/l, while the mean haemocytometer EDTA white cell count was 12.0 (± 5.7) x 10^9/l and the mean automated CTAD white cell count was 12.7 (± 6.2) x 10^9/l. However haemocytometer white cell counts in EDTA and automated white cell counts in CTAD were significantly different (p = 0.008). In 14 of the 30 cat blood samples the automated EDTA white cell count was higher than the reference interval (>15.5 x 10^9/l); however, in 8 of these, both the haemocytometer EDTA and automated CTAD white cell counts were less than 15.5 x 10^9/l. In only one case was the automated CTAD white cell count above the reference range while the haemocytometer EDTA white cell count was not.

Automated red cell counts in CTAD were significantly different (P < 0.001) when compared with EDTA anticoagulated samples, however the difference was small. The mean (± sd) automated EDTA red cell count was 7.84 (± 1.93) x 10^{12}/l compared with a mean of 8.08 (± 1.97) x 10^{12}/l in CTAD. This was accompanied by a significant difference in MCV between EDTA and CTAD anticoagulated samples, with the mean (± sd) MCV of 42.7 (± 4.3) fl in EDTA samples compared with a mean of 40.9 (± 4.0) fl in CTAD samples (P < 0.001). MCV was also significantly lower (P = 0.009) in citrate samples than in EDTA anticoagulated samples. The mean differences (± s_{diff}) are tabulated in Table 5.1.

Mean platelet volume was significantly lower in CTAD anticoagulated samples compared with EDTA (P < 0.001), mean (± sd) value of 10.5 (± 1.1) fl in EDTA and mean of 10.2 (± 1.0) fl in CTAD. Similarly, MPV of citrate anticoagulated samples was significantly lower (P = 0.008) than EDTA anticoagulated samples, but did not differ significantly (P = 1.000) from CTAD samples. The platelet-crit was significantly higher in CTAD samples than in EDTA (P < 0.001), mean (± sd) of 0.194 (± 0.167) l/l in EDTA and mean of 0.325 (± 0.184) l/l in CTAD. The mean differences (± s_{diff}) are tabulated in Table 5.1.

No difference in cell morphology or staining characteristics was seen with any of the anticoagulants.

Table 5.1 Mean differences and standard deviations of the differences (s_{diff}) for various parameters comparing anticoagulants and techniques. Mean differences approach zero as the agreement between methods of analysis increases. n = number of paired samples for comparison.

parameter and technique	anticoagulant	n	mean difference	s_{diff}
automated platelet count ($\times 10^9$/l)	CTAD - EDTA	51	137.7	113.6
	citrate - EDTA	12	59.7	100.5
	CTAD - citrate	12	73.4	172.8
hemocytometer platelet count ($\times 10^9$/l)	CTAD - EDTA	16	187.3	203.0
smear estimate of platelet count ($\times 10^9$/l)	CTAD - EDTA	51	404.3	358.3
	citrate - EDTA	12	202.5	299.9
	CTAD - citrate	12	227.6	522.5
platelet aggregation grade	CTAD - EDTA	51	-2.47	2.02
	citrate - EDTA	12	-0.83	2.33
	CTAD - citrate	12	-2.50	2.02
MPV (fl)	CTAD - EDTA	51	-0.32	0.56
	citrate - EDTA	11	-0.62	0.48
	CTAD - citrate	11	0.07	0.46
platelet-crit (l/l)	CTAD - EDTA	51	0.131	0.110
	citrate - EDTA	11	0.025	0.015
	CTAD - citrate	11	-0.064	0.088
total white cell count ($\times 10^9$/l)	automated CTAD - automated EDTA	51	-3.26	3.61
	automated EDTA - hemocytometer EDTA	30	5.15	3.90
	automated CTAD - hemocytometer EDTA	30	0.66	2.92
	automated CTAD - hemocytometer CTAD	18	0.10	1.63
RBC ($\times 10^{12}$/l)	CTAD - EDTA	51	0.23	0.29
	citrate - EDTA	12	0.06	0.17
	CTAD - citrate	12	0.13	0.20
MCV (fl)	CTAD - EDTA	51	-1.71	1.54
	citrate - EDTA	12	-1.17	0.83
	CTAD - citrate	12	-0.33	0.78

5.4. Discussion

Regardless of the laboratory method used for assessment, platelet counts were higher when CTAD was used as an anticoagulant compared with the more traditionally used EDTA. In many cases the difference in platelet counts was clinically relevant. Whereas one third of cats had platelet counts apparently low enough to indicate potentially important thrombocytopenic disease (<100 x 10^9/l) in EDTA anticoagulated samples, all but one had platelet counts over 100 x 10^9/l in CTAD. Decrease in the subjective degree of platelet aggregation in CTAD samples suggests that the improvement in platelet counts was a result of decreased platelet aggregation. Significant reductions in human platelet activation have been demonstrated by use of CTAD instead of EDTA. The proportion of activated platelets increases progressively with time in human blood collected in EDTA as measured by expression of membrane glycoproteins which accompany platelet activation. They reach over 30% by 4 hours and 79% at 48 hours. However in blood collected into CTAD, the percentage of activated platelets increases from 7.5% at 4 hours to 10.2% at 24 hours and 16% at 48 hours (Kuhne *et al.*, 1995).

Citrate anticoagulant may itself offer some advantages over EDTA. In another study severe platelet aggregation was seen less frequently in feline blood samples when anticoagulated with citrate, than when anticoagulated with EDTA (Morittz and Hoffmann, 1997). When held at 37 °C, 94% of citrated canine platelets retained their smooth discoid shape after half an hour, whereas only 34% of EDTA platelets retained this shape under the same conditions. 55% of EDTA platelets developed pseudopods and 11% developed both pseudopods and underwent a change in shape from discs to spheres (Handagama *et al.*, 1986). This difference in effect between the two anticoagulants persisted over time and at different storage temperatures. The mechanism for this effect of EDTA on platelets has not been established. Development of pseudopods and spherical transformation are early morphological changes associated with activation of platelets due to reorganisation of the cytoskeletal actin filamentous network (Tuffin, 1991). Hence, it is likely that EDTA itself leads to a degree of platelet activation by mechanisms unknown. Examination of Figure 5.2b reveals a positive bias with most samples having higher automated platelet counts in citrate than in EDTA. This trend is also apparent in the smear estimated platelet counts (not shown), however is only slightly apparent in the subjective assessment of platelet aggregation grade (Figure 5.3.b), in which almost equal numbers of samples have more platelet aggregation in citrate than in EDTA (n = 3) or equal degrees of aggregation (n = 4) as have less platelet aggregation in citrate than in EDTA (n = 5). While no significant differences were found in platelet counts or aggregation scores between EDTA and citrate samples, the possibility that citrate is in fact, associated with less platelet activation than EDTA cannot be rejected because of the low power of the study.

Figure 5.2.c shows that in most samples the automated platelet counts in citrate and CTAD were in close agreement and although there is a statistically significant difference between automated platelet counts in the two anticoagulants, in most samples the difference is unlikely to be clinically relevant. However a consistent bias is seen in Figure 5.2.c with CTAD counts tending to be higher

than those in citrate, and a similar pattern is seen in the smear estimates of platelet count (data not shown). Accordingly, the subjective degree of platelet aggregation was less in CTAD than in citrate as shown by Figure 5.3.c. Although the presence of the outlying citrate result in Figure 5.2.c enlarges the mean difference between the two anticoagulants and influences the statistical conclusions, when this result is removed from the data set, automated counts remain statistically significantly higher and aggregation grades remain significantly lower in CTAD anticoagulant than in citrate. In addition, neither gross clotting, nor severe aggregation rendering the platelet count very low, occurred in CTAD samples during the study, but each occurred once in citrate samples. These findings suggest that the platelet inhibitory properties of CTAD are of clinical relevance and confer an additional advantage to the use of citrate alone in feline blood samples. However further study of a larger number of samples would be desirable to confirm the trends seen here.

A difference between citrate anticoagulant and CTAD is predicted from the actions of the platelet inhibitory constituents of CTAD. The use of CTAD solution resulted in significantly less human platelet activation than citrate solution alone as measured by the degree of heparin inactivation by released platelet factors (Contant *et al.*, 1983). In studies of feline platelets using PGE_1, which also increases platelet cAMP, Welles *et al.* (1994b) found that spontaneous aggregation of feline platelets never occurred in blood samples collected in citrate with added PGE_1, and the platelets became nonreactive to aggregatory agonists whereas aggregation was observed when citrate alone was used. However species variations have been found in the action of various inhibitors of platelet aggregation. Low concentrations of adenosine which inhibit ADP-induced aggregation of human and rabbit platelets do not inhibit feline platelets (Philp and Bishop, 1970). However the concentration of adenosine in CTAD is high, and falls within the range at which inhibition of feline platelet aggregation by adenosine begins to be seen (Tschopp, 1969). Dipyridamole, at concentrations higher than those present in CTAD did not inhibit ADP-induced platelet aggregation in cats (Philp and Bishop, 1970). The relevance of these differences in inhibition of ADP-induced aggregation is unknown. Although ADP has been proposed as a principal nucleotide inducing aggregation in physiological situations (Mustard and Packham, 1970), platelet activation during stirring has been attributed to serotonin as opposed to ADP in the cat (Tschopp, 1969), and hence serotonin may be an important agonist during handling of feline blood samples. Serotonin-induced feline platelet aggregation is inhibited by very low concentrations of adenosine (Tschopp, 1969). Theophylline has been shown to enhance the de-aggregatory action of prostacyclin (PGI_2), an adenyl cyclase activator, *in vivo* in cats (Gryglewski *et al.*, 1978).

Lower automated white cell counts were seen when CTAD was used compared with EDTA. The CTAD white cell counts closely approximated manual EDTA white cell counts in 30 cats in which both were performed, although there was a statistically significant difference between them (Figure 5.4). Because the impedance type analyser differentiates cell types by size alone, white cell counts may be over-estimated when platelet aggregation is present because the size of platelet aggregates approximates that of white cells. The lower white cell counts in CTAD therefore would be expected

to accompany the decrease in platelet aggregation found with this anticoagulant compared with EDTA. Concurrent falsely increased white cell counts (pseudoleukocytosis) and falsely decreased platelet counts (pseudothrombocytopenia) have been demonstrated in human blood samples in EDTA as a result of in vitro platelet aggregation (Savage, 1984; Schrezenmeier *et al.*, 1995) and previously have been noted to occur in feline EDTA blood samples (Byrne *et al.*, 1994; Mischke *et al.*, 1995). The differences in white cell count were clinically relevant. Half of the cats with an automated EDTA white cell count above the reference interval had normal white cell numbers when manually counted and when an automated count was done on CTAD anticoagulated blood. The degree of change in platelet count and white cell count was poorly correlated (data not shown) which may be due to the fact that aggregation was not completely inhibited in all samples, combined with inaccuracies in the platelet counting method.

Changes were observed in red cell parameters with CTAD samples. Although statistically different, the increase in red cell count in CTAD samples is not clinically relevant. Mean red cell volume tended to be lower in both CTAD and citrate samples than in EDTA samples. Swelling of human red cells has been noted with storage at room temperature. Although this change occurs with all anticoagulants, it is greatest with EDTA (Dacie and Lewis, 1991b).

Mean platelet volume was higher in EDTA samples than in corresponding CTAD and citrate samples. Increased MPV detected by impedance counters has been demonstrated in human and canine platelets in EDTA but is not apparent in citrate based anticoagulants (Bull and Zucker, 1965; Holme and Murphy, 1980; Thompson *et al.*, 1983; Handagama *et al.*, 1986). This is attributed to EDTA induced platelet swelling; an increase in the fluid volume with little or no change in dry weight (Holme and Murphy, 1980), the mechanism for which has not been elucidated. Shape change of platelets from discoid to spherical, as occurs during activation, is also associated with an increase in MPV (Holme and Murphy, 1980) and hence any factor which prevents platelet activation may be expected to produce a lower MPV. In a recent study by Zelmanovic and Hetherington (1998) the MPV of unnaggregated platelets in feline EDTA blood samples was lower than the MPV of free, activated platelets in aggregated samples. Thus, it is likely that the platelet inhibitors in CTAD by reduction of platelet activation contributed to the lower MPV in these samples compared with the EDTA samples in this study. However, no statistically significant difference in MPV was found when the two citrate based anticoagulants were compared. Although the sample size is too small to be certain that a difference is unlikely, this suggests that it is the citrate base of the anticoagulant which leads to the change in MPV. The increase in platelet-crit in CTAD samples is an expected consequence of increased platelet count.

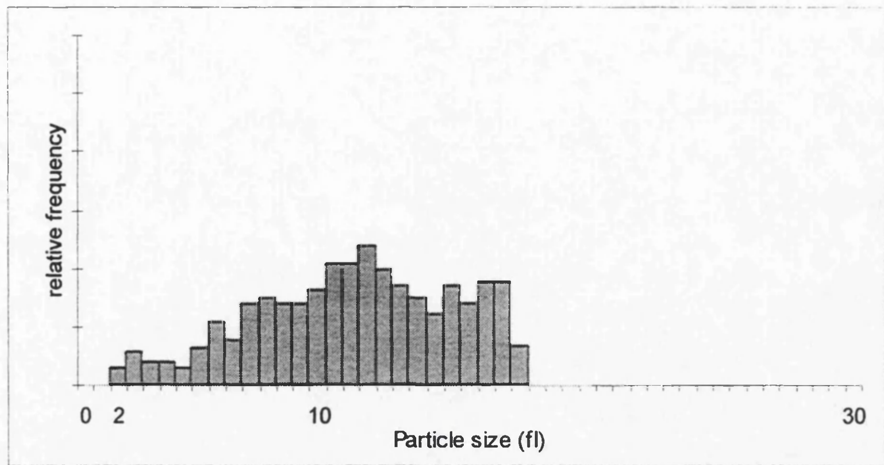
It is not possible to assess how close to the true platelet count the platelet counts in this study may be. In general, automated cell counts are associated with fewer errors than manual ones because a much higher number of cells are counted. The coefficient of variation for human platelet counting by haemocytometer is 8-10% and by automated methods is 3-4% (Dacie and Lewis, 1991c). However,

platelet counts by all methods will be underestimated when platelet aggregation is present. Aggregation interferes with impedance counting by changing perceived cell size, and interferes with optical cell counting because aggregates have a different light scatter pattern than do individual platelets and are not counted as platelets (Zelmanovic and Hetherington, 1998). The 0.1 mm depth of a haemocytometer will exclude large platelet aggregates from the counting area, and clumps are often seen collecting at the edges of the cover glass. Smaller platelet clumps may be seen within the chamber, but the numbers of platelets within them may be impossible to accurately count. Platelet counts estimated from smears are made inaccurate because the distribution of platelets on the smear is not uniform, with clumps accumulating at the sides and the feathered edge of the smear (Tvedten, 1994b). and platelet numbers within aggregates cannot be counted accurately. The overlapping size of feline platelets and erythrocytes causes further inaccuracies in impedance counting. Optical cell counters, which differentiate platelets and red cells by their light scatter pattern, rather than by size alone do not have this difficulty. An automated platelet count performed on unaggregated blood by one of these analysers is likely to provide the closest to a true platelet count. It would be of great value to compare samples collected into CTAD anticoagulant with EDTA and citrate samples using an optical cell analyser. However one source of error that will always be present with CTAD is the dilutional effect of using a liquid anticoagulant. Furthermore, while it reduces feline platelet aggregation, CTAD does not prevent it completely and a more optimal combination of platelet inhibitory agents may be found in future.

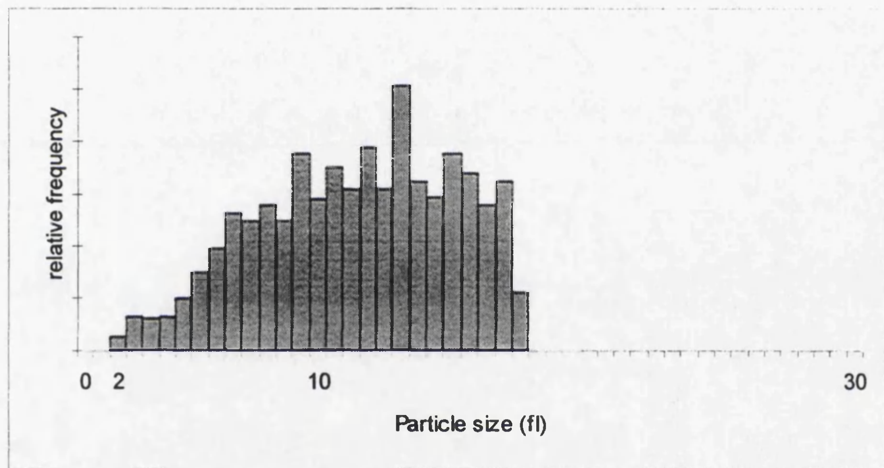
In conclusion, the use of CTAD anticoagulant offers several advantages over EDTA and result from a reduction in platelet aggregation. Although similar results were often obtained, the use of CTAD anticoagulant was associated with less platelet aggregation than citrate. The platelet inhibitors dipyridamole, adenosine and theophylline are likely to be responsible for this effect. Clinically relevant improvements in the accuracy of platelet counts and white blood cell counts are seen with its use. There are no detectable effect on cell morphology or staining characteristics, and little effect on red cell parameters.

Figure 5.1 (following page) *Example of the platelet volume distribution curves generated by impedance counter (Minos® Vet, Abx Hematologie) for aliquots of blood from the same cat which were anticoagulated with three different anticoagulants: a) EDTA (platelet count 80 x 10^9/l), b) citrate (platelet count 155 x 10^9/l prior to correction for dilution) and c) CTAD (platelet count 180 x 10^9/l prior to correction for dilution). Particles larger than 17.5 fl are assumed to be erythrocytes and are not shown. Particles smaller than 2 fl are assumed to be debris and the remaining particles are assumed to be platelets.*

a)



b)



c)

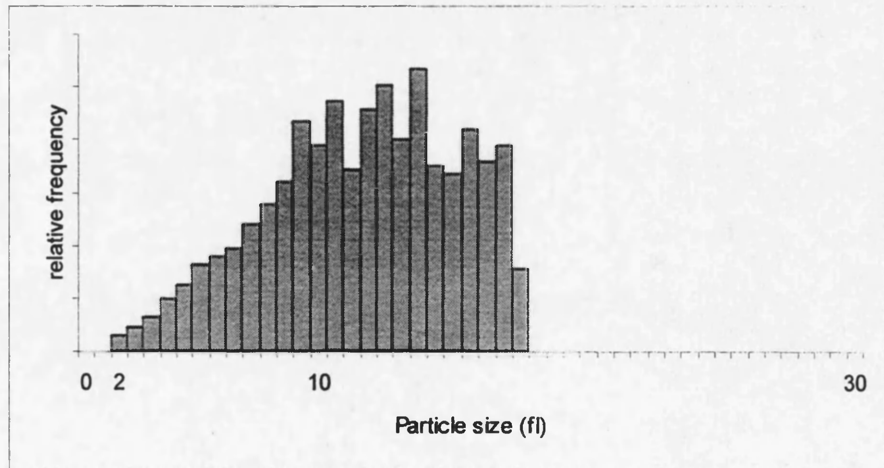
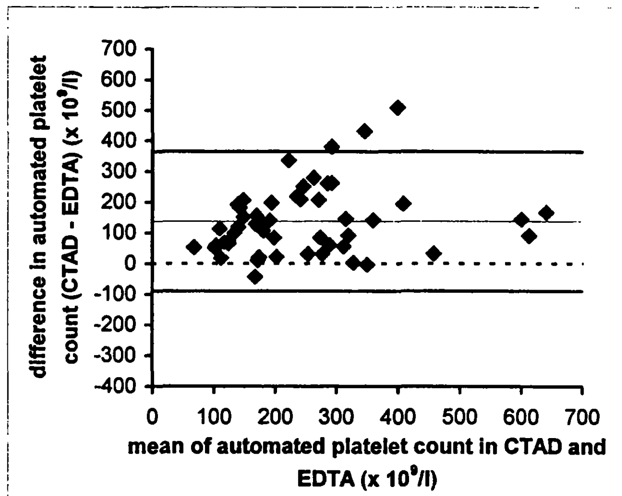


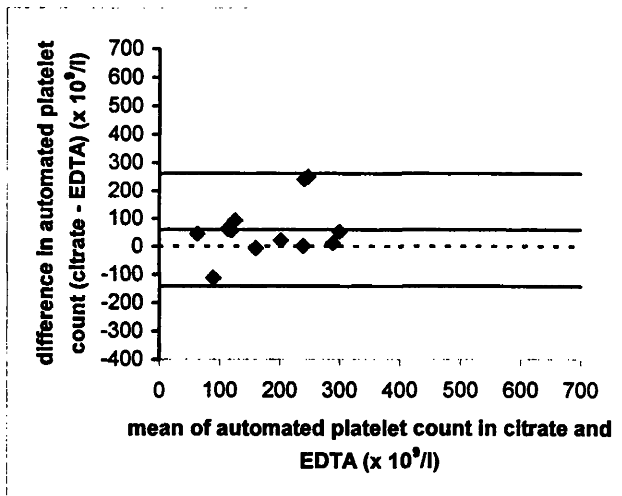
Figure 5.2 (following page) *Difference between automated platelet counts in one of three anticoagulants [count in anticoagulant 1 - count in anticoagulant 2] plotted against average [(count in anticoagulant 1 + count in anticoagulant 2)/2]. The middle solid line represents the mean of the difference and the outer solid lines represent the upper and lower limits of agreement ($\pm 2s_{diff}$). The dashed line represents a difference of zero (ie: perfect agreement between the results in the two anticoagulants).*

a) *Difference against mean for CTAD and EDTA. Mean difference $\pm s_{diff}$ is $137.7 \pm 113.6 \times 10^9/l$.*
b) *Difference against mean for citrate and EDTA. Mean difference $\pm s_{diff}$ is $59.7 \pm 100.5 \times 10^9/l$.*
c) *Difference against mean for CTAD and citrate. Mean difference $\pm s_{diff}$ is $73.4 \pm 172.8 \times 10^9/l$.*

a)



b)



c)

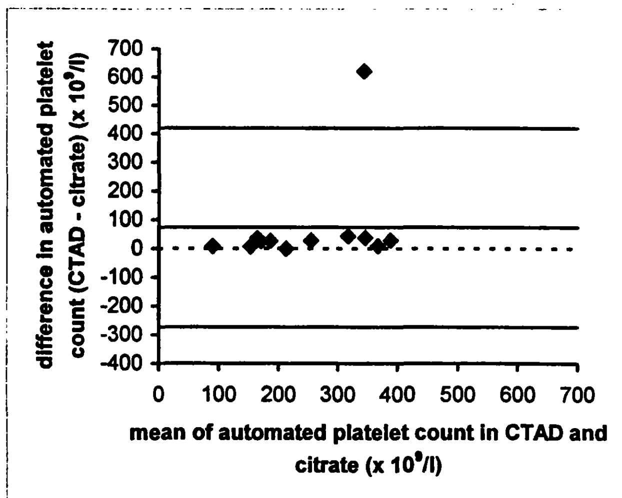


Figure 5.3 (following page) *Difference between platelet aggregation grade in one of three anticoagulants [grade in anticoagulant 1 - grade in anticoagulant 2] plotted against average [(grade in anticoagulant 1 + grade in anticoagulant 2)/2]. The middle solid line represents the mean of the difference and the outer solid lines represent the upper and lower limits of agreement ($\pm 2\ s_{diff}$). The dashed line represents a difference of zero (ie: perfect agreement between the results in the two anticoagulants).*

a) Difference against mean for CTAD and EDTA. Mean difference $\pm s_{diff}$ is -2.47 $\pm$ 2.02.
b) Difference against mean for citrate and EDTA. Mean difference $\pm s_{diff}$ is -0.83 $\pm$ 2.33.
c) Difference against mean for CTAD and citrate. Mean difference $\pm s_{diff}$ is -2.50 $\pm$ 2.02.

a)

difference in platelet aggregation grade (CTAD - EDTA)

mean of platelet aggregation grade in CTAD and EDTA

b)

difference in platelet aggregation grade (citrate - EDTA)

mean of platelet aggregation grade in citrate and EDTA

c)

difference in platelet aggregation grade (CATD - citrate)

mean of platelet aggregation grade in CTAD and citrate

<u>Figure 5.4 (following page)</u> *Difference between white cell counts by differing methods [count 1 - count 2] plotted against average [(count 1 + count 2)/2]. The middle solid line represents the mean of the difference and the outer solid lines represent the upper and lower limits of agreement (± 2 s_{diff}). The dashed line represents a difference of zero (ie: perfect agreement between the two counts).*

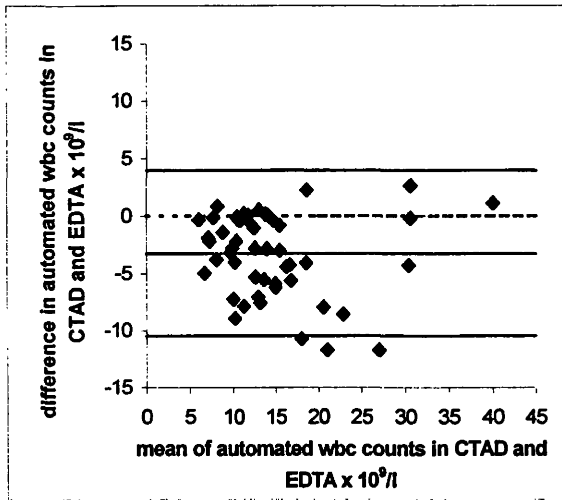
a) Difference against mean for automated white cell counts in CTAD anticoagulant and EDTA. For most samples the white cell count is lower in CTAD anticoagulant than in EDTA by a mean ± s_{diff} of 3.26 ± 3.61 x 10^9/l. In some samples white cell count in CTAD was as much as 8-12 x 10^9/l less than in EDTA.

b) Difference against mean for automated white cell count and haemocytometer white cell count in EDTA. In almost all the EDTA samples the automated white cell count is much greater than the haemocytometer white cell count. Mean difference ± s_{diff} is 5.15 ± 3.90 x 10^9/l.

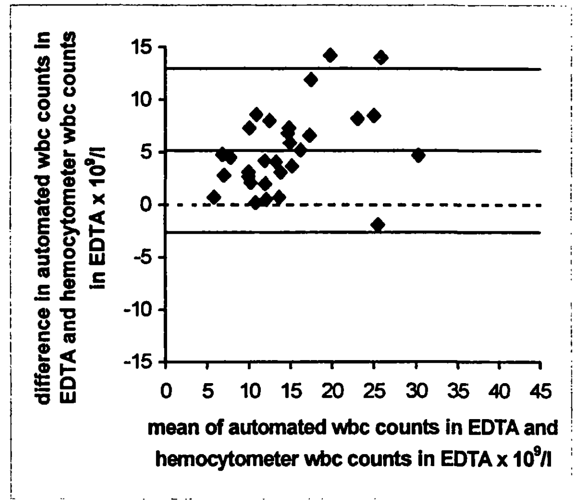
c) Difference against mean for automated white cell count in CTAD anticoagulant and haemocytometer white cell count in EDTA. There is close agreement between the automated white cell count in CTAD anticoagulant and the haemocytometer white cell count in EDTA for most samples. Mean difference ± s_{diff} is 0.66 ± 2.92 x 10^9/l).

d) Difference against mean for automated white cell count and haemocytometer white cell count in CTAD. There is close agreement between the automated white cell count and haemocytometer white cell count in CTAD. Mean difference ± s_{diff} is 0.10 ± 1.63 x 10^9/l).

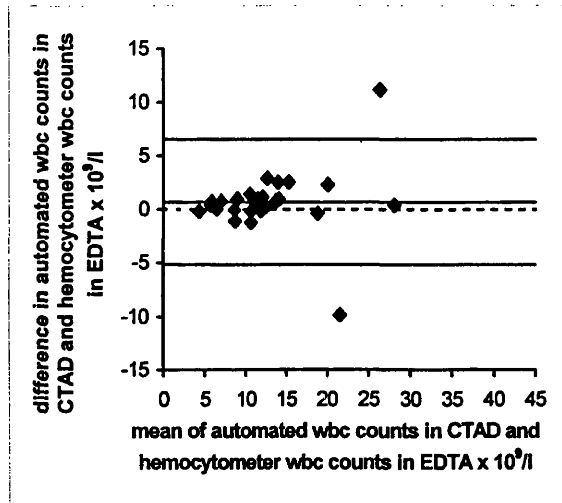
a)



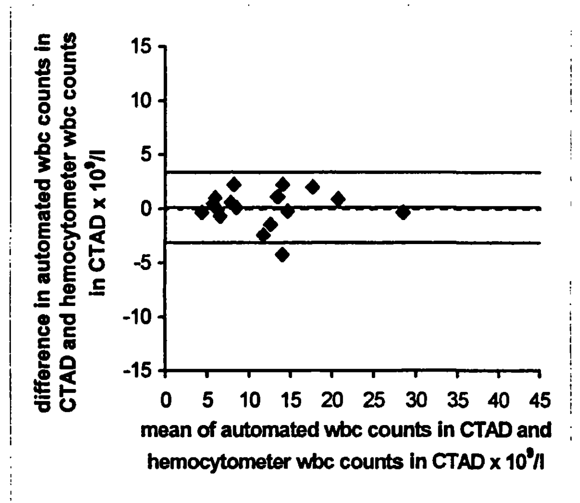
b)



c)



d)



6. Discussion

The difficulties in laboratory evaluation of platelet number have inhibited the accurate study of disorders of platelet number in the cat. Quoted reference ranges for platelet numbers may be inaccurate. The thrombocyte response in various disease states has not been well defined and is frequently extrapolated from the human literature.

Disorders of platelet number are infrequently recognised in the cat, and they are unlikely to represent an important primary disease. The prevalence of thrombocytopenia in the cat is low, and associated spontaneous bleeding occurs even less frequently (Jordan *et al.*, 1993), (Chapter 4). However, severe thrombocytopenia is an important and potentially life threatening clinical finding, of which the clinician needs to be aware. Unfortunately, thrombocytopenia may be frequently missed, as occurred in more than half of the cats with true thrombocytopenia as reported in Chapter 4, and this is likely to be due to the high frequency of false thrombocytopenia in the cat. The recognition of thrombocytosis in the cat is also hindered by the technical difficulties of feline platelet counting. Although the massive increases associated with essential thrombocythaemia should not escape detection, milder thrombocytosis may be obscured, with the loss of a potentially useful diagnostic marker for neoplastic or inflammatory disease.

An ideal method for platelet counting in the cat would fulfil the following criteria:

1. A small sample volume would be required (1-2 mL).
2. A large number of platelets would be counted to limit sampling errors.
3. Platelets would be accurately differentiated from other cell types and debris.
4. Aggregation of platelets would be prevented or reversed so that individual platelets could be counted, or a method for enumerating platelets within aggregates used.
5. The method would retain its accuracy after storage of blood samples for a reasonable period (24 hours).
6. The method would not be unduly time consuming or expensive.
7. The method would enable counting of all blood cells and morphological examination from the same sample.

Automated cell counting is generally more accurate than manual counts because of the large numbers of cells counted (Kjeldsberg, 1993). In addition small sample volumes can be used, and the processing time for each sample minimised, allowing cost minimisation. Haematology analysers vary in their method of cell type determination, as reviewed in Chapter 2. Newer analysers using laser technology, are able to differentiate platelets from other cell types and debris with greater accuracy than older style impedance analysers. In addition, although platelets within aggregates cannot be counted, they are not mistaken for white cells (or other cell types) as occurs with

impedance analysers (Chapter 5). However these analysers remain expensive and their use is limited to some veterinary laboratories.

Flagging of the presence of platelet aggregation is performed by many analysers but is of limited usefulness when the majority of samples are aggregated. The ability to estimate the number of platelets within an aggregate is inhibited by the variation is size of feline platelets, however assessment of platelet-crit rather than platelet number, may give a realistic reflection of the functional platelet mass. This may be a useful parameter since larger platelets are functionally more active than smaller ones, and hence it is the total volume of platelets present which determines the haemostatic potential.

The platelet-crit value determined by analysers such as the Minos is calculated from the platelet count and mean platelet volume and thus is prone to the same inaccuracies as the platelet count itself. However in some methods of analysis the platelet crit is actually measured. Using a method of differential separation of the buffy coat (quantitative buffy coat (QBC) analysis), measurement of the erythrocyte PCV, total WBC count and platelet count can be made in a similar manner to that used to measure PCV in a microhaematocrit tube. A cylindrical plastic float is used to expand the lengths of the buffy coat layers to allow their measurement. In a study of canine and feline samples, platelet aggregation caused more interference with the reference method (haemocytometer platelet counts) than with the QBC method (Levine *et al.*, 1986). In another study of canine blood samples in which platelet aggregation was induced by the addition of ADP, the QBC platelet counts were minimally affected, whereas the platelet counts by impedance counter were decreased by a mean of 53% (Koplitz *et al.*, 2000). However when platelet aggregation is severe, platelets clump on top of the QBC float and cannot be measured (Levine *et al.*, 1986). Correlation of platelet counts calculated from platelet-crit with direct counts will be adversely affected by the variability in platelet size (platelet heterogeneity). However platelet-crit is a potentially more useful parameter, as previously mentioned.

Inaccuracies in platelet count caused by the presence of the float when platelets are aggregated are avoided by use of a centrifugal-based laser analyser, the VS2000 (HemaTechnologies Ltd, Glasgow, UK) which is still under development. In this analyser a laser is used to locate interfaces between air, plasma, platelets, white blood cells, red blood cells and the haematocrit seal and this allows accurate measurement of the lengths of the various layers without the use of a float to expand the buffy coat layers. However platelet aggregation also leads to falsely low platelet counts with this method as large platelet aggregates may be trapped amongst the red cell layer and not measured.

Overcoming the problem of platelet aggregation in feline blood samples would thus be a great aid to increasing the accuracy of feline platelet counts, by all methods developed to date. Although platelet aggregation may well begin as soon as the vein is punctured, the practicalities of sampling make

this a difficult area to improve. The presence of a substance in the sample tube, which reversed any platelet aggregation already present in the sample would be of greatest benefit.

When human platelets aggregate without the release of granule contents deaggregation can be readily induced and the fibrin that has been bound during aggregation readily dissociates (Cattaneo *et al.*, 1987). The ability of a particular stimulus to induce only primary, reversible aggregation or both primary and secondary, irreversible aggregation depends on the species from which the platelets are derived, there being variation in sensitivity to various agonists amongst species; and the concentration of the agonist. Synergism occurs when platelet stimuli are combined resulting in stronger platelet aggregation responses than those observed with the individual stimuli (Adams, 1985). The particular stimulus or stimuli occurring during blood sampling and sample handling in cats is unknown. Thus the possibility exists that aggregation may be reversed in at least some samples, especially those in which aggregation is mild.

Deaggregation of platelets which have undergone primary aggregation has been demonstrated in human and rabbit platelets (Kinlough-Rathbone *et al.*, 1983), and occurs readily and immediately with the addition of EDTA, PGE_1 or PGI_2. The addition of a proteolytic enzyme is required to deaggregate human platelets that have undergone secondary aggregation induced by thrombin (Kinlough-Rathbone *et al.*, 1985). Collagen aggregated platelets can be deaggregated with PGI_2 in an in vivo model in the cat (Gryglewski *et al.*, 1978). Theophylline enhances this deaggregatory effect (Gryglewski *et al.*, 1978).

Even if aggregation cannot be reversed, prevention of further *in vitro* aggregation would also be of benefit. Several products prevent aggregation of feline platelets if present before the aggregatory stimulus occurs (Table 6.1). Platelet inhibitory drugs continue to be developed and investigated for use in prevention of human thrombotic disease, but published investigations of their efficacy in feline platelets are lacking.

CTAD was chosen for study because it is stable at room temperature, commercially available and inexpensive. Although both PGI_2 and PGE_1 have demonstrated efficacy in inhibiting feline platelet aggregation (Gryglewski *et al.*, 1978; Hwang, 1985; Welles *et al.*, 1994b), their instability and handling requirements makes them unsuitable for clinical use (Welles *et al.*, 1994b; Narayanan, 1995).

Unfortunately the practical application of CTAD is limited by its liquid form and the dilutional errors that result from its use. Other anticoagulants which have been recommended to reduce platelet activation include a mixture of acid-citrate-dextrose (ACD), aspirin (acetyl salicylic acid) and PGE_1 (Files *et al.*, 1981; Narayanan, 1995), and a mixture of aspirin (acetyl salicylic acid), formaldehyde and EDTA (Valli *et al.*, 1980), but both of these are also liquids. A dry anticoagulant containing pyridoxal-5'-phosphate (PLP) has been developed for use in human haematology which avoids the

platelet aggregation induced by EDTA or citrate and is suitable for measurement of routine haematological parameters (Lippi *et al.*, 1990). PLP inhibits primary aggregation by ADP, and leads to rapid disaggregation of ADP-aggregated rabbit platelets (Lam *et al.*, 1980). Its mechanism of action remains unclear but is not the result of increasing cAMP concentrations within the platelet (Lam *et al.*, 1980). The PLP containing anticoagulant has not yet been evaluated in feline blood, but it warrants further investigation. However, because of the morphological stability of cells in EDTA (Tvedten, 1994b), development of an EDTA-based anticoagulant with platelet inhibitory activity may have advantages for simultaneous performance of the full blood count. Repeated analysis of samples over time is needed to evaluate this.

The methods used in this evaluation of CTAD were simple and demonstrate the clinical application of the anticoagulant. However, a more thorough evaluation of the advantages of a particular anticoagulant could be made using specific techniques to quantify platelet aggregation. Platelet aggregometers measure the size of platelet aggregates by either analysis of light transmission or impedance. The size of aggregates is proportional to the extent of platelet aggregation (McNicol, 1996). As aggregation progresses, light transmission through a stirred platelet suspension increases and is compared to a control solution (McNicol, 1996). This method is, in general, qualitative rather than quantitative (Adams, 1985). The impedance method is more suited to comparing platelet aggregation in different anticoagulants as it allows measurement of platelet aggregation in whole blood as well as platelet suspensions (McNicol, 1996). Following activation, platelet aggregates form on two electrodes placed in the platelet solution impairing the electrical conduction (McNicol, 1996). Both methods correlate well with each other (McNicol, 1996), but require specific equipment. Other techniques which can be used to quantify secondary (irreversible) platelet aggregation include assay of substances released from platelets following granule release such as β-thromboglobulin, serotonin, ATP, β-glucuronidase, and thromboxane A_2 (McNicol, 1996). These methods cannot be used to quantify the degree of platelet activation or primary, reversible aggregation which may occur with a mild stimulus. In addition the plasma separation procedures required may further activate platelets.

The activation of platelets, as occurs prior to aggregation, can be detected by several methods. The expression of surface antigens on the platelet following activation can be detected using monoclonal antibodies that bind only to activated and not to resting platelets (Shattil *et al.*, 1987; Michelson and Shattil, 1996). Activation dependent antigens that have been studied include P-selectin and $\alpha_{IIb}\beta_3$ integrin (glycoprotein IIb/IIIa) (Michelson and Shattil, 1996). Fluorescence-conjugated antibodies are detected using flow cytometry and only small volumes of whole blood and minimal manipulation of samples is required (Shattil *et al.*, 1987; Welles *et al.*, 1994b; Michelson and Shattil, 1996). However, this technique cannot be used to measure the amount of fluorescence per individual platelet if the platelets are aggregated (Michelson and Shattil, 1996). Exposure of fibrinogen binding sites following activation can be detected using radioactive labelled fibrinogen (McNicol, 1996). Likewise this technique cannot be applied to aggregated samples in

which native fibrinogen is already bound. In density-gradient analysis fractionation of platelets is performed by centrifugation in special media, the lowest density platelets being those which have undergone the most activation (Zelmanovic and Hetherington, 1998). This process is expensive, technically difficult and requires large volumes of blood (Zelmanovic and Hetherington, 1998).

A new platelet parameter, mean platelet component concentration (MPC) is measured by the ADVIA® 120 Hematology System (Bayer Corporation, Tarrytown, NY). Using a method of two-angle laser light scattering flow cytometry, the refractive index of individual platelets can be determined which is related to both platelet density and activation state (Zelmanovic and Hetherington, 1998). Thus MPC provides a simple measure of platelet activation state but only in the unaggregated platelets within a sample (Zelmanovic and Hetherington, 1998). However in their study of feline blood samples, Zelmanovic and Hetherington, (1998) found that in aggregated samples, the remaining, free, unaggregated platelets also has an increased MPC, suggesting that they too were activated. Unfortunately MPC measurements are only accurate in blood samples collected in EDTA (Zelmanovic and Hetherington, 1998). This is because the spherical transformation of platelets in EDTA, which does not occur with citrate based anticoagulants, is necessary for application of the Mie scattering theory used to analyse the scattering signals (Zelmanovic and Hetherington, 1998). Therefore development of an EDTA-based anticoagulant with platelet inhibitory activity may be ideal for use with this equipment.

Table 6.1 *Action of various antagonists in preventing feline platelet aggregation induced by various agonists in vitro.*

antagonist	agonist					
	ADP	**serotonin**	**collagen**	**adrenaline**	**U-46619 (TXA_2 analogue)**	**arachidonate**
prostacyclin (PGI_2) (adenyl cyclase activator)	complete inhibition[*]		inhibition[†]			
prostaglandin E_2 (PGE_2)	complete inhibition[1]					
prostaglandin D_2 (PGD_2)	partial inhibition[1]					
adenosine (adenyl cylcase activator)	no effect[1],[‡•]	inhibition[§]				
dipyridamole (phosphodiesterase inhibitor)	no effect[1],[3]					
aspirin (acetyl salicylic acid)	no effect[**]		inhibition[5†]	no effect[5]		inhibition[††]
imidazole (thromboxane synthetase inhibitor)	no effect[1]			no effect[1]		
indomethacin (cyclooxygenase inhibitor)	no effect[1]		moderate inhibition[‡‡] no effect[1]	no effect[1]		
ketanserin (serotonin antagonist)	no effect[7]	inhibition[7]	inhibition[7]		inhibition[7]	
BM-13177 (TXA_2 antagonist)		no effect[7]	minimal inhibition[7]		inhibition[7]	

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[*] (Hwang, 1985)
[†] (Gryglewski *et al.*, 1978)
[‡] (Philp and Bishop, 1970)
[•] Adenosine begins to inhibit ADP-induced aggregation at concentrations between 100 and 1000 μM.(Tschopp, 1969)
[§] (Tschopp, 1969)
[**] (Allen *et al.*, 1985)
[†] Aggregation induced by low concentrations of collagen could be inhibited by aspirin, but when high concentrations of collagen (1.5 μg/mL) were used, aspirin did not inhibit aggregation.(Hart and Nolte, 1991)
[††] (Greene, 1985)
[‡‡] (Ogawa *et al.*, 1998)

7. APPENDICES

7.1. ABBREVIATIONS

ACD .... acid-citrate-dextrose
ADP .... adenosine diphosphate
AMP .... adenosine monophosphate
ATP .... adenosine triphosphate
cAMP .... adenosine 3', 5'-cyclic monophosphate
cGMP .... guanosine 3', 5'-cyclic monophosphate
CHS .... Chédiak-Higashi syndrome
CPD .... citrate-phosphate-dextrose
DDAVP .... desmopressin
DIC .... disseminated intravascular coagulation
DNA .... deoxyribonucleic acid
EDRF .... endothelium-derived relaxing factor
EDTA .... ethylenediamine tetra-acetic acid
FDP .... fibrin degradation products
FeLV .... feline leukaemia virus
FIP .... feline infectious peritonitis
FIV .... feline immunodeficiency virus
G proteins .... guanine nucleotide-binding proteins
G6PD .... glucose-6- phosphate dehydrogenase
(GM)-CSF .... granulocyte-macrophage colony stimulating factor
GMP .... guanosine monophosphate
GP .... glycoprotein
GTP .... guanosine triphosphate
IL .... interleukin
KL .... c-kit ligand
MCV .... mean cell volume (of red cells)
ML .... Mpl ligand
MPV .... mean platelet volume
mRNA .... messenger ribonucleic acid
PAF .... platelet-activating factor
PCV .... packed cell volume
PDGF .... platelet derived growth factor
PF .... platelet factor
PG .... prostaglandin
PGD_2 .... prostaglandin D_2

PGI_2 .... prostacyclin, prostaglandin I_2
PRP .... platelet rich plasma
RBC .... red blood cell
SLE .... systemic lupus erythematosis
t-PA .... tissue plasminogen activator
TXA_2 .... thromboxane A_2, thromboxane
vWD .... von Willebrand's disease
vWf .... von Willebrand's factor
WBC .... white blood cell

7.2. PREPARATION OF BLOOD SMEARS USING THE MODIFIED MAY-GRÜNWALD-GIEMSA STAIN

Preparation of the stain:

A new batch of stain should be prepared every 3-5 days

Buffered distilled water pH 6.8

Buffer tablets pH 6.8: BDH GURR® Prod 33199 *

1 tablet dissolved in distilled water and made up to 1 litre.

May and Grünwald's stain: BDH GURR® Prod 35025S (1 litre).

Freshly diluted to 50% with buffered distilled water and filtered before use.

Giemsa's stain improved R66: BDH GURR® Prod 35086 5P (1 litre).

Freshly diluted to 7.5% with buffered distilled water and filtered before use.

Blood smear staining method:

- Fix air-dried films in methanol for 5 minutes.
- Immerse slides in May-Grünwald stain for 6 minutes.
- Transfer slides, without washing, into Giemsa stain for 6 minutes.
- Repeat step 3. using separate bath of fresh stain.
- Wash slides in buffered distilled water, rapidly, 3 times.
- Allow slides to stand in buffered distilled water for 1 minute to allow differentiation to take place.
- Stand slides upright to dry.

This is a Romanowsky stain and its properties depend on the interaction of methylene blue, methylene blue azure and eosin components. The methylene blue, a basic dye, is taken up by the acidic groupings of the nucleic acids and proteins of the cell nuclei and primitive cytoplasm, ie DNA and RNA. Basophil granules, contain heparin, which is acidic, also have an affinity for this dye, and stain blue. Methylene blue azure stains the azurophil granules of leukocytes. Eosin, which gives a red colour, is an acidic dye which reacts with basic groupings e.g. the haemoglobin molecule. The granules of eosinophils contain a spermine derivative which reacts strongly with eosin.

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* BDH Laboratory supplies, Poole, England BH15 1TD, telephone: (01202) 669700

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