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IMMUNE-MEDIATED THROMBOCYTOPENIA IN DOGS:
ASSAY DEVELOPMENT, A ROLE FOR COMPLEMENT, AND ASSESSMENT
IN A TOXICOLOGIC STUDY

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Michael A. Scott

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Kenneth A. Schwartz
Major professor

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IMMUNE-MEDIATED THROMBOCYTOPENIA IN DOGS: ASSAY DEVELOPMENT, A ROLE FOR COMPLEMENT, AND ASSESSMENT IN A TOXICOLOGIC STUDY

Volume I

By

Michael A. Scott

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ABSTRACT

IMMUNE-MEDIATED THROMBOCYTOPENIA IN DOGS: ASSAY DEVELOPMENT, A ROLE FOR COMPLEMENT, AND ASSESSMENT IN A TOXICOLOGIC STUDY

By

Michael A. Scott

The diagnosis of primary immune-mediated thrombocytopenia (IMT) in dogs is generally based on clinical criteria, the exclusion of other identifiable causes of thrombocytopenia, and response to therapy. The diagnosis of secondary IMT associated with certain drug exposures, infections, and neoplasms has also been based on indirect evidence. The role of humoral immunity in these conditions remains speculative without reliable direct assays for canine platelet surface-associated immunoglobulin (PSAIg).

We therefore developed and characterized a sensitive immunoradiometric assay (IRMA) for canine PSAIg and platelet-bindable immunoglobulin (PBIg). Immunoglobulins were reproducibly detected in a dose-response fashion with ^{125}I-labeled staphylococcal protein A (SpA), which we found to be unreactive with about 20% and 33% of canine IgG and IgM, respectively. ^{125}I-labeled antibodies to canine IgG and IgM were used to define the antibody class and to detect SpA-nonbindable immunoglobulins. False positive results due to nonspecific PSAIg and PBIg, mostly IgM, were avoided by using fresh 37°C blood samples and 37°C plasma incubation temperatures.

The PSAIg assay was applied to investigations of the pathogenesis of sporadic cytopenias occurring in dogs during a toxicologic study with a proprietary drug. Significant amounts of drug-dependent antibodies were not consistently detected during

screening or rechallenge studies, so humoral immunity did not appear to be responsible for the cytopenias.

In human patients, drug-induced IMT may occur when immune complexes bind to platelet Fcγ receptors. We tested canine platelets for a response to heat-aggregated IgG (HAIgG) analogous to the Fcγ receptor-mediated aggregation of human platelets, and found that they lacked a functional Fcγ receptor analogue. Instead, canine platelets were agglutinated by the complement-mediated (C3) interplatelet bridging of HAIgG. This progressed to aggregation and release under certain conditions, suggesting the possibility of a receptor-linked signal transduction pathway.

The importance of PSAIg and complement in the pathogenesis of canine thrombocytopenic conditions associated with toxicants, infections, neoplasms, and autoimmunity can now be investigated.

To my parents, Dr. and Mrs. Earle S. Scott,
who instilled in me the desire to learn and the will to question

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KEY TO ABBREVIATIONS

ACD	acid citrate dextrose
ADP	adenosine diphosphate
ATP	autoimmune thrombocytopenia
BSA	bovine serum albumin
CBC	complete blood count
CIC	circulating immune complexes
CVF	cobra venom factor
CVs	coefficients of variation
D-MIFA	direct megakaryocyte immunofluorescence assay
DMIFT	direct megakaryocyte immunofluorescence testing
DEA1.1	dog erythrocyte antigen 1.1
DX	drug X
E	eluate
ELISAs	enzyme-linked immunosorbent assays
ESAIg	erythrocyte surface-associated immunoglobulin
FC-PIFA	flow cytometric platelet immunofluorescence assay
FT	flow through
GFP	gel-filtered platelets

HAIgG	heat-aggregated IgG
HAIgM	Canine heat-aggregated IgM
HIT	heparin-induced thrombocytopenia
IA	immune adherence
IC	immune complex
IMT	immune-mediated thrombocytopenia
IMTP	immune-mediated thrombocytopenic purpura
IRMA	immunoradiometric assay
ITP	idiopathic thrombocytopenic purpura
IVIgG	human gamma globulin
K9HAIgG	canine HAIgG
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M-PIFA	microscopic platelet immunofluorescence assay
MAIPA	Monoclonal Antibody-specific Immobilization of Platelet Antigens
MPS	mononuclear phagocyte system
MPVs	mean platelet volumes
NBT	nitroblue tetrazolium
PAF	platelet activating factor
PAIg	platelet associated immunoglobulin
PBIg	platelet-bindable immunoglobulin
PBS	phosphate buffered saline
PC	positive control

PF3	platelet factor-3
PFA	paraformaldehyde
PgE_1	prostaglandin E_1
PMA	phorbol 12-myristate 13 acetate
PRP	platelet-rich plasma
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SpA	staphylococcal protein A

REVIEW OF THE LITERATURE

The following definitions are provided to clarify how several terms relating to immunological thrombocytopenias will be used in this dissertation. The use of these terms has been variable and sometimes imprecise.

platelet associated immunoglobulin (PAIg) = any immunoglobulin detected in or on platelets including internal and surface immunoglobulin, specifically or nonspecifically bound in any way; context is required to determine if PAIg refers only to surface immunoglobulin, as is often the case, or to total immunoglobulin including internal and surface fractions

platelet-bindable immunoglobulin (PBIg) = any immunoglobulin in serum, plasma, or fractions thereof that remains bound to platelets after suitable incubations and washes

antiplatelet antibodies = immunoglobulins (PAIg and PBIg) with specificities for platelet epitopes; the term usually applies to antibodies reactive with surface epitopes, but antibodies to internal epitopes are also antiplatelet antibodies

antiplatelet autoantibodies = antiplatelet antibodies with reactivity to platelets from the same individual that is producing the antibodies

autoimmune thrombocytopenia (ATP) = thrombocytopenia caused by antiplatelet autoantibodies; ATP is also used for autoimmune thrombocytopenic purpura

idiopathic thrombocytopenia = thrombocytopenia from unknown causes

immune-mediated thrombocytopenia (IMT) = thrombocytopenia resulting from immunological factors, usually antibody-mediated destruction

> **primary IMT** = IMT unassociated with an underlying condition; while known to be immune-mediated, cases of primary IMT are also idiopathic in that their inciting causes are unknown
>
> **secondary IMT** = IMT associated with underlying diseases such as neoplasia, systemic lupus erythematosus, hyperthyroidism, or drug exposure; these may or may not be autoimmune

immune-mediated thrombocytopenic purpura (IMTP) = IMT with mucocutaneous hemorrhage; many patients have IMT without purpura

idiopathic thrombocytopenic purpura (ITP) = a disease where mucocutaneous hemorrhage is associated with thrombocytopenia from unknown causes; this term is also used by some to mean IMTP because the original terminology persisted after the immunological nature of many cases of "ITP" was demonstrated; "ITP" is also the abbreviation often used for "immune thrombocytopenic purpura" which is equivalent to IMTP

I. Introduction

Platelets, the smallest formed elements of blood, are anucleate cytoplasmic fragments of the largest hematopoietic cells, the megakaryocytes. Megakaryocytes residing primarily in the bone marrow but also in such sites as the spleen and lung,[1] shed their discoid platelets into the bloodstream. In normal dogs, platelets circulate at a concentration of about 200,000 - 500,000/μl.[2] Megakaryocytes also circulate in canine blood at very low concentrations, perhaps in transit from marrow to lungs,[3] but the significance of this is unknown.

The maintenance of normal circulating platelet concentrations requires effective megakaryocytopoiesis and thrombopoiesis. These complex processes are under cytokine control such that decreases in circulating platelet mass normally result in increased platelet production.[4] Thrombopoietin is the name given to the cytokine primarily controlling these processes. Thrombopoietin was recently purified and cloned, and its receptor, c-Mpl, has been identified as the product of the c-mpl proto-oncogene which is a member of the cytokine receptor superfamily.[5,6] Canine thrombopoiesis appears to be governed similarly, as sera from dogs made aplastic by irradiation contain a humoral

factor, presumably thrombopoietin, that binds to c-Mpl and transmits a mitogenic signal to megakaryocytes.[7,8] While this signal is pivotal to thrombopoiesis, other cytokines such as interleukin 6 have been shown to influence the production and function of canine platelets.[9]

Circulating platelets participate in numerous physiologic and pathologic processes including maintenance of vascular integrity, coagulation, fibrinolysis, inflammation, thrombosis, tumor metastasis, neovascularization, and repair. These metabolic activities involve the participation of platelet membrane glycoproteins and phospholipids, signal transduction pathways, internal storage granules and their secretory pathways, metabolic pathways for the de novo generation and release of cytoplasmic products, muscular and microtubular components, other cells, and numerous plasma factors.[10] Despite their anucleate status, mammalian platelets are clearly very active, complex, and important elements of blood.

Of primary relevance to this review, platelets are essential for normal hemostasis. With damage to vascular endothelium, normal platelets quickly adhere to exposed subendothelium where they spread, aggregate, and form a primary hemostatic platelet plug. Subendothelial collagen is important as an initial platelet agonist in this process. Platelet activation leads to the secretion of ADP from dense granules and to the generation and release of thromboxane A2, both of which result in the recruitment and activation of more platelets to strengthen the plug. Concomitant activation of the coagulation system leads to the generation of another potent platelet agonist, thrombin, which cleaves fibrinogen to form a stabilizing fibrin network within and around the platelet plug patching the vascular defect.[10]

Severely impaired primary hemostasis results in reduced vascular integrity and hemorrhage characterized most frequently by mucocutaneous petechiae and ecchymoses, epistaxis, melena, hematochezia, hematuria, scleral hemorrhage, hyphema, and retinal hemorrhage. This pattern of hemorrhage occurs in dogs with congenital and acquired platelet functional defects, and in dogs with marked reductions in the circulating platelet mass. Thrombocytopenia, defined by a platelet concentration below the normal range, is generally considered the most frequent cause of nontraumatic hemorrhage in dogs.

II. Thrombocytopenia

A. Pseudothrombocytopenia

True thrombocytopenia must always be differentiated from pseudothrombocytopenia, a laboratory artifact occurring when a substantial proportion of the platelets in a sample are not recognized as such. With human samples, pseudothrombocytopenia may occur when platelets are clumped due to:[11] 1) anticoagulant-dependent IgG, IgA, or IgM antibodies reactive to GPIIb/IIIa in the presence of EDTA or sometimes other anticoagulants;[12,13] 2) cold agglutinins; 3) platelet rosetting around neutrophils or monocytes (platelet satellitism), which is generally due to EDTA-dependent interactions at room temperature, possibly involving IgG[14] or thrombospondin;[15] 4) platelet activation during or after venipuncture; or 5) to a significant number of the platelets in a sample being larger than the high threshold setting for platelets on automated counters. The latter two are common concerns for the accurate enumeration of canine platelets.

As a breed, normal cavalier King Charles spaniels have the same circulating concentration of platelets as other canine breeds, but automated counters underestimate

their platelet concentrations and often indicate thrombocytopenia.[16,17] This pseudothrombocytopenia is due to a bimodal distribution of platelet size with one platelet population similar in size to platelets of other breeds, and a second larger population that is apparently gated out of the platelet size range by at least some automated cell counters. The cause of this breed difference is unknown.

Greyhounds generally appear to have lower circulating platelet concentrations than other breeds.[18,19] The mean ($\pm$ S.D.) platelet concentration of 36 clinically normal greyhounds was 154,000 $\pm$ 43,000, while that of non-greyhounds was 238,000 $\pm$ 22,000 ($p < 0.001$).[19] It has been suggested, but not proven, that bipotential marrow stem cells responsive to erythropoietin and thrombopoietin generate erythrocytes in greyhounds at the expense of platelets[19] This would be consistent with the elevated hematocrits in these dogs as well as the low platelet concentrations. Platelet sequestration and low-grade immune-mediated or infectious destruction have also been suggested causes of apparent thrombocytopenia in greyhounds.[18,19] If no pathologic or clinical significance can be linked to this breed difference, a greyhound-specific reference range for platelet concentration will be required to prevent the misdiagnosis of thrombocytopenia in this breed.

Anticoagulant-induced pseudothrombocytopenia has also recently been reported in dogs.[20,21] In a carefully studied case, EDTA-anticoagulated blood from a healthy, untreated dog had an apparent platelet concentration of 50,000/μl as assessed by a Technicon H-1 hematology analyzer.[20] The platelet concentrations of several previous samples ranged from 248,000 to 316,000/μl. Platelet clumps were also noted by direct examination of blood smears made at the time of apparent thrombocytopenia. Platelet

clumping was not apparent when citric acid or heparin were used as anticoagulants, and the platelet concentrations with these two anticoagulants were 105,000 and 191,000/μl, respectively.

Recent studies of the phenomenon with human blood indicate that for any given sample, several anticoagulants may induce pseudothrombocytopenia, but EDTA consistently has the greatest effect.[13] Interestingly, increasing concentrations of EDTA appear to increase the effect initially but eventually inhibit and in fact prevent platelet clumping in susceptible samples.[13] While this finding led to the suggestion that calcium chelation may not be solely responsible for inducing antibody interactions with GPIIb/IIIa, it is also possible that calcium chelation is critical but that high concentrations of EDTA inhibit necessary metabolic platelet processes. The expression of activation antigens on clumped platelets indicates that platelet activation and metabolic processes are involved.[13]

Differentiation of true thrombocytopenia from pseudothrombocytopenia is best done by the direct evaluation of a blood smear;[13] the presence of many large platelets should suggest the need for a manual method of platelet enumeration, and the presence of platelet clumps indicates the need for another sample. The method of platelet enumeration significantly affects the degree of pseudothrombocytopenia with any given sample, and automated counters cannot be relied upon to indicate the presence of platelet clumps.[13]

B. Causes of Thrombocytopenia in Dogs

While the focus of this dissertation is on canine IMT, all known causes of thrombocytopenia in dogs will be discussed because: 1) the diagnosis of IMT is largely one of exclusion, necessitating a firm knowledge of potential causes, and 2) immune-mediated platelet destruction may contribute to thrombocytopenia in many diseases. What is known about the mechanism of thrombocytopenia in these diseases will be discussed in an attempt to document any potential immune-mediated contributions, and to differentiate clearly nonimmune thrombocytopenias from immune or potentially immune-mediated thrombocytopenias.

General reviews of thrombocytopenia and specific reviews of IMT and drug-induced thrombocytopenia in dogs have often intermixed a significant amount of knowledge about the human conditions with a relatively small amount of documented knowledge about the canine conditions.[2,22-24] While similar causes are likely operant in analogous human and canine conditions, they are not necessarily identical. In this review, an attempt has been made to focus principally on canine thrombocytopenia, omitting several diseases associated with thrombocytopenia in people that have not been identified in dogs. Human conditions are discussed where they may be useful models to better understand canine diseases, primarily those with an immune-mediated pathogenesis. Discussions pertaining to the human model will be clearly identified as such.

1. incidence and general disease associations

The incidence of thrombocytopenia in dogs is probably best estimated by a retrospective survey of dogs examined at the College of Veterinary Medicine, North

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Carolina State University from 1983 to 1989.[25] Of the 18,910 dogs for which a complete blood count was obtained, 987, or about 5%, were thrombocytopenic (<200,000/μl), though 48% of these had platelet concentrations over 150,000/μl. This incidence is biased for a referral institution as well as for the geographic region which, compared to many other parts of the country, has a higher prevalence of infectious diseases associated with thrombocytopenia.

The 987 thrombocytopenic dogs were categorized by general disease type such that thrombocytopenia was attributed to the following: IMT in 5%, cytopathologically or histopathologically confirmed neoplasia in 13%, inflammation or infection in 23%, and miscellaneous causes in 59%.[25] Miscellaneous conditions included trauma (70), acute hemorrhage (29), drug therapy including cancer chemotherapy (70), coagulopathies such as DIC of unknown origin (8), platelet clumping (70), renal and liver disease (29), sequestration (3), and bone marrow disease such as hyperestrogenism (6). Also placed in the miscellaneous category were 304 (31%) dogs for which the cause of thrombocytopenia was unknown. Inclusion of thrombocytopenia due to platelet clumping may have slightly overestimated the incidence of true thrombocytopenia, as pseudothrombocytopenia may have accounted for many of these 70 cases.[25]

Overall, IMT was diagnosed in 0.26% of all admissions including dogs with diagnoses of primary IMT (27, 0.14% of all admissions), systemic lupus erythematosus (SLE) (13), Evans syndrome (6), pemphigus (1), and rheumatoid arthritis (1). The diagnosis of IMT was made clinically on the basis of a positive antinuclear antibody test, a positive LE cell preparation, thrombocytopenia with megakaryocytic hyperplasia, or response to immunosuppressive drug therapy or splenectomy.

In another study of 60 thrombocytopenic dogs, the general diagnoses were categorized as immune-mediated in 33 (55%), neoplasia-associated in 11 (18%), infectious or inflammatory in 8 (13%), and miscellaneous (including DIC and unknown causes) in 8 (13%).[26] The population included only those thrombocytopenic dogs for which platelet volume analyses and bone marrow evaluation were done within 48 hr of each other. Since platelet volumes and fragmentation indices were considered useful in making a diagnosis of IMT, the population was likely biased toward this diagnosis. This may explain the large difference between the 2 studies with respect to the percentage of thrombocytopenias attributable to immune-mediated mechanisms.

Clearly, thrombocytopenia is frequently associated with neoplasia and infection or inflammation, each of which can be occult. This emphasizes the importance of thorough evaluations in an attempt to exclude such causes before a diagnosis of primary IMT is reached. Confirming antibody-mediated platelet destruction with reliable tests for PAIg may be useful for guiding treatment in IMT as well as in the thrombocytopenic conditions associated with neoplasia, infections, and inflammation.

2. pathophysiologic mechanisms

While numerous specific disease states are associated with thrombocytopenia, only a few general pathophysiologic mechanisms are ultimately responsible for the condition. The total body platelet mass at any given time is dependent upon a balance between the rate of platelet production and the rate of platelet destruction or consumption. The platelet concentration of peripheral blood is similarly affected by these factors, and additionally by the degree of platelet sequestration outside the peripheral vascular system.

When the balance tips toward production, the result is an increased platelet concentration, called thrombocytosis; when the balance tips toward consumption, destruction, or sequestration, thrombocytopenia may ensue. Intensified platelet production may partially or completely compensate consumptive and destructive states, thus maintaining relatively normal peripheral platelet concentrations.

Many thrombocytopenic states are likely caused by the cumulative effects of multiple pathophysiologic mechanisms, but others appear to have a predominant cause. Wherever possible, the following causes of canine thrombocytopenia will be grouped by the predominating pathophysiologic mechanism thought to produce the thrombocytopenia. The pathophysiology of thrombocytopenias associated with neoplasia and infectious diseases are often multifactorial, complex, or only tenuously defined. Consequently, some of their discussion will be separate from specific mechanistic categories.

a. decreased production

Adequate platelet production is dependent upon the maintenance of a healthy megakaryocyte population. Conditions which reduce the number of viable megakaryocytes or hematopoietic cells in general may lead to thrombocytopenia, bicytopenia, or pancytopenia. In most cases of bone marrow injury, multiple cell lines are affected.[27] Thrombopoiesis may also be decreased when adequate numbers of megakaryocytes are present if they are functionally impaired, though this is difficult to evaluate.

(1) chemical toxicants (drugs) with predictable effects

Predictable and dose-dependent thrombocytopenias have been well-documented for several common myelosuppressive drugs in dogs. Other chemicals have been implicated by clinical reports, but a firm cause-and-effect relationship has not been established.

(a) chemotherapeutic agents

The bone marrow suppression occurring with many antineoplastic chemotherapeutic agents is a well-recognized toxicity that is not restricted to any particular class of drug.[28-30] Myelosuppression, which may include thrombocytopenia, can occur with the use of: 1) alkylating agents (busulfan, chlorambucil, cyclophosphamide, melphalan,[31] dacarbazine); 2) mitotic inhibitors (vinblastine); 3) antimetabolites (pyrimidine analogues including cytosine arabinoside, 5-fluorouracil, and 5-aza-2'-deoxycytidine,[32] purine analogues including 6-mercaptopurine and azathioprine,[33] and the folic acid antagonist methotrexate); 4) antibiotics (doxorubicin, mitomycin, mithramycin), and 5) other agents such as cisplatin, hydroxyurea, and carmustine.

The sometimes severe thrombocytopenia caused by doxorubicin in dogs[34] has been associated with decreased platelet survival.[35] It is not known if the decreased platelet survival is just the result of a more rapid turnover of platelets due to the low platelet concentrations, or if doxorubicin has more direct effects on the platelets which contribute to their early removal.[35] Myelosuppression and thrombocytopenia were profound and intolerable when doxorubicin was encapsulated in glutaraldehyde-treated erythrocytes before administration, perhaps because fixation altered the drug's effect.[36] With the

related drug mitoxantrone, thrombocytopenia does not appear to be a clinical problem at dosages up to 6.5 mg/m^2.[37,38] Cisplatin, as a single agent, induced severe thrombocytopenia with or without granulocytopenia in 10% of 41 dogs treated for a variety of malignancies.[39] Azathioprine has also been associated with pancytopenia in dogs.[40]

(b) estrogens

Estrogens, from either endogenous or exogenous sources, can have profound effects on hematopoiesis in such sensitive species as ferrets,[41-43] mice,[44] and dogs.[45] In dogs, the effects of natural or synthetic estrogens have been well-studied. Hematologically, a marked initial leukocytosis is followed by leukopenia, progressive anemia, and a persistent thrombocytopenia.[45,46] These findings are associated with initially stimulated but later depressed granulopoiesis, depressed erythropoiesis, and depressed thrombopoiesis accompanied by a hypoplastic bone marrow.[47,48] The severity of the hematologic changes are dose-dependent and vary with the individual affected; a spectrum of conditions, from aplastic pancytopenia to transient thrombocytopenia, may be seen.

While great individual variation in the toxicity of estrogens has been frequently noted, others have consistently induced experimental thrombocytopenia in dogs.[48,49] A single intramuscular injection of estradiol valerate (1 mg/kg) into 8 healthy dogs led to mean platelet concentrations that were statistically significantly reduced by day 7, 9.1% of initial value (351,000/μl) on day 11, and at a nadir of 12,000/μl on day 14.[48] Recovery began on day 17 and platelet concentrations were 125,000/μl in the 3 dogs evaluated on day 23. No dogs bled spontaneously, and mean platelet volumes did not

change. Megakaryocyte numbers in the bone marrow were markedly reduced, and ^{111}In-kinetics were indicative of suppressed thrombopoiesis. The maintenance of minimum platelet concentrations after all radiolabeled platelets had disappeared indicated persistence of a basal rate of thrombopoiesis. Predictable thrombocytopenia of similar severity and time-course was also induced in 4 dogs by a single intramuscular injection of estradiol cypionate at a dose of 1 mg/kg.[49]

The synthetic ethynylestradiol has also induced a dose-dependent thrombocytopenia.[50] At a dose of 1 mg/kg per os once daily, thrombocytopenia developed with a nadir of 71,000/μl during week 4, with partial recovery before a second low of 85,000/μl on week 10. At a dose of 5 mg/kg, the mean platelet concentration fell to 3,000/μl by week 3 and was persistently low, resulting in severe hemorrhage and death.

Studies in mice have shown that estrogens do not directly inhibit myelopoiesis; rather, they stimulate the murine thymus to produce an inhibitor of granulocyte-macrophage progenitor cell replication.[51] Canine thymic stromal cells studied in vitro have also been shown to produce an inhibitor of myelopoiesis, more inhibitory than the murine analogue,[52] while estrogen has no significant direct effect on canine marrow progenitors.[46,52] This and the presence of estrogen receptors on nonlymphoid thymic cells suggests that the thymus may mediate estrogen-induced myelosuppression in vivo.[52] In vivo studies have shown that some dogs injected with estrogen had a serum factor inhibitory to GM-CFU formation.[53] It is not yet known if this factor is the same as that produced by thymic stromal cells in culture, but it may play an important role in the development of the cytopenias produced in some animals exposed to estrogen.[53]

Variations in the amount produced may explain the variations among dogs in susceptibility to estrogen myelotoxicosis.[52]

Clinically, estrogen-induced thrombocytopenia develops from endogenous and exogenous estrogen sources.

i) endogenous

Thrombocytopenia with bone marrow hypoplasia occurs secondary to the high concentrations of endogenous estrogens produced by some testicular sertoli cell neoplasms[54,55] and by some ovarian granulosa cell neoplasms.[56] Myelosuppression with thrombocytopenia due to hyperestrogenism has also occurred with testicular interstitial cell tumors.[57]

ii) exogenous

Thrombocytopenia has also occurred as a result of the use of synthetic estrogens to terminate pregnancies[58] or as therapies for such conditions as estrogen-responsive urinary incontinence, prostatic hyperplasia, and perianal hepatoid adenomas.[59] Marrow suppression is not unique to any particular synthetic estrogenic compound, though there appear to be differences in toxicity.

While many believe that the chance for success in treating dogs with estrogen-induced myelosuppression are too low to be justified, therapy has been successful when repeated platelet transfusions were used.[47] However, considerable effort and expense may be required to provide the necessary long-term support. Surgical extirpation of

estrogen-producing neoplasms is certainly necessary when endogenous sources are present.

(c) drugs with idiosyncratic effects

Phenylbutazone and meclofenamic acid are nonsteroidal anti-inflammatory drugs which have been associated with idiosyncratic myelosuppression and hypoplastic bone marrows in dogs.[41,47,60,61] Bicytopenias and pancytopenia have been reported, but the pathophysiology of the suppression has not been studied in dogs.

Scattered clinical case reports have implicated other drugs as causes of thrombocytopenia due to myelosuppression. These include thiacetarcemide which was associated with pancytopenia,[62] trimethoprim-sulfamethoxazole associated with thrombocytopenia and anemia,[63] and trimethoprim-sulfadiazine with fenbendazole which was associated with aplastic anemia.[47,64]

In the case of trimethoprim-sulfadiazine, it appears that myelosuppression can occur when administered to dogs on poor diets with poor folate status.[65] In such dogs, potentiated sulfonamides further decrease folate concentrations by inhibiting sequential steps in folate metabolism. This can lead to suppressed DNA synthesis and reduced hematopoiesis. However, folate deficiencies are rare in dogs, so more studies are needed to understand the significance of this potential. It is important to recognize the potential myelosuppressive effects of the potentiated sulfonamides because these antimicrobials have also been implicated in causing immune-mediated platelet destruction in dogs (see below). The two conditions require differentiation.

(2) irradiation

Ionizing radiation from sources including x-rays, ^{60}Co, ^{198}Au, ^{226}Ra, ^{239}Pu, ^{90}Sr, ^{210}Po, and ^{50}Y has long been used to induce generalized bone marrow aplasia in dogs for the study of the effects of irradiation and as models for treatment of radiation-induced disease.[66] Irradiation, be it x-irradiation,[67,68] γ-irradiation (^{60}Co),[69-71] or internal ß-irradiation from exposure to and absorption of ^{90}Sr[72,73] or ^{32}P[74] may cause thrombocytopenia or pancytopenia with terminal aplastic anemia or myeloproliferative disease. Because ^{90}Sr and ^{32}P translocate quickly to the skeleton after exposure, myelosuppression by these ß-emitters is similar to that of acute external radiation. This myelosuppression has been used to advantage in the therapy of such conditions in dogs as polycythemia vera (primary erythrocytosis) and essential thrombocytosis.[74]

Recently, marrow-ablative total body irradiation (^{60}Co) has been used in dogs prior to autologous bone marrow transplantation.[75,76] These dogs required as long as 8 weeks for their platelet concentrations to exceed 20,000/μl, and platelet transfusions were necessary for up to 7 weeks to prevent significant hemorrhage.

(3) myelophthisis

Marrow replacement by primary or metastatic neoplastic proliferations can cause myelophthisic thrombocytopenia or multiple cytopenias in dogs when substantial marrow infiltration is present. It may not be possible, however, to definitively incriminate physical marrow replacement as the principal cause of suppressed hematopoiesis in any given case. Myelophthisic suppression has been reported to occur in dogs with acute myelogenous and lymphocytic leukemias,[77] chronic lymphocytic leukemia,[78,79]

lymphocytosis of large granular lymphocytes,[80] lymphoma,[78,81] multiple myeloma,[82] some cases of megakaryoblastic leukemia[83] and other myeloproliferative disorders involving megakaryocytes.[84] The spectrum of marrow involvement by canine lymphoproliferative malignancies, from focal to packed patterns, has been described.[85] The precise mechanisms of myelophthisic-induced myelosuppression are not known, but competition for nutrients, occlusion of blood supply, lysis of marrow cells, and secretion of inhibitors have been considered potential contributing factors.

Myelofibrosis is also a cause of marrow replacement and thus may potentially cause or contribute to thrombocytopenia. In dogs, myelofibrosis has been associated with neoplasia, hemolytic anemias, irradiation, and myelonecrosis, though the cause is unknown in many cases.[86,87] Thrombocytopenia has specifically been associated with myelofibrosis in dogs with neoplasia,[78,88] DIC,[89] myelonecrosis,[88] pyruvate kinase deficiency,[90,91] and experimental irradiation.[73,92] Myelonecrosis without apparent myelofibrosis has also been associated with thrombocytopenia.[89,93] Causes of myelonecrosis have recently been reviewed for dogs and include infections, neoplasia, and drugs.[87]

Thrombopoiesis may not be as sensitive to myelofibrosis as is erythropoiesis. In one series of 14 dogs with myelofibrosis, 13 had nonregenerative anemias (mean Hct = 18%) but only 3 were thrombocytopenic, and not markedly so.[86] In fact, 5 nonthrombocytopenic dogs actually had an increased number of megakaryocytes, consistent with the hypothesis that megakaryocyte-derived growth factors have a pathophysiologic role in at least some cases of myelofibrosis. The relative effects of myelofibrosis on different marrow cell lines may depend on the underlying cause.

(4) immune-mediated suppression

Thrombocytopenia associated with marked marrow hypoplasia or aplasia has been reported in a dog with a positive result for possible antiplatelet antibodies using a platelet factor-3 (PF3) assay.[94] IMT was strongly suspected, but response to therapy could not differentiate the effects of oxytetracycline from those of corticosteroids. Other weaknesses of the case study included failure to examine a core biopsy of the marrow and use of serum for the PF3 assay (discussed below). However, the case suggests that amegakaryocytic IMT may occur in dogs, similar to the better studied canine cases of antibody-mediated pure erythrocyte aplasia.[95]

Acquired amegakaryocytic thrombocytopenia in people is a rare disorder characterized by the absence or extreme paucity of megakaryocytes. It probably has multiple causes, but the general lack of recognizable megakaryocytes or of marrow cells reactive with anti-[platelet glycoprotein] antibodies suggests a defect in megakaryocyte development at a stage preceding the acquisition of platelet glycoproteins.[96] In some cases, it has been caused by antibodies reactive to platelets and megakaryocytes[96,97] or to cytokines[98] that normally participate in megakaryocytopoiesis. While such cases are actually a type of IMT, their pathogenesis involves an obvious production failure and so they are addressed here.

In one carefully studied human patient with amegakaryocytic IMT and elevated PAIg, IgG isolated from the patient's plasma after she relapsed with the disease markedly inhibited the formation of megakaryocyte CFU, especially in the presence of complement.[97] This inhibition could be removed by incubating the IgG with autologous or allogeneic platelets. IgG isolated from a sample obtained when the patient was in

remission did not inhibit megakaryocytopoiesis. These findings indicated the presence of antibodies that bound to platelets and also inhibited normal megakaryocytopoiesis. In another patient, GM-CSF activity was suppressed by anti-[GM-CSF] antibodies such that the patient had amegakaryocytic thrombocytopenia in the absence of elevated PAIg.[98] While these reports are rare, antibodies may more commonly react with megakaryocytes in such a way as to alter their development and thrombopoietic activity but not their number[99-101] (see below).

Cell-mediated immunity has also been implicated in the pathogenesis of human acquired amegakaryocytic thrombocytopenia, with suppression of megakaryocytopoiesis being thought to result from T lymphocytes in one case and cells of the monocyte/macrophage line in another.[102] The contribution of cell-mediated immunity to canine amegakaryocytic thrombocytopenic conditions remains to be shown.

Increased PAIgG has also been detected in human patients with aplastic pancytopenia, reportedly unrelated to transfusion history.[103,104] This suggests that aplastic pancytopenia may have an autoimmune or immune-mediated component in some people, and that platelet destruction and decreased platelet production may occur simultaneously. Since some cases of pure erythrocyte aplasia in dogs appear to have an immune-mediated pathogenesis,[95,105] it would seem reasonable to hypothesize that some cases of aplastic pancytopenia in dogs may also be immune-mediated.

(5) hereditary disorders

(a) cyclic hematopoiesis

Cyclic hematopoiesis of grey collies is a rare autosomal recessive condition characterized by regular cyclic fluctuations in leukocyte, reticulocyte, and platelet concentrations with a periodicity of about 11-14 days.[106,107] Periodic decreases in cell production are due to a stem cell disorder that is correctable by bone marrow transplantation.[108] Cyclic depressions in platelet concentrations may be great enough to induce thrombocytopenia in affected dogs.

(b) hereditary thrombasthenic thrombopathia

A variable but moderate thrombocytopenia was reported in otterhounds with hereditary thrombasthenic thrombopathia, but the cause of the thrombocytopenia is unknown.[109,110] The presence of a large population of bizarre giant platelets and the deficiency in surface glycoproteins in affected dogs suggests that the thrombocytopenia may be the result of production abnormalities.

(6) infections

Decreased platelet production may contribute to the thrombocytopenia of infections when megakaryocytes are damaged by direct infection, immune-mediated mechanisms, or presumably by the local effects of marked intramedullary inflammation. For example, canine distemper virus can infect megakaryocytes and may contribute to thrombocytopenia in distemper.[111] Chronic canine monocytic ehrlichiosis is associated with bone marrow hypoplasia and cytopenias of unknown cause.[112,113] And infections

with such organisms as *Leishmania spp.* can be associated with granulomatous marrow infiltrates of organism-laden macrophages that may affect hematopoiesis.[114] The thrombocytopenia of these diseases is multifactorial and will be discussed in more detail below.

Canine parvovirus infects rapidly dividing cells leading most notably to acute enteritis and often panleukopenia.[115] Parvoviral infections have reportedly been associated with suppression thrombocytopenia.[116] However, viral antigen has minimally and inconsistently been found in the bone marrow of experimentally infected dogs, and, when present, it was not apparent in recognizable cells of the megakaryocytic series.[115,117] Moreover, the neutropenia appears to be secondary to severe enteritis rather than to marrow suppression.[115] Other studies have failed to identify significantly suppressed erythropoiesis.[118] Overall, there is no compelling evidence to indicate that suppressed hematopoiesis causes thrombocytopenia in canine parvoviral infections. When thrombocytopenia occurs, it is more likely related to septicemia/endotoxemia associated with severe enteritis.[115]

(7) idiopathic

Bone marrow aplasia or hypoplasia can occur without an obvious cause, as can megakaryocytic aplasia or hypoplasia. Such cases of platelet production failure must be classified as idiopathic, though they may in fact be the result of unidentified myelosuppressive toxicants, infections, or immunologic disorders.

(8) other

Defective platelet production and thrombocytopenia reportedly occur with uremia,[119] but the precise pathogenesis and frequency in dogs are not clear.

b. abnormal distribution (sequestration)

Conditions falling into this category occur when the total body platelet mass is normal, but the circulating platelet concentration is reduced because of reversible platelet redistribution out of the circulation. Redistribution into organs has been commonly referred to as sequestration. However, the term "sequestration" has also commonly been used in the context of irreversible organ trapping due to platelet destruction by the mononuclear phagocyte system.[120] In this dissertation, irreversible organ trapping falls under the category of decreased platelet survival. Sequestration is used to describe reversible platelet redistribution out of the circulating pool. Increased platelet destruction may occur to some degree as a secondary phenomenon.

Approximately one third of human platelets usually reside in the spleen.[121] Reports in dogs have been similar,[2,122] as about a third of ^{111}In-labeled platelets quickly distribute to the spleen on infusion into normal dogs.[122] However, there may be some handling damage of infused platelets that leads to selective trapping and destruction by the splenic mononuclear phagocyte system (MPS), so this value could be high. Epinephrine injections in normal dogs caused a rapid transient increase in platelet concentrations that amounted to 33% of the peak concentrations.[123] When epinephrine was given to a splenectomized dog, the mean increment in platelet concentration was 17% of the peak concentration. If the data for the single asplenic dog are largely

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applicable to other dogs, then these data indicate that dogs have a rapidly mobilizable platelet pool split nearly equally between the spleen and elsewhere.

Human patients with enlarged spleens from such diseases as lymphoma, polycythemia vera, and infectious mononucleosis were shown to have thrombocytopenia associated with normal total platelet masses.[121] Splenic platelet concentrations were disproportionately high compared to the amount of splenic blood, and the spleens of hypersplenic patients contained a higher percentage of the total platelet mass than did spleens of normal patients. Epinephrine infusions caused increased platelet concentrations in normal and splenomegalic subjects but not in asplenic patients.[121] Together, the data support an increased pooling of exchangeable platelets in enlarged spleens. Splenic platelet destruction was considered a small but additive contributor to the thrombocytopenia.

Similar effects of splenomegaly have been presumed to occur in dogs, being associated with mild to moderate thrombocytopenias of little clinical significance.[27] Generally, whenever unexplained thrombocytopenia accompanies splenomegaly in dogs, splenic sequestration is considered a likely contributor to the thrombocytopenia. Thrombocytopenia was attributed to sequestration in 3 of 987 thrombocytopenic dogs in one review, but the details were not provided.[25] Splenic pooling reportedly can occur in dogs with splenic or hepatic neoplasia, splenic torsion, hemolytic crisis, barbiturate anesthesia, and hepatomegaly.[27,124] However, splenic trapping in these cases is unproven, and if present, it could relate to either reversible (redistribution) or irreversible (destruction) mechanisms.

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Splenic platelet sequestration with hepatomegaly and liver failure is reportedly secondary to shunting of hepatic blood to the splenic circulation due to portal hypertension.[125] However, if thrombocytopenia occurs through this mechanism in liver failure, it would appear to be uncommon and clinically less important than platelet dysfunction. In a platelet study of 20 dogs with confirmed hepatic disease, 6 dogs had mucocutaneous bleeding associated with decreased platelet function but no dog was thrombocytopenic.[126]

Mild to moderate thrombocytopenia and severe nonregenerative anemia were attributed to splenomegaly in a dog with a hypocellular bone marrow and splenic hematopoiesis.[127] The dog was treated with tetracycline and corticosteroids for suspected ehrlichiosis or immune-mediated disease. Because it did not respond favorably to therapy, its spleen was removed and found to weigh 15% of its total body weight. By 8 days post-splenectomy, the dog's hematocrit had doubled and the platelet concentration was over $1x10^6/\mu l$. Fifty days after splenectomy the bone marrow was hypercellular without abnormalities. No cause for the cytopenias other than splenomegaly were identified, and splenectomy resolved the cytopenias.

Dogs with massive splenomegaly due to lymphoma are often thrombocytopenic, and splenectomy may resolve the thrombocytopenia.[128] While this suggests that sequestration may have a mechanistic role in the thrombocytopenia, other factors such as immune dysfunction or the tumor burden may be involved.

Experimentally, the marked thrombocytopenia induced by intravenous endotoxin in dogs is associated with transient pulmonary platelet sequestration with progressive translocation of the pulmonary platelets to predominantly the liver where they are

destroyed.[129] Severe hypothermia, defined by rectal temperatures of 20°C, may induce significant platelet sequestration in the lungs, liver, and spleen of dogs.[130]

Experimental infusion of a combination of factor Xa and phosphatidylcholine/phosphatidylserine vesicles into dogs was used to mimic the thrombin generation that occurs in DIC. It led to selective loss of high molecular weight multimers of von Willebrand's factor (vWF), and a rapid, reversible thrombocytopenia that was marked and associated with circulating platelet aggregates.[131] The specific cause of the thrombocytopenia is unknown but appeared to be due to transient sequestration, possibly of platelet aggregates in the microcirculation or of platelets and vWF at endothelial cell surfaces.

Several relatively old studies of the acute in vivo effects of heparin on canine platelets have been reported. Platelet concentrations in 3 dogs given 200 U/kg were transiently decreased at 5-10 minutes, at 5-90 minutes, and at 5-75 minutes after heparin injection.[540] Quick[541] had similar results in 4 dogs, using 5 different heparins. When at least 55 U heparin were injected, there was always a transient, severe thrombocytopenia that was usually maximal at 5 minutes and gone by 30 minutes. He further noted that at 1 minute after injection, all platelets were in clumps; at minute 2, single platelets were appearing but clumps were present; and by 5 minutes, agglutination was gone. The authors felt that the return of platelet concentrations to normal was due to platelet release from clumps, interestingly, while still in the presence of a marked heparinemia.

Fidlar[542] had similar results, with platelet concentrations dropping transiently, sometimes to 10% of preheparin values, with intravenous heparin infusions. The degree of thrombocytopenia varied among dogs, and was associated with clumping in all dogs

from 2.5 minutes to more than 1 hour after injection. Continuous infusions maintained depressed counts, though not as low as the initial drop. Leukocyte concentrations tended to drop similarly to platelets.

Rowsell[132] more recently reported that 50, 90, and 220 U/kg heparin subcutaneously every 8 hours had no effect on platelet concentrations in dogs, though a transient thrombocytopenia in the first hour would not have been detected by the protocol. Platelet survivals in the heparinized dogs were assessed by diisopropyl fluorophosphate-^{32}P. While 220 U/kg heparin decreased platelet survival without affecting platelet concentrations, 50 and 90 U/kg heparin actually prolonged platelet survival. The findings suggest that there may be an acute platelet redistribution after heparin injection that is associated with platelet clumping but not necessarily shortened platelet survival. At high doses of heparin, platelet survival may be shortened, indicating a consumptive or destructive mechanism.

Lastly, sequestration has been considered a possible contributor to the thrombocytopenia of some infectious diseases to be discussed in later sections.

c. severe blood loss

Experimentally induced severe acute blood loss has caused moderate thrombocytopenia in dogs when accompanied by volume replacement.[133,134] Thrombocytopenia was associated with regenerative thrombopoietic responses characterized by increases in mean platelet volume and platelet density. Milder thrombocytopenia has developed 6 to 10 hours after severe acute hemorrhage without volume replacement.[135]

Clinically, thrombocytopenia has been attributed to blood loss in dogs.[25] In general, however, clinical hemorrhage alone is unlikely to cause clinically significant thrombocytopenia.[119,136,137]

d. pathologically decreased platelet survival

The average lifespan of canine platelets in the peripheral blood is approximately 5-7 days whether measured with radioactive[2] or newer biotinylation techniques.[138] When this lifespan is shortened by an accelerated removal of platelets from the circulation, thrombocytopenia will occur if there is no compensatory increase in thrombopoiesis. Accelerated removal may be due to an increased utilization of platelets, often called consumption, and/or to a destruction of platelets by immunologic or nonimmunologic means. Platelet survival studies can be used to confirm accelerated platelet removal, but they have not been applied clinically to dogs.

(1) nonimmunologically decreased platelet survival

(a) drugs and foreign materials

Nonimmunologic, consumptive, drug-induced thrombocytopenias have been reproducibly caused by several pharmacologic agents used clinically or experimentally in people and in dogs. Other drugs have been noted to cause such thrombocytopenias during toxicity trials in drug development studies. It is likely that some of these go unreported and published reports underestimate the number of pharmacologic compounds found to induce thrombocytopenia. The following serve as examples.

i) heparin

As discussed previously, platelet survival may be decreased in dogs given 220 U/kg heparin subcutaneously every 8 hours.[132] However, thrombocytopenia did not occur, so the clinical significance of this finding is questionable. There have been no reports of clinically significant heparin-induced thrombocytopenia in dogs.

ii) protamine sulfate

Protamine sulfate is used to reverse the effects of heparin. In heparinized or nonheparinized dogs, it can induce a relatively severe thrombocytopenia[139-141] with platelet accumulation in the lungs.[142] Though protamine-heparin complexes activate complement, complement appears to have no role in the thrombocytopenia induced by protamine in dogs.[143] The thrombocytopenia may relate to the direct and immediate proaggregatory effect of protamine on washed human platelets.[144]

iii) human IVIgG

Human gamma globulin (IVIgG) preparations have recently been evaluated for use in treating immune-mediated thrombocytopenia and hemolytic anemia in dogs. A single infusion of 1 mg/kg IVIgG to dogs induced an unexplained mild thrombocytopenia (148,000/μl) with a nadir on days 7 and 8.[145] This effect would be antagonistic to the desired effect if it also occurs in dogs with IMT. Therefore, while a proven therapy for people with IMT, further studies are indicated before human IVIgG can be recommended for use in dogs.

iv) GM-CSF

Canine[146] and human rGM-CSF[147] were shown to cause a reproducible, dose-dependent, mild to marked thrombocytopenia in normal dogs. Thrombocytopenia was associated with normal or increased numbers of marrow megakaryocytes, large circulating platelets, and decreased platelet survivals. While production may have been affected to some degree, the findings were more consistent with a consumptive thrombocytopenia.

v) monocrotaline pyrrole

The pyrrolizidine alkaloid monocrotaline is toxic to endothelial cells and causes acute and persistent thrombocytopenia in experimentally exposed dogs.[148] The thrombocytopenia is associated with platelet deposition in the lungs, and while it has been termed sequestration, it is likely not a passive process.

vi) MDL 19,669

Thrombocytopenia, apparently associated with decreased platelet survival, occurred in dogs receiving the antidepressant compound MDL 19,660.[149] Thrombocytopenia reached maximal severity (10,000 - 40,000) by 1 week, and platelet concentrations returned to at least baseline values by 4-7 days after cessation of dosing.[150] Megakaryocytic hyperplasia, increased numbers of large platelets with abundant rough endoplasmic reticulum, and improvement in platelet concentrations while dogs were still receiving the compound suggested a responsive productive capacity of the bone marrow. Platelet vacuolation was felt to be suggestive of possible direct damage to the platelets. There

was no evidence of DIC or splenomegaly.[150] The precise mechanism of this drug-induced thrombocytopenia was not determined, but immune mechanisms and direct drug effects were considered. However, direct proaggregatory effects were not detected, and prednisone did not protect from the thrombocytopenia. PAIg was not measured. These studies demonstrate the difficulties encountered in understanding the precise mechanism of drug-induced thrombocytopenia, even in an experimental setting.

vii) foreign surfaces

Dogs have commonly been used to test the biocompatibility of biomaterials such as plastics used for various sorts of catheters. Polyvinyl-coated polyethylene tubing is an example of such a material which induced thrombocytopenia in test dogs.[151]

viii) cationized albumin

Experimental use of cationized albumin has induced thrombocytopenia in dogs.[152] This may, in part, be due to direct effects since cationized albumin caused platelet aggregation in vitro.

ix) other

In people, bleomycin, desmopressin, mitomycin, ristocetin, and hematin may also cause nonimmune destruction of platelets.[11] These and other drugs may do the same in dogs.

(b) disseminated intravascular coagulation

Consumptive coagulative processes such as disseminated intravascular coagulation (DIC) are, by definition, associated with the consumption of platelets, fibrinogen, and coagulation factors. In dogs, such excessive activity of the coagulation system has been reported in association with numerous conditions including sepsis, necrosis, neoplasia, overheating, severe trauma, pancreatitis, snake envenomation, hepatic disease, gastric dilation and volvulus, congestive heart failure, and heartworm disease.[153-159] In DIC, evidence of systemic thromboembolism and organ failure may be present. Platelet and fibrinogen consumption may be evidenced by decreased circulating concentrations, though survival studies may be necessary to document increased consumption when production partially compensates or even exceeds increases in consumption. When consumption of coagulation factors occurs at a rate greater than compensatory production, and plasma concentrations of platelets and coagulation factors are dramatically reduced, hemorrhage may ensue.

Clinical and laboratory evidence for DIC indicate a nonimmunologic consumptive contribution to thrombocytopenia, but other mechanisms responsible for thrombocytopenia in dogs with DIC depend on the underlying disease. An immunologic component may be present. Platelets from a dog with DIC of unknown etiology were positive for PAIg in one report.[160] Dogs with DIC of unreported cause have had positive results for possible antiplatelet antibodies using the PF3 test or the direct megakaryocyte immunofluorescence assay.[161] The relationship of such findings to DIC itself versus the underlying condition requires further studies.

Experimentally, consumptive coagulation with mild to moderate thrombocytopenia has occurred in dogs autotransfused after massive intraperitoneal hemorrhage.[162] Compared to control dogs transfused with banked blood, autotransfused dogs had prolonged PT and PTTs, elevated FDPs, and decreased concentrations of factors II, V, VIII, fibrinogen, and platelets. Very low platelet concentrations in the blood processed for autotransfusion probably contributed to the greater thrombocytopenia as compared to that occurring with banked blood transfusion, but the reasons for the consumptive coagulopathy are not clear.

(c) infections/sepsis

While thrombocytopenia associated with infection may result from any combination of pathophysiologic mechanisms, decreased platelet survival is likely to contribute in many cases. Thrombocytopenia may occur sporadically with many infections, especially in terminal patients with widespread sepsis. In such patients, thrombocytopenia may relate as much to the patient's status as to the disease. There are some infectious agents, however, that regularly induce thrombocytopenia by decreasing platelet survival.

i) gram-negative bacteria

Endotoxemia from gram-negative bacterial infections is frequently associated with thrombocytopenia.[163] Experimental infusions of endotoxin to dogs results in marked thrombocytopenia[49,164-166] which may be the result of numerous factors including the generation of platelet activating factor,[167] direct effects on the platelets, and the initiation

of DIC. Platelet depletion to under 5% of control dogs by estrogen administration did not alter the hemodynamic responses, mortality, or gross necropsy findings induced by endotoxin injection.[49] Thrombocytopenia, therefore, appears to be secondary and not a primary cause of the salient clinical features of canine endotoxic shock.

Direct effects of endotoxin on canine platelets have been evaluated by in vitro aggregometry. The studies to date indicate that endotoxin binds to canine platelets, apparently nonspecifically,[168] and causes them to passively agglutinate[168] without granule release[169,170] or thromboxane production.[171] This direct effect appears to be dependent upon the complement system,[169] which is also necessary for endotoxin-induced aggregation and release of rabbit platelets.[172,173] The contribution of these interactions to in vivo platelet clearance is unknown.

Endotoxin can also stimulate procoagulant activity on monocytes leading to the surface generation of thrombin which may accelerate platelet clearance.[174] This mechanism may contribute to thrombocytopenia in many infections accompanied by histiocytic/granulomatous proliferations.

ii) leptospirosis

Thrombocytopenia is a typical finding in dogs with leptospirosis.[175] Experimental infections of dogs with *Leptospira interrogans* serovars have produced variable thrombocytopenias and associated hemorrhage in concert with increased FDPs.[176,177] The findings have suggested that low grade DIC may cause or contribute to the thrombocytopenia, possibly secondary to endothelial cell damage. Leptospiral toxins may be responsible for the endothelial cell damage as well as acting directly on platelets, plasma

proteins, or other tissues. Leptospiral extracts have been shown to clump platelets in vitro in a manner similar to *E. coli* endotoxin, but how much this may contribute to the observed thrombocytopenia in infected dogs is not known.[178]

iii) nematodes

Parasitism by the lungworm *Angiostrongylus vasorum* has been associated with mild to moderate thrombocytopenia that occurred in conjunction with laboratory evidence of a consumptive coagulopathy.[179] However, an immune-mediated mechanism involving immune complexes and accelerated platelet destruction was entertained as a reasonable cause or contributor to the thrombocytopenia. No data were generated to support or refute this hypothesis.

Canine heartworm disease, caused by the filarial nematode *Dirofilaria immitis*, has frequently been associated with thrombocytopenia.[25,180,181] The pulmonary arteritis responsible for the clinical signs of heartworm disease is undoubtedly also directly responsible for part of the thrombocytopenia.[181] Damaged endothelium and dying heartworms are associated with platelet consumption and formation of thromboemboli, and platelet hyperaggregability has been documented.[180] Direct platelet interactions with parasites and their products may also be important contributors to thrombocytopenia. Thrombocytopenia may be worsened by accompanying hemolysis or DIC.

DIC has been established as a cause or contributor to thrombocytopenia in some dogs with heartworm disease, including dogs with and without the vena cava syndrome.[158] However, where low-grade DIC has been suggested to contribute to thrombocytopenia in some dogs with heartworm infections, the consumption of fibrinogen

and coagulation factors has not been proven,[181] so the thrombocytopenia may have other origins as well. Thrombocytopenia sometimes occurs or worsens with adulticide treatment, presumably due in part to activation and consumption in thromboemboli associated with dying heartworms.

One dog with heartworm disease and clinical IMT has been reported to have increased PAIg,[160] though the two may have been unrelated. PAIg findings for a large number of heartworm-infected dogs have not been reported, but infected dogs have been shown to have increased concentrations of circulating immune complexes[182] which might contribute to thrombocytopenia. Direct Coombs' testing of heartworm infected dogs has failed to indicate a major immunologic contribution to the anemia of heartworm disease.[183]

iv) Rocky Mountain spotted fever

Thrombocytopenia is the most consistent hematologic finding in Rocky Mountain spotted fever (RMSF) caused by *Rickettsia rickettsii*.[184] The organism is distributed by the vector ticks the American dog tick (*Dermacentor variabilis*) and the wood tick (*Dermacentor andersoni*) which are found in the eastern and western United States, respectively. While thrombocytopenia is usually mild, it may be severe, especially if DIC occurs as a terminal event.[184] Hemorrhage may occur in infected dogs, even without marked reductions in platelet concentrations. In a prospective study of 28 dogs with confirmed naturally-acquired RMSF,[185] 18% had petechial or ecchymotic hemorrhages, and some of these dogs had platelet concentrations greater than 50,000/μl.

Other common clinical findings include anorexia, lethargy, fever, lymphadenomegaly, and cutaneous edema and necrosis.[185,186]

Thrombocytopenia has consistently occurred in experimental RMSF infections,[186,188,538] though clinical hemorrhage has not.[538] When thrombocytopenia was associated with petechial and ecchymotic hemorrhages, there was microscopic evidence of necrotizing vasculitis involving capillaries, veins, and small arteries.[186] Vascular lesions were thought to be of two forms. Dogs dying acutely had endothelial damage suggestive of direct effects of the organism. Later lesions were similar to immune-mediated vasculitides, suggesting the possibility of an immune-complex pathogenesis. In human RMSF patients, vasculitis leads to increased vascular permeability, microthrombus formation, and hemorrhage such as focal retinal hemorrhages.[187] Increased retinal vascular permeability has also been documented in experimentally infected dogs.[188] This generalized vascular endothelial damage is presumably the cause of thrombocytopenia in RMSF,[184,189] but whether it results from a direct effect of the organism, immune-complex disease, or some other mechanism is unknown.[188]

The fact that the organism replicates in endothelial cells of small blood vessels and capillaries is suggestive that direct damage contributes to the vasculitis.[187] Human platelets have been shown to adhere much more to R. rickettsia-infected cultured human endothelial cells than to uninfected controls, even when cell damage was minimally detectable by transmission electron microscopy. Thus, endothelial cell infection in vivo may alter the cells in such a way as to attract and consume platelets leading to thrombocytopenia. With endothelial cell damage, platelets may also be consumed as they patch vascular defects by binding to subendothelial collagen.

Platelets from dogs with experimental RMSF tended to respond more to ADP than did those of uninfected controls; the aggregation slopes, maximum amplitudes, and the percentage of dogs with irreversible aggregation were greater in the RMSF group. This may have been related to their increased mean platelet volumes.[190] Hyperaggregability and increased mean size are consistent with the proposed peripheral consumptive process. However, DIC does not seem to be operative in typical natural or experimental infections.[185,186,188] There is no evidence to date to incriminate a role for endotoxin.

While immune complexes may play a role in the pathogenesis of RMSF vasculitis, immune complex vasculitis was considered an unlikely cause of increased retinal vascular permeability in experimentally infected dogs.[188] This was because a humoral response was not detected until post-inoculation day 8, but platelet concentrations began to decrease on day 4 and retinal vascular permeability increased on day 6. Also, vascular repair occurred as titers continued to rise. Others have investigated the plasma from dogs with naturally-acquired RMSF for PBIg[185] using the indirect ELISA of Campbell.[191] All 6 samples tested were negative. While these findings offer no evidence for an immunologic contribution to the vasculitis and thrombocytopenia, they do not exclude the possibility.

v) infectious canine hepatitis

Dogs with experimentally-induced infectious canine hepatitis (canine adenovirus-1) developed mucosal and cutaneous petechiae in association with platelet concentrations less than 50,000/μl.[192] Thrombocytopenia was accompanied by decreased platelet retention on glass beads, prolonged PT and PTT, depressed coagulation factor VIII concentrations,

increased FDPs, and increased numbers of circulating megaplatelets. While bone marrows of surviving dogs were hyperplastic, those of fatally infected dogs were hypoplastic with megakaryocytic hypoplasia. Erythrocyte fragmentation, decreased platelet survivals, decreased aggregatory responsiveness to ADP with less secondary aggregation, and ultrastructural evidence of vacuolation and dilation of the open surface canalicular system were also noted.[193] Together, the findings suggest the development of DIC, most likely secondary to widespread endothelial cell damage by the virus. Direct effects of the virus on the platelets and megakaryocytes could not be excluded as contributory to the thrombocytopenia.

vi) herpesvirus

Puppies infected with canine herpesvirus may have marked thrombocytopenia associated with diffuse necrotizing vasculitis and widespread petechial hemorrhages.[194] Thrombocytopenia is presumably secondary to the vasculitis which may also lead to DIC.

vii) septic peritonitis

Experimentally, marked thrombocytopenia occurred secondary to septic peritonitis in dogs given intraperitoneal processed human feces.[195] Lack of complement consumption suggested that the thrombocytopenia was not mediated by complement. Decrements in platelet concentration were associated with increments in fibrinogen concentration, but fibrinogen kinetics, coagulation times, and fibrin/fibrinogen degradation products were not assessed to determine if increased consumption of coagulation factors was present. The role of endotoxins was not investigated.

Other infections associated with thrombocytopenia that may be partially attributable to decreased platelet survival will be discussed below.

(d) neoplasia

The thrombocytopenia of neoplasia may be attributable to many mechanisms including several associated with pathologically decreased platelet survival. Because of the multifactorial nature of the thrombocytopenia and the difficulty in defining the principal cause in any particular case, neoplasia-associated thrombocytopenia will be discussed below.

(e) envenomation

The venom of some snakes contains platelet-activating factors that can produce a marked thrombocytopenia in the absence of DIC, while others induce thrombocytopenia associated with DIC.[156]

(f) extracorporeal circulation

Extracorporeal circulation of human blood during open heart surgery causes a platelet function defect and thrombocytopenia with the formation of platelet micro-particles, possibly due to activation by foreign surfaces and turbulent flow. Canine platelets are similarly affected.[196]

(g) zinc-induced hemolytic anemia

Thrombocytopenia has been reported in association with some cases of zinc-induced hemolytic anemia in dogs,[197,198] and in some cases it was apparently a manifestation of DIC.[197,199] However, the release of ADP from lysed erythrocytes may be contributory to platelet consumption in this and other forms of hemolytic anemia. In human infants with erythroblastosis fetalis, a moderate thrombocytopenia may occur. The platelets do not share the antigenic target that is responsible for the immune-mediated erythrocyte destruction; rather, it appears that platelets are destroyed as a result of breakdown products of erythrocytes.[11]

(h) hypophosphatemia

Hypophosphatemia produced by hyperalimentation in dogs has caused thrombocytopenia associated with shortened platelet survival and decreased clot retraction.[200] Affected dogs had megaplatelets, megakaryocytic hyperplasia, and hemorrhage. Platelet ATP concentrations were decreased, perhaps due to the inhibition of anaerobic glycolysis induced by hypophosphatemia, since phosphate supplementation prevented these changes. The contribution of concurrent hypophosphatemia-induced erythrocyte hemolysis to the thrombocytopenia was neither discussed nor evaluated.

(i) thrombotic thrombocytopenic purpura/hemolytic uremic syndrome

Thrombotic thrombocytopenic purpura (TTP) is a clinical syndrome characterized by hemolytic anemia with erythrocyte fragmentation, thrombocytopenia, neurologic

abnormalities, and usually fever and renal dysfunction.[11] The syndrome has been associated with autoimmune diseases and infections, but the pathogenesis of the syndrome is largely unclear and likely multifactorial. It is possible, however, that altered vascular endothelium may be the unifying disorder in TTP. Hemolytic uremic syndrome (HUS) is a related syndrome characterized by acute renal failure, fever, hemolytic anemia with erythrocyte fragmentation, and thrombocytopenia. HUS is more common in children than in adults, and it differs from TTP in that renal failure predominates and neurologic abnormalities are usually absent. The syndrome has been associated with infections, certain drugs, and antibodies reactive with endothelial cells. As with TTP, it seems likely that the vascular endothelium, especially of the renal vasculature, is altered and dysfunctional.

A syndrome similar to HUS has been reported in 4 dogs, each less than 2 years old.[201] These dogs had acute onset of vomiting and lethargy, and they developed severe thrombocytopenia with renal failure. Other findings included a moderate number of schizocytes, increased FDPs, sometimes mild hemolysis, fibrin thrombi in arterioles and capillaries (including those of the kidneys), and in 2 dogs, widespread petechiae and generalized seizures late in the course of the disease. More cases and more work on this syndrome will be necessary to define it and compare it with the human syndromes.

(j) vasculitis

Diseases associated with thrombocytopenia and vasculitis include RMSF, canine herpesvirus infection, and HUS and have been discussed.

(k) other

The human model would suggest that several other nonimmunological, consumptive thrombocytopenias are likely to occur in dogs, but they have not been specifically reported. For example, human burn patients may have thrombocytopenia whether or not they become septic, and it appears from studies in rats that thermal injury may cause localization of platelets in damaged tissues.[11] Mild thrombocytopenia occurs in about 20% of human patients with aortic valvular stenosis, presumably due to turbulent blood flow or interactions with the deformed valve.[11] Fat embolism is also commonly associated with thrombocytopenia in people.[11] Thrombocytopenia may occur in these and similar conditions in dogs, but to date, confirmed associations have not been reported.

(2) immunologically decreased platelet survival

(a) anaphylaxis

Severe thrombocytopenia has occurred in association with anaphylaxis in people and laboratory animals.[11] To the extent that this is a hypersensitivity reaction, the mechanism of the thrombocytopenia is immune-mediated. However it is not a type II hypersensitivity as is typical of IMT. The thrombocytopenia may relate to immune complex interactions with platelets, DIC, or other factors. Similarly, mild to moderate decreases in platelet concentrations have been associated with exposure to allergens in sensitized people. This may be due to the interaction of IgE with platelets, a population of which appears to bear Fc receptors for IgE.[202]

Thrombocytopenia has been a consequence of Hymenoptera stings in some dogs, but the precise mechanism is unknown.[203] Nonimmunologic effects of the venom and allergic responses have been suggested causes, with DIC likely occurring in at least some cases.

(b) secondary IMT

IMT may sporadically occur independently but concurrently with other diseases. However, when it occurs in association with other conditions at a frequency greater than would be expected by just coincidence, IMT has been considered a secondary process induced by the underlying disease.[204] The presence of elevated PAIg indicates a probable immunologic component to the thrombocytopenia and suggests that the thrombocytopenia is due to premature clearance of immunoglobulin-coated platelets from the circulation. In human medicine, secondary IMT has been associated with many conditions, and in some cases, IMT precedes other signs of the underlying disease. A list of human conditions associated with secondary IMT may be a useful guide to anticipate canine diseases that may lead to IMT.

Numerous drugs can induce secondary IMT in people, and specific antibody binding to platelet glycoproteins has been documented in many cases.[205,206] IMT also frequently occurs simultaneously with other autoimmune diseases including immune-mediated hemolytic anemia, immune-mediated granulocytopenia, and systemic lupus erythematosus.[204,207,208] Neoplasms commonly associated with IMT include Hodgkin's disease and non-Hodgkin's lymphoma, acute and chronic lymphocytic leukemia, and a variety of solid tumors.[207,209,210] Immune-mediated platelet destruction may contribute to

the consumptive/destructive thrombocytopenia frequently seen in human septicemic patients without laboratory evidence of DIC.[211-213] Other human diseases with well established ties to IMT are thyrotoxicosis and infectious mononucleosis.[204] Less clear associations with IMT have been reported for scleroderma, Hashimoto's thyroiditis, Gaucher's disease, Crohn's disease, sarcoidosis, rheumatoid arthritis, myasthenia gravis, ulcerative colitis, tuberculosis, brucellosis, and histoplasmosis.[204]

Some of these or similar relationships may occur in dogs, but because adequate testing for IMT is rare, our knowledge in this area is limited. Without widespread use of direct assays for PAIg in dogs, it is difficult to make strong statements about which canine diseases can cause secondary IMT. Such associations are spotty and have usually been made through insensitive or nonspecific indirect assays for PBIg, direct megakaryocyte immunofluorescence assays, or by analogy to the human model. Only a few cases of a few diseases have been substantiated with direct assays for PAIg.

i) drug-induced

From an antibody detection perspective, two classes of drug-induced platelet-reactive antibodies need to be considered: 1) drug dependent antibodies which require the presence of the drug or its metabolite in the assay mixtures for their detection (e.g. quinidine), and 2) drug-induced but drug-independent antibodies which behave like autoantibodies in that the drug's presence is not required for binding (e.g. gold).[214] Drug-dependent and drug-independent antibodies may be present concurrently after exposure to some drugs.[215]

In people, numerous drugs have been associated with the production of drug-dependent antibodies that bind to platelets and appear to be instrumental in producing thrombocytopenia. Drug-induced IMT has been most clearly established for allylisopropylacetylurea (Sedormid), quinine, quinidine, sulfonamides, heparin, cimetidine, organic arsenicals, methyldopa, and gold salts.[204] Evidence that thrombocytopenia is immune-mediated and drug-induced in these patients includes: 1) the development of thrombocytopenia and PAIg after drug exposure, with a delay consistent with an immune response; 2) with some drugs, demonstration of in vitro drug-dependency of the binding of serum or plasma antibodies to platelets; 3) resolution of thrombocytopenia and PAIg levels after drug withdrawal; and 4) relapse after re-exposure to the drug. Either the parent drug or a metabolite may be the offending agent.[216]

In dogs, drug-induced IMT has been suspected in association with several drugs. However, good evidence has often been lacking due to inadequate assays for PAIg. Drugs that have been incriminated include auranofin (an organic gold compound) and gold sodium thiomalate, high-dose cefonicid and cefazedone (second generation cephalosporins), trimethoprim-sulfadiazine, levamisole, and possibly diethylstilbestrol and dapsone.

a) gold salts

In the case of gold salts, 3 of 14 and 2 of 14 dogs respectively receiving high doses of oral auranofin or injectable gold sodium thiomalate developed thrombocytopenia after 45 to 72 months of dosing in long-term drug toxicity trials.[217] The platelet concentration nadirs ranged from 4,000 to 124,000/μl, and thrombocytopenia was

associated with large circulating platelets and megakaryocytic hyperplasia. Two of the 5 dogs also had hemolytic anemia with spherocytosis, and one of these dogs was Coombs'-positive for IgG. With cessation of dosing, thrombocytopenia spontaneously and completely resolved in 2 of the dogs within 1 week, and by day 12 in a third. The other two dogs required prednisolone therapy to which they initially responded quickly, though one of the two dogs suffered several relapses as attempts were made to reduce the dose of prednisolone which was finally discontinued after 34 weeks.

Compared to healthy, nonthrombocytopenic controls, all 4 of the 5 gold-treated dogs tested for PAIg with a SpA-based radioimmunoassay were positive at least once while thrombocytopenic. In the dog with recurrent relapses, the amount of PAIg correlated inversely with the platelet concentration ($r=0.82$). While the difference between negative and positive was small, the radioimmunoassay results provided evidence that the thrombocytopenias were immune-mediated. It is not known why the disorder occurred in only the high-dose group.

Variable and sometimes prolonged persistence of gold-induced thrombocytopenia and PAIg has also been reported in people where there is evidence that gold salts can induce the production of autoantibodies reactive with platelets in the absence of gold.[218] As in the dogs, people receiving gold salts may not become thrombocytopenic for many months. This gold-induced thrombocytopenia may actually be an autoimmune phenomenon as opposed to most well-studied, drug-induced IMTs in people which do not persist after drug withdrawal.

In a single case report, a dog with bullous pemphigoid developed marked thrombocytopenia with accompanying hemorrhage during therapy with aurothioglucose.[219]

Recovery was spontaneous after cessation of aurothioglucose therapy. When the dermatosis recurred, aurothioglucose was used at 1/4 the dosing frequency, and thrombocytopenia did not develop. Testing for IMT was not done.

b) sulfonamides

IMT has frequently been associated with sulfonamide antimicrobials in people,[205,216] and evidence is mounting for a similar toxicity in dogs. Positive PAIg was reported in a dog that developed thrombocytopenia secondary to treatment with the potentiated sulfonamide trimethoprim/sulfadiazine.[160] This dog also had an increase in serum PBIg that apparently bound without the addition of drug to the assay. It was not clear from the report if drug therapy was causative or simply coincidental in this case.

A more thoroughly examined case of thrombocytopenia in a dog treated with trimethoprim/sulfadiazine has also been reported.[220] The assessment of the pathogenesis of thrombocytopenia in this case was indirect and based on detection of an undefined humoral factor (considered to be most likely an antiplatelet antibody) capable of lysing and fragmenting platelets. The 7-month-old dog developed epistaxis after about 1 week of trimethoprim/sulfadiazine therapy for vaginitis. The dog was severely thrombocytopenic with megakaryocytic and granulocytic hyperplasia and a mean platelet size of about 3 times normal. Coagulation times were normal and rickettsial titers were negative. The platelet fragmentation index, a measure of the percentage of platelets less than 2.19μm^3 in volume, was about 5 times normal. Antibiotic therapy was discontinued and the platelet concentration rose from 15,000 to 80,000/μl over the next 2 days.

Six days later, with no therapy, the platelet concentration had climbed to 182,500/μl, the mean platelet volume had dropped to 10.62μm^3, and the fragmentation index had dropped to about 2 times the mean of normal. At day 24, the platelet concentration was normal, the mean platelet volume was mildly elevated, and the fragmentation index was essentially unchanged. All 3 parameters were normal at 7 months. In further tests, acute patient plasma (presumably citrated) induced significantly more fragmentation of normal platelets when incubated with them for 1 hr at 37°C than did normal citrated plasma or citrated plasma from a normal dog treated with trimethoprim/sulfadiazine. The acute plasma therefore contained a thrombolytic factor that the controls did not, and this was considered to be, most likely, an antibody. Proof that the thrombolytic factor was an antibody was lacking in this case, but such a hypothesis is consistent with the findings. Besides an antiplatelet antibody, however, other possibilities for the nature of the humoral thrombolytic factor include immune complexes, endotoxin, or other activators of complement.

A report often cited in discussions of drug-induced thrombocytopenia in dogs relates to a systemic allergic response to trimethoprim/sulfadiazine in 6 Doberman Pinschers.[221] However, only 1 of the 3 dogs tested was thrombocytopenic, and no details specific to the thrombocytopenia were reported. The drug reaction appeared to involve an immune-mediated vasculitis with complement consumption and may have been a type III hypersensitivity reaction. One dog was rechallenged and redeveloped the condition with sulfadiazine but not trimethoprim; this dog did not have thrombocytopenia. This thrombocytopenia probably related more to a type III than a type II hypersensitivity reaction.

In another case report, a single dog treated with the sulfone dapsone for 5 days developed a regenerative anemia, hypoproteinemia, and a marked thrombocytopenia associated with petechial and ecchymotic hemorrhages.[222] No megakaryocytes were detected in an otherwise cellular bone marrow, and the dog's platelet concentration was unresponsive to several transfusions with platelet-rich plasma over the next few days. The dog died, and at necropsy there was evidence of pancreatitis and DIC. A PF3 assay was later done in the presence and absence of dapsone and was negative. The pathogenesis of the thrombocytopenia is unclear in this case, but could have been due to a dapsone-induced immune response directed against megakaryocytes or megakaryocytes and platelets.

Reference has also been made to a dog that developed marked thrombocytopenia and epistaxis in apparent relation to sulfonamide antibiotic therapy. In this case, the condition resolved without therapy.[2] Another dog clinically diagnosed with IMT had been treated with sulfonamides/penicillin within 2 weeks of the diagnosis.[40]

c) cephalosporins

In toxicity trials with the second generation cephalosporins cefonicid and cefazedone, dogs developed dose-dependent anemia, neutropenia, and thrombocytopenia.[223-225] As first described, it was considered likely that the hematologic changes were the result of multiple mechanisms including peripheral destruction and bone marrow damage. An immune-mediated component was suggested by the delayed onset of cytopenias, a shortened period of induction on rechallenge, evidence of increased hemophagocytosis, erythrocyte spherocytosis, and positive direct antiglobulin tests.

Studies were done to assess for ^{125}I-SpA-detectable antibodies associated with platelets (direct assay), erythrocytes (direct assay), and granulocytes (indirect assay) in dogs developing cytopenias after treatment with high-dose cephazedone. Of the 14 treated dogs, 7 became anemic, 7 became neutropenic, and 11 became thrombocytopenic. Of the affected dogs examined, 6/7 with anemia, 9/9 with thrombocytopenia, and 7/7 with neutropenia had positive results with the respective assays compared to controls.

The assay for PAIg was very similar to that described in the studies of gold-induced thrombocytopenia in dogs.[217] Concerns about this assay, and therefore the validity of the results, are more thoroughly addressed in the section on veterinary assays for PAIg and PBIg. It is important to note the absence of a positive control and the weakness of the positivity of test samples. While the highest positive result was elevated 3 times the cut-off value of 2 standard deviations above the mean of at least 4 controls run in the same assay, the next 2 most elevated results were just 1.7 and 1.4 times the cut-off values. Two of the positive samples were less than 2% above the cut-off values. Furthermore, the range of cut-off values for different runs of the assay was 1.13 to 1.64, and 5 of the positive results fell within this range (though the values were higher than the control cut-offs for the day). While the samples reported as positive may well have been, confidence is low for those values falling within or close to the range of interassay variation.

Similar comments can be made about the weak positivity of results for the erythrocyte assay where positive results ranged from 1.2-2.4 times the mean of 3 controls. As for the indirect assay for granulocyte-bindable immunoglobulins, the positive results were higher, ranging from 1.6-6.6 times the mean of 4 controls. It is

important to mention that serum samples were heated to 56°C for 60 minutes prior to assaying. Such heating will produce aggregated immunoglobulins which may bind to leukocytes via Fc receptors. While samples from normal controls were also heated, the effects of heating may or may not be similar among samples.

While immune-mediated mechanisms may have contributed to the thrombocytopenia in these dogs, the assays do not provide compelling evidence that IMT was of primary importance. Evidence against drug-induced IMT was the recovery of platelet concentrations during ongoing drug administration in 5/11 dogs.[226] Perhaps the evidence of myelotoxicity was of greater significance. This included persistent deficits of CFU-E and CFU-M progenitor cells,[226] as well as reversible ultrastructural damage to marrow cells including mitochondrial damage in megakaryocytes.[227]

High doses of cefmetazole have also been shown to induce mild thrombocytopenia in dogs, reversible after cessation of dosing.[228] The mechanism of the thrombocytopenia was not reported and apparently not pursued.

d) diethylstilbestrol

Diethylstilbestrol (DES), a nonsteroidal synthetic with estrogenic activity, was implicated in the etiology of IMT in a dog treated with it for 3-4 years.[229] Thrombocytopenia was associated with petechial and ecchymotic hemorrhages, an active bone marrow, and a PF3 assay result that was "probably positive" in the presence of DES but negative in its absence. These results were considered diagnostic in light of the fact that corticosteroid treatment had been initiated before the sample was collected. However, in the single control sample, DES alone caused a significant shortening of the clotting

time (the end point of the assay), from 98.5 to 71.0 seconds, so the additional 8 seconds of shortening in the test sample is of questionable significance. DES was discontinued and the dog responded to corticosteroid treatment but later relapsed upon steroid discontinuation. A second PF3 assay at that time reportedly "confirmed the previous results implicating diethylstilbestrol". When the dog relapsed a third time, it was splenectomized and then remained asymptomatic for at least 24 months.

The findings of an active bone marrow and the favorable response to corticosteroids were good evidence that estrogenic myelosuppression was not the problem. However, a definite cause-and-effect relationship between the drug and the thrombocytopenia was not established. The fact that this dog relapsed twice in the absence of continued DES administration is not supportive of a typical drug-dependent IMT in which cases there is rapid recovery with drug withdrawal and recurrence of thrombocytopenia only with drug rechallenge.[204] Only if DES had induced a true autoimmune response which persisted after drug removal, or if DES had remained in the dog's system for a prolonged period of time after it was discontinued, could a drug-induced IMT be considered. As for the assay results, the initial PF3 results were borderline, and the second set of data was not presented. Overall, the conclusion that this dog had a drug-dependent IMT is not particularly compelling.

e) levamisole

A heartworm infected dog treated with levamisole for about 4 weeks developed thrombocytopenia and petechial hemorrhages in association with megakaryocytic hyperplasia in the bone marrow.[230] The drug was discontinued and the condition resolved

without therapy. Based on these findings, the authors felt that immune mechanisms probably contributed to the thrombocytopenia, and that the lack of large circulating platelets and an immaturity of the megakaryocytes suggested impaired thrombopoiesis as well. It was also mentioned that 2 other dogs were known to have become thrombocytopenic while on levamisole therapy, but in no case was specific evidence presented to implicate an immune pathogenesis.

f) other

The following drugs have been said to cause immune-mediated thrombocytopenia in dogs, but reports to adequately substantiate these claims are lacking: phenylbutazone, styrid caracide, amphetamines, and dilantin.[136]

ii) systemic immune-mediated disease

There is not a clear division between primary IMT and secondary IMT associated with systemic immune-mediated conditions, since many human patients with IMT also have high titers of antinuclear antibodies but do not develop SLE.[208,231,232] The thrombocytopenias associated with human SLE and IMT are felt to share a common pathogenesis; decreased platelet survival is secondary to increased PAIg caused by autoantibodies or immune complexes. Generally, the systemic immune-mediated diseases with which IMT may be associated are characterized by general B-cell activation with the production of autoantibodies directed toward multiple targets. Immune complexes are often abundant leading to widespread organ injury by type III hypersensitivity.[233]

Primary IMT and SLE may represent different clinical presentations on a spectrum of these autoimmune disorders. The same may be true in dogs.[234,235]

a) systemic lupus erythematosus

The occurrence of thrombocytopenia in association with canine SLE was first reported in 1965,[236] and it is now an expected potential finding.[233] The role of PAIg in SLE has been presumed,[235] but recent positivity for PAIg in 2 dogs with SLE is direct evidence that PAIg mediates the thrombocytopenia in canine SLE.[160] Positive results with direct megakaryocyte immunofluorescence or with indirect assays have been reported in many dogs with SLE.[160,161,191,237-241]

Thrombocytopenia has accompanied juvenile-onset polyarthritis syndrome in Akitas, an immune-mediated syndrome which can be very similar to SLE.[242]

b) Evans' syndrome

Evans' syndrome, as first described, was the co-occurrence of thrombocytopenia or neutropenia with immune-mediated hemolytic anemia.[243] The term now defines the co-occurrence of IMT and immune-mediated hemolytic anemia. In his original 1949 description of the syndrome,[243] Evans showed great foresight when he wrote: "If thrombocytopenic purpura is due to the formation of an antibody-like substance similar to that found in acquired hemolytic anemia both deficient formation and excessive destruction may be important in producing the extreme degrees of destruction sometimes observed. Sensitized platelets may be susceptible to agglutination, and phagocytosis and the presence of an anti-platelet antibody in the circulation may damage the cytoplasm of

the megakaryocyte so as to inhibit the formation of platelets." He also suspected that if such were the case, multiple antibodies rather than one with a broad spectrum of reactivity would account for the multiple cytopenias, because there was no correlation between the severity of hemolysis and the degree of thrombocytopenia. Indeed, the antibodies responsible for the concurrent cytopenias in at least some human patients have since been shown to be distinct, since those eluted from one cell type do not react with the other cell type.[244] The canine situation awaits further studies.

The occurrence of thrombocytopenia in association with canine immune-mediated hemolytic anemia was first reported in 1965.[245] The canine syndrome was felt to mimic Evans' syndrome in people, but studies of PAIg in dogs with Evans' syndrome have not been reported. An immune-mediated pathogenesis has been supported, however, by positivity of indirect assays for PBIg or by direct megakaryocyte immunofluorescence assays in some tested dogs.[161,191,239-241,246,247]

Positive indirect results for PBIg have also been reported in a dog with steroid responsive idiopathic neutropenia and thrombocytopenia, and in a dog with thrombocytopenia and red cell aplasia.[241]

c) other

IMT has occasionally been clinically diagnosed or suspected in dogs with rheumatoid arthritis or pemphigus.[25,237]

iii) neoplasia

Thrombocytopenia is common in dogs with neoplasia, and it may occur for numerous reasons, both immunologic and nonimmunologic (see below). It has been suggested that immune-mediated mechanisms may be an under-recognized contributor to thrombocytopenias associated with human neoplasms.[210] In a review of 2,059 dogs with neoplasia, 10% were thrombocytopenic but IMT was diagnosed in none of them.[248] However, appropriate tests were not available. In other reports, immune-mediated platelet destruction has been repeatedly incriminated in dogs with a variety of neoplastic processes.

Foremost on the list of associated neoplasms is probably lymphoma. A retrospective review of the Veterinary Record Data Program, a data repository for veterinary patients visiting teaching hospitals across North America, showed a significant association between diagnoses of lymphosarcoma and diagnoses of IMT.[249] That is, dogs with IMT more commonly had lymphoma than did dogs without IMT. Which occurred first and what the mechanistic nature of the relationship is was not assessed. It should be noted that the data spanned 27 years, and the diagnostic criteria were not standardized in any way. Moreover, the diagnosis of IMT in most cases was likely based on just clinical criteria.

Positive indirect assays for PBIg and direct megakaryocyte immunofluorescent assays have been used to support the diagnosis of lymphoma-associated IMT in several reports.[161,241,250,251] Similar assays have also incriminated hemangiosarcoma,[241] lymphangioma,[252] multiple myeloma, and myelogenous leukemia.[161] One dog has been reported with elevated PAIg and myelodysplasia.[160]

The possibility that immune mechanisms play a role in the thrombocytopenia of some canine nonhematopoietic neoplasms was specifically assessed in 7 thrombocytopenic dogs with cancer and positive direct megakaryocyte immunofluorescence assay (D-MIFA) results.[253] Cancers included benign mixed mammary neoplasms, mast cell neoplasia, hemangiosarcoma with metastases, nasal adenocarcinoma, and a fibrosarcoma. As will be discussed below, the reliability of D-MIFA results is uncertain, so the immunological nature of the thrombocytopenias in this study is also uncertain. D-MIFA results were slightly positive in 2 of the nonthrombocytopenic controls with neoplasia, suggesting that these dogs had compensated thrombocytopenias or that positive results were somewhat nonspecific. Each dog had increased marrow megakaryocytes, but only 1 of 6 tested had a positive PF3 assay result. Platelet concentrations increased in 4 of 4 dogs whose cancers were treated without immunosuppressive therapy, suggesting that the thrombocytopenia was related to the cancer. The positive D-MIFA in thrombocytopenic but not nonthrombocytopenic cancer patients, and the conversion to negative after treatment and normalization of platelet concentrations implicated an immune pathogenesis. Unfortunately, there were no control dogs with regenerative but nonimmune thrombocytopenia. It was felt that immune complexes may have contributed to the IMT because they were at higher concentrations in dogs with neoplasia than in the same dogs during remission. DIC was excluded by laboratory findings, and only 1 dog had splenomegaly.

iv) infectious diseases

The potential immune-mediated contribution to the thrombocytopenia of infectious diseases will be discussed in this section with primary reference to human diseases.

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While similar contributions are likely and should be sought for canine diseases, there are currently little supportive data. The human situation may serve as a useful guiding model for canine studies.

Many bacterial, viral, fungal, and protozoal diseases have been associated with IMT in people.[11,211] In general, infection-associated IMT may be related to cross-reactive antibodies, to bacterially-induced production of autoantibodies, to the exposure of platelet cryptantigens by the presence of the organisms, to the binding of antibodies to infectious agents (or their parts) attached to the platelets, or to the induction of platelet-associated immune complexes.[11,211]

Some thrombocytopenic human patients with bacterial sepsis have had elevated PAIg which has been considered indicative of an immune-mediated component to the thrombocytopenia.[213,254] Kelton used a direct complement lysis inhibition test and showed that PAIgG was increased in 16 of 21 episodes of thrombocytopenic gram-positive or gram-negative septicemia but in only 1 of 31 episodes of nonthrombocytopenic septicemia.[213] Others have shown that serum from neonates with bacterial or viral infections frequently contained PBIg, though the assay used was not quantitative.[255]

Lelie[256] measured PAIg with a platelet immunofluorescence assay in septicemic human patients and suggested that the PAIg represented specific autoantibodies. Increased PAIgG was present in 8/13 (62%) patients with thrombocytopenic septicemia and also in 11/17 (65%) nonthrombocytopenic, septicemic patients. After successful therapy, 6-29 days after acute testing, 10/14 (71%) patients still had elevated PAIgG. Ether eluates of PAIg or positive sera were not reactive with Glanzmann type I platelets, indicating specificity of the PAIgG for GPIIb/IIIa. However, the eluate IgG did not react

well or at all with platelets prepared in citrate instead of EDTA. These findings are consistent with antibody reactivity to cryptantigens induced in vivo by bacteria and in vitro by EDTA.

Of relevance to PAIgG in sepsis are the reports of direct bacterial binding to platelets. *Staphylococcus aureus* binds to washed human platelets in a rapid, saturable, and reversible manner independent of protein A and plasma cofactors.[257] Moreover, the binding can be blocked by incubation with a monoclonal antibody to FcγRII indicating a role for the platelet Fc receptor. Similar direct binding to human platelets and evidence for a specific receptor-ligand interaction of viridans group streptococci has been shown.[258] In this case, however, binding was not Fc-mediated. These findings are relevant to the discussion of elevated PAIg in sepsis because if bacteria are bound to platelets, antibodies bound to the bacteria could also be associated with the platelets leading to elevated PAIg. This mechanism is also relevant to the IMT that complicates some human malarial infections, in which case specific IgG binding to platelet-bound malarial antigen has been shown to be Fab-mediated.[259] The presence of platelet-bound organisms is also likely to contribute to platelet removal through such mechanisms as platelet activation, facilitated phagocytosis, and complement activation. The relative contribution of immunologic and nonimmunologic platelet destruction in sepsis remains to be determined.

As for viruses, specific antiplatelet antibodies identified in some thrombocytopenic patients with concomitant viral infections are evidence for a virally-induced autoimmune component to the thrombocytopenia.[260] Specific autoantibodies, usually directed against GPIIb/IIIa have been reported in association with such viral diseases as measles, acquired

rubella, HIV infections,[261] varicella, and infectious mononucleosis.[260] The factors involved in the inconsistent production of these antibodies remain to be determined.

The thrombocytopenia associated with HIV infection appears to have a multifactorial and stage-dependent pathogenesis. Platelet survival studies indicate that decreased platelet survival is of primary importance relatively soon after infection, but bone marrow suppression is generally more prominent at later stages in patients with AIDS-related complex or frank AIDS.[262] Elevated PAIg is thought to be responsible for platelet destruction, and roles for specific autoantibodies,[261] cross-reactive anti-viral antibodies,[263,264] immune complexes,[265] and complement[265] have all been described. Megakaryocyte infection may be responsible for decreased production.[266,267]

Until PAIg is evaluated in a considerable number of dogs with infectious diseases, the role of immune-mediated platelet destruction in such dogs will go uncharacterized. A direct test for PAIg was positive in 1 dog each with concurrent *Ehrlichia canis*/*Babesia canis* infection and dirofilariasis.[160] Otherwise, IMT has been associated with infections by indirect evidence for platelet-reactive antibodies or by test results suggesting concurrent immunologic disorders related to other cells. For example, positive Coombs' tests[268] and antinuclear antibodies[268] reported in dogs with bacterial endocarditis suggest the potential for IMT in canine bacteremias. Diseases for which immune-mediated contributions have been repeatedly considered include canine monocytic ehrlichiosis, RMSF, histoplasmosis, leishmaniasis, and distemper. Only in the case of canine distemper are there currently enough data to support a major immunologic component to the thrombocytopenia.

Canine distemper virus (CDV), a moribillivirus (family *Paramyxoviridae*) antigenically related to human measles virus, has been associated with marked thrombocytopenia in experimentally infected puppies,[111] though thrombocytopenia has not been consistently recognized in older or spontaneously infected dogs.[269] Thrombocytopenia also develops in dogs vaccinated with modified live virus (MLV) distemper vaccines.[270-272]

In the experimentally induced infections, inoculated puppies were thrombocytopenic by postinoculation day (PID) 5.[111] On PID 7, platelet-associated CDV was detected by direct immunofluorescence on acetone-fixed, air-dried cytocentrifuge preparations. IgG but not IgM or C3 was found on the same platelets as CDV antigen from day 7 on, and the amount of PAIgG and platelet-associated CDV antigen increased as viremia progressed. Surprisingly, titers of CDV-specific IgG were never detected. An indirect protein A-colloidal gold technique was used to show that from PID 7 on, dense aggregates labeling specifically for CDV were present on the surface and in the open canalicular system of platelets, though whole virions were not identified. Phagocytosis of platelets by Kupffer's cells was prominent from PID 5 onward. These findings suggest that a complement-independent, immune complex-induced destruction of platelets may cause or contribute to the thrombocytopenia of experimental distemper virus. However, the nature of the proposed complexes and their orientation with respect to the platelet surface are not known. Fc receptor binding was suggested, but there is no evidence that dogs have a platelet Fc receptor. Antibody binding to platelet-bound CDV antigen could also account for the findings.

In the same studies, CDV antigen was associated with megakaryocytes from PID 4 on, but it was most abundant after PID 8.[111] The density of megakaryocytes in the marrow of infected dogs was constant. The absence of a responsive megakaryocytic hyperplasia suggests that impaired megakaryocytopoiesis and thrombopoiesis may also have contributed to the thrombocytopenias.

The thrombocytopenias reported in dogs after vaccination with MLV distemper vaccines have been transient and of variable degree.[270-272] Puppies vaccinated at 4 and again at 7 weeks had decreased platelet concentrations that were lowest 1 week after vaccination.[272] The average reduction in platelet concentrations after primary and secondary immunizations were 33 and 30% respectively. However, no age-matched nonvaccinated controls were concurrently evaluated. Others[273] have found that dogs boostered with a MLV distemper-hepatitis virus vaccine tended to have mild to moderate decreases in platelet concentration about 1 week later, though none became thrombocytopenic. No adverse effects on ADP-induced platelet (PRP) aggregation were detected in these vaccinated dogs.

In another study, mature dogs vaccinated with a MLV vaccine for distemper, hepatitis, and parainfluenza had significantly reduced platelet concentrations 2 days after vaccination, averaging a 48% decrease from baseline values.[271] Platelet concentrations appeared to be normal by 96 hours after vaccination. Significant platelet decrements did not occur in 3 of the 9 dogs, but thrombocytopenia was marked in 1 dog (15,000/μl). Levamisole given to another group of nine dogs at the time of vaccination prevented the decrease in platelet concentrations. This protection may relate to levamisole's immunomodulatory activity. Distemper infections are known to induce marked

immediate and long-term effects on the structure and function of lymphoid tissue, sparing neither B nor T lymphocytes, but the contribution of these changes to the thrombocytopenia is unknown.[274] Levamisole may modulate these effects.

Reportedly, deer, fox, bear, and raccoons vaccinated with MLV measles or canine distemper vaccines also have a consistent drop of about 100,000 platelets/μl 3-5 days after vaccination and lasting for as long as a week.[22]

While experimental distemper vaccinations have generally not induced marked thrombocytopenia, clinical purpura has reportedly followed 1-21 days after routine clinical vaccinations with MLV vaccines, including those for canine parvovirus.[22,275] Detailed reports or further studies of these clinical observations have not been published. A fever and platelet destruction immediately following vaccination were considered sufficient to show that the "reactions clearly were vaccine related".[22]

Support for clinical purpura following vaccination comes from the human model. Acute thrombocytopenic purpura was the most frequent complication requiring hospitalization of Finnish people vaccinated with a live-virus measles, mumps, and rubella vaccine.[276] Of the 1.5 million people vaccinated over 12 years, thrombocytopenic purpura was reported in about 50 of them, usually 2-3 weeks after vaccination. PAIg was increased in many of these patients, and circulating antibodies with specificity to GPIIb/IIIa were present in some.[277]

v) alloimmune neonatal

Alloimmune neonatal thrombocytopenia occurs in human infants when the mother has become immunized by fetal platelets possessing paternally-derived antigenic

determinants recognized as foreign by the mother. In people, transplacentally acquired maternal immunoglobulins cause platelet destruction in utero. Neonatal alloimmune thrombocytopenia has also been described in pigs, in which case the sensitized dam passes the platelet-reactive antibodies to the newborn pig through her colostrum.[278] While not described in dogs, this form of IMT may certainly occur.

vi) posttransfusion purpura

Posttransfusion purpura (PTP) is a distinct entity in human patients occurring about 1 week after a transfusion.[204] It is associated with severe thrombocytopenia and high titers of platelet-reactive alloantibodies which mediate destruction of transfused as well as autologous platelets despite the fact that autologous platelets, after recovery, lack the reactive epitope for the alloantibody in question. This destruction of autologous platelets, apparently by an alloantibody, is incompletely understood.

Posttransfusion purpura may also occur passively if alloantibodies are inadvertently transfused. In this case, thrombocytopenia occurs within a few hours.[11]

Destruction of autologous platelets has been reported for 2 dogs given multiple transfusions of allogenic platelets.[279] While thrombocytopenia did not occur with the first 25 and 34 transfusions, the next transfusion for each dog caused dramatic thrombocytopenia, in either 3 days or 2 hours. Platelet concentrations rose over the following few days, faster in one dog than in the other, and subsequent transfusions were associated with repeated, but milder, depressions in platelet concentrations. The authors considered an immune complex mechanism the most likely.

While details were not provided, elevated PAIg has been reported in a single dog that developed thrombocytopenia 14 days after a blood transfusion.[160] This dog may have had posttransfusion purpura.

Unrelated is the marked but transient post-transfusion thrombocytopenia that has been reported to occur in canine recipients of DEA1-incompatible erythrocytes or plasma containing anti-DEA1 alloantibodies.[280] The thrombocytopenia occurred and resolved within a few hours of transfusion, and was associated with poor clot retraction. The rapid recovery and transient nature of the thrombocytopenia suggests that sequestration occurred, and it was hypothesized to be due to immune adherence to the antibody-coated erythrocytes.[281]

vii) hypothyroidism

Preliminary studies have suggested that as a group, hypothyroid dogs have higher platelet concentrations and lower mean platelet volumes than do euthyroid controls.[282] On the basis of these altered indices and suspicions that hypothyroidism may predispose to IMT in dogs, it was suggested that dogs of known thyroid status be assessed for PAIg and platelet survival to determine if hypothyroidism is accompanied by accelerated immune-mediated platelet destruction.[282]

viii) other

IMT has been diagnosed in 6 canine recipients of DLA-incompatible fetal liver hematopoietic cell transplants.[283] These dogs developed thrombocytopenia after recovering from postirradiation nadirs of platelet concentration, and their bone marrows

were hypercellular with megakaryocytic hyperplasia. All 6 were positive for megakaryocyte-associated immunoglobulin by a direct megakaryocyte immunofluorescence test, and 5 of the 6 had positive PF3 results. The cause of post-transplantation induced IMT in these dogs was undetermined.

(c) primary IMT

A great deal of progress has been made over the last 30 years toward understanding primary IMT in people, but many questions remain. Efforts to characterize and understand canine IMT are comparatively only preliminary. In this section, the human condition will be reviewed first to provide a solid basis for comparison with the canine analogue which will be reviewed second.

i) human

a) acute IMT

Acute IMT may be better classified as a secondary form of IMT[284] because it usually occurs in the winter and spring 1-21 days following viral infections.[204] However, the relationship between infection and thrombocytopenia is unclear and often inapparent.[204] It will therefore be considered a primary form of IMT for this discussion.

Acute IMT is a gender-neutral disorder occurring in people of all ages, but usually in children from 1 to 10 years old.[285] Clinically, there is typically a sudden onset of petechiae and mucocutaneous hemorrhages. Mild splenomegaly and lymphadenomegaly are often found, but they are thought to relate to recent infections. Thrombocytopenia is often marked ($< 10{,}000/\mu l$) and accompanied by a normal to increased number

of megakaryocytes in the bone marrow. PAIg may be elevated[204] but circulating titers of GPIIb/IIIa and GPIb/IX-bindable IgG are often undetectable or only mildly elevated.[286] A predominance of circulating IgM has been reported to bind to GPIb, GPIIb, GPIIIa, and several undefined proteins.[287]

Acute IMT usually resolves spontaneously within 1-2 months, though about 10% of children with presumed acute IMT develop chronic IMT.[284] The best mode of therapy for acute IMT is somewhat controversial since most patients recover regardless of the therapeutic protocol, and some recover even if untreated.[204,284,288] Treatment options include gamma globulin, glucocorticoids, anti-D globulin, and splenectomy in selected cases. On an individual basis, it is not always possible to differentiate acute IMT from chronic IMT. People with an initial diagnosis of acute IMT are considered to have chronic IMT if they do not recover within 6 months.[284]

Several pathogenic mechanisms have been proposed to explain acute IMT, including roles for immune complexes, cross-reactive antibodies, anti-idiotype antibodies, altered platelet surfaces, inappropriate immune responses, and immune stimulation by superantigens.[284] The mechanism of superantigens has recently been reviewed.[289] The occurrence of acute IMT in the convalescent stage of viral infections would seem to argue against viral/antiviral immune complexes which would presumably have been present much earlier. Anti-idiotypic antibodies formed in response to antiviral antibodies may mimic platelet antigens and induce more antibody production; this phenomenon occurs in a murine model of SLE.[290]

b) chronic IMT

Chronic IMT appears to be an autoimmune disease analogous to autoimmune hemolytic anemia.[208] It is primarily a disease of adults, with women being affected 3 to 4 times as frequently as men.[204,285] HLA type may influence susceptibility to chronic IMT.[204] As opposed to the acute condition, chronic IMT usually has an insidious onset and does not resolve spontaneously. Platelet concentrations usually range from 5,000 to 100,000/μl, and accompanying clinical signs, if present, include petechiae and hemorrhages associated with the skin and mucous membranes. As with acute IMT, megakaryocytes are typically in normal to increased numbers in the bone marrow. Splenomegaly is mild when it occurs, and it occurs only occasionally; its presence should suggest an alternative diagnosis.[204,284]

Various combinations of increased PAIgG (all subclasses), IgM, IgA, and complement are usually demonstrable, with IgG predominating.[204,291-296] Specific platelet-associated or platelet-bindable autoantibodies reactive with the major platelet glycoproteins are currently detectable in about 80% of patients.[297-299] The detection of serum titers of antiplatelet antibody is less consistent and quite variable among studies and assays. Recently, at least one platelet-reactive immunoglobulin was detected in 85% of sera tested from human patients with chronic IMT.[299] The high percentage detected was attributed to detecting IgA and IgM classes in addition to IgG, and to using an expanded panel of membrane glycoproteins compared to most studies.

The target epitopes of autoantibodies in chronic IMT are generally public antigens shared by normal platelets from most people.[101,296] Targets have been identified by several approaches including immunoblotting, immunoprecipitation, reactivity with

platelets congenitally deficient in particular surface glycoproteins, and by techniques based upon the immobilization of potential surface targets by use of murine monoclonal antibodies. The epitopes most frequently identified are on glycoproteins IIb, IIIa, the IIb/IIIa complex, Ib/V/IX, IV, and Ia/IIa,[285,296-302] but other targets possibly including glycosphingolipids[303] and phospholipids[304] have been reported. In a recent study, GPs IV and Ia/Iia were never reactive to serum antibodies without reactivity of either GPIIb/IIIa or GPIb/IX.[299]

Some patients have clinical hemorrhagic signs but platelet concentrations sufficiently high that normal hemostasis would be expected. Several platelet membrane glycoproteins including GPIIIa, GPIIa, P-selectin, and possibly GPIb are present on endothelial cells, so it is possible that antibodies react with both targets in some IMT patients.[305-308] This could compromise vascular integrity and allow hemorrhage at apparently protective platelet concentrations.

In others patients with IMT, antibodies are directed against important functional platelet epitopes on GPIb, GPIIb/IIIa, and GPIIIa.[286,309-311] In one patient, clinical thrombasthenia occurred in the absence of thrombocytopenia due to specific platelet autoantibodies directed against GPIIb/IIIa.[312] In a study of another 49 IMT patients with normal platelet concentrations after treatment, 3 (6%) had continued bleeding tendencies and 18 (37%) had in vitro platelet aggregation defects, perhaps related to functional impairment of platelets by circulating antibodies[310]

Usually, however, platelets in patients with chronic IMT tend to be young and large with an increased average functional capacity that is associated with shorter bleeding times than those of patients with similar platelet concentrations from decreased

platelet production.[313,314] Also, an increase in circulating platelet microparticles (PMPs) has been reported in IMT patients, as well as in patients with other platelet destructive states, and these PMPs appear to provide significant hemostatic activity.[315]

Antibody-coated platelets are generally thought to be removed from circulation by mononuclear phagocytic cells in the spleen and liver,[293,316] though the bone marrow may play a significant role as well.[317] Increased PAIgG apparently leads to an increased hepatic role of platelet destruction.[318] Kinetic studies have usually indicated shortened platelet survivals consistent with accelerated platelet destruction.[100] However, kinetic studies must be interpreted carefully because in thrombocytopenic conditions from any cause, a greater percentage of the total platelet mass will be consumed by normal physiologic maintenance of vascular integrity. Thus platelet survivals may be decreased despite primary production problems.[100,319] This is similar in dogs as evidenced by shortened survival of transfused platelets in dogs made thrombocytopenic by lethal irradiation as compared to normal recipients.[67]

Platelet production may be decreased in some patients with IMT,[100,320] possibly because megakaryocytes are functionally damaged by antibodies. Because most patients have a normal or increased number of megakaryocytes indicative of adequate or increased megakaryocytopoiesis, thrombopoiesis has generally been considered to be accelerated. However, an increased number of marrow megakaryocytes may not reflect increased platelet production if megakaryocyte function is impaired.[320] Megakaryocytopoiesis must be differentiated from thrombopoiesis.

Extensive antibody cross-reactivity between human platelets and megakaryocytes is well recognized and includes reactivity to GPIb, GPIIb, and GPIIIa.[99,321-328] However,

the techniques employed may not have differentiated exosolic binding from cytosolic binding, and may not have controlled for the potential binding of detecting antibodies to megakaryocytes by megakaryocyte Fc receptors. Immune complexes have been shown to bind to human megakaryocytes identified by concurrent staining for GPIIb and IIIa, suggesting the possibility that megakaryocytes may share the FcγRII of platelets.[324] Recently, human megakaryocytes were shown to synthesize and express FcγRII receptors, and similarly to platelets, these receptors appeared to be much less abundant than GPIIb/IIIa.[329] It has been hypothesized that these receptors may be important in the uptake of immune complexes, including, perhaps, such viruses as HIV. In any case, their presence must be considered when interpreting megakaryocyte immunostaining.

While cross-reactivity between platelets and megakaryocytes exists, the contribution of megakaryocyte-associated immunoglobulin to thrombocytopenia in IMT has not been determined. Rats made thrombocytopenic with rabbit anti-[rat platelet] serum appeared to have severely impaired megakaryocyte maturation, whereas rats made thrombocytopenic by repeated platelet pheresis had accelerated maturation, though their platelet concentrations were 50% higher.[330] Megakaryocyte maturation and health were assessed by morphological appearance, proportions of maturation stages, rate of transit of tritiated thymidine through each maturation stage, and by megakaryocyte number. Dogs injected with rabbit anti-[canine platelet] antiserum developed marked thrombocytopenia associated with changes in the megakaryocyte populations.[331] These changes included a shift toward immaturity but with no apparent decrease in numbers, and morphologic abnormalities including cytoplasmic foaminess, vacuolation, karyorrhexis, nuclear swelling, and karyolysis. Occasional damaged megakaryocytes were surrounded

by mononuclear cells. Together, the findings suggest the potential for impaired thrombopoiesis despite stimulated megakaryocytopoiesis.

As for natural disease, megakaryocyte fluorescence was present in samples from human patients with chronic IMT but not normal controls when air-dried marrow smears were evaluated with an equine anti-[human IgG] serum.[332] Unfortunately, the presence of fluorescence from similarly treated normal platelets, and the inability to reproduce the results with a rabbit anti-[human IgG] serum confused the interpretation of these findings. Others[333] found that the megakaryocytes from 10/16 patients with chronic IMT, but from 0/28 thrombocytopenic or healthy controls reacted positively to a fluorescein-conjugated anti-[human IgA] serum. Reactions were only considered positive if fluorescence could be blocked by coincubation of the smears with fluorescent and nonfluorescent detector.

The megakaryocyte-reactivity of platelet-reactive immunoglobulin has also been assessed in two human patients with IMT; radiolabeled IgG obtained from their cultured splenic lymphocytes bound to normal donor megakaryocytes at least five times as much as IgG fractions of controls, and binding was prevented by prior incubation of the samples with normal platelets.[334] Moreover, many megakaryocytes from people with IMT have ultrastructural evidence of damage, primarily in the form of dilated demarcation membrane systems which is seen as vacuolation by light microscopy.[321]

The presence of normal to increased numbers of megakaryocytes in most bone marrow samples of IMT patients indicates that if antiplatelet antibodies commonly react with megakaryocytes, they react with only a subset of them and/or they do not cause complete cell destruction as they do for platelets. Levene[335] used a panel of monoclonal antibodies to show that megakaryocytes change their phenotype (antigen expression) as

they mature. The cross-reactivity is not complete between immature and mature megakaryocytes or between megakaryocytes and platelets, some antigens being lost with increasing maturity.[335] This may explain why some platelet-reactive antibodies react with only a subset of megakaryocytes.[99,326] Freeze-fracture and electron microscopic studies of megakaryocytes and platelets also suggest that the platelet membrane is structurally different from that of megakaryocytes.[336] Most platelets, and therefore their membranes, appear to originate from the interior and not from the external surface of megakaryocytes.[336] When megakaryocytes begin active thrombopoiesis, the internal portions are exposed and their antigenic properties are likely changed.

Further evidence of maturation-dependent reactivity was described by Stahl.[321] A rabbit antiserum to murine platelets reacted with the mature megakaryocytes releasing platelets but not with the smooth megakaryocytes lacking evidence of thrombopoiesis. And in people with IMT, 50-75% of the megakaryocytes were considered damaged, but only those whose demarcation membrane systems had opened to the exterior to elaborate platelets.[321] Reactivity with these late-stage megakaryocytes may explain why marrows of IMT patients often contain many megakaryocytes but little active platelet shedding. Such megakaryocyte reactivity may also explain the impaired platelet production, despite increased megakaryocyte cell cycle activity, suggested by autologous platelet survival studies in people with chronic IMT.[100] A better understanding of the contribution megakaryocyte-reactive antibodies make to thrombocytopenia in people with IMT awaits further studies. However, it is important to emphasize that the presence of a normal or increased number of megakaryocytes provides no information as to whether each cell is actually releasing a normal, increased, or decreased number of platelets.

Successful treatment of chronic IMT has been achieved with glucocorticoids, splenectomy, intravenous gamma globulin, anti-D immunoglobulin, azathioprine, cyclophosphamide, vincristine, cyclosporine, colchicine, ascorbate, danazol, and combinations of these.[285,288] Patients with platelet concentrations above 30,000/μl may not require more than careful monitoring.[285,310] Platelet transfusions should be used only in life-threatening situations where transient increases in platelet concentrations may prevent serious sequelae. Routine transfusions are not justified because the ubiquity of target antigens makes rapid destruction of transfused platelets a predictable outcome. Despite all therapies, over one third of 1,761 reported affected adults from 1928 to 1989 did not have a continuous and complete remission.[285] The search for better therapeutic alternatives for chronic refractory IMT continues.

Another form of chronic IMT which may go unrecognized is the compensated form when PAIg is present in the absence of thrombocytopenia. Increased thrombopoiesis maintains the platelet concentration in the normal range despite the presence of PAIg and increased immune-mediated platelet destruction. That the PAIg is pathogenic is supported by the development of thrombocytopenia in neonates born to mothers with this "hidden" disease.[337] Moreover, circulating antibodies in the mothers and their offspring react with identical glycoproteins.

ii) canine

a) introduction

As with people, dogs may have thrombocytopenia with no detectable abnormalities besides those related to low platelet concentrations. A recent direct assay for canine

PAIg has shown that some of these dogs have IMT, as evidenced by elevations in PAIg.[160] Acid eluates of platelets from some of these dogs contain immunoglobulins that rebind to allogeneic platelets.[338] Radioimmunoprecipitations using antibodies in the acid eluates have shown that some of these antibodies have specificity for GPIIb/IIIa.[339] These dogs presumably have IMT which is induced by the presence of surface PAIg. The underlying reason for elevated PAIg in these dogs is unknown, making the condition idiopathic but immune-mediated and autoimmune if the antibody binding is Fab-mediated.

Historically, many dogs with steroid-responsive thrombocytopenias have been diagnosed as having IMT or even autoimmune thrombocytopenia. In reality, diagnoses have been uncertain, being based on clinical findings or indirect antibody assays. Current indirect assays for canine PBIg generally have limited sensitivity and specificity and do not constitute definitive diagnostic tests for IMT (see below). Clinical diagnoses are also indirect and require the systematic elimination of other known causes of thrombocytopenia, something which has not always been thoroughly done. Response to therapy is diagnostically weak because thrombocytopenias may be due to unrecognized factors that are steroid-responsive, antibiotic-responsive, or transient and resolving despite therapy. Consequently, reports of IMT in dogs may include cases of nonimmune thrombocytopenia. Also, secondary and primary IMT have not always been clearly differentiated or discussed separately. Therefore, the following review of canine IMT based on reports in the literature is best considered a review of primary and secondary IMT and probably some nonimmune conditions as well. It serves as a record of the current understanding of the composite picture of what has been believed to be IMT in dogs.

b) history of canine IMT

Early case reports describing idiopathic thrombocytopenia in dogs were likely reports of IMT, but no supportive testing was available.[340-342] The occurrence of thrombocytopenia in association with canine systemic lupus erythematosus[236] and with canine immune-mediated hemolytic anemia[245] was first reported in 1965, and the latter situation was felt to mimic Evans' syndrome. The first report of studies on a series of dogs with IMT was published by Wilkins et al. in 1973.[239] Using the PF3 release assay with globulin fractions of canine patients, this was the first laboratory evidence that idiopathic thrombocytopenia in dogs may be immune-mediated. Work on improved assays for diagnosing canine IMT has continued, and a direct clinical assay for canine PAIg has been described.[160] Accurate differentiation of immune from nonimmune canine thrombocytopenic conditions awaits more routine use of this and other direct assays for PAIg.

c) signalment

It is probably safe to say that dogs of any breed, age, or gender may suffer from primary or secondary IMT, so the signalment of any particular patient is not useful diagnostically. While some investigators have found no breed predisposition,[40,239] others have reported that the following breeds are likely to suffer from IMT: toy poodles, miniature poodles, standard poodles, old English sheepdogs, cocker spaniels, and German shepherds.[22,25,161,343] Using a direct ELISA for PAIg, Lewis recently found that while 8 (24%) of the dogs with IMT were cocker spaniels, none of the 21 dogs in the non-IMT thrombocytopenic group was a cocker spaniel.[160] While based on submissions of samples

for Coombs' and PF3 testing rather than on results of these assays, the following additional breeds have been reported to be predisposed to immune-mediated blood diseases in general: Irish setters, miniature and standard dachshunds, Shetland sheepdogs, Scottish terriers, and vizslas.[22] Familial predispositions for IMT and/or IMHA have been reported in the following breeds: vizsla, American cocker spaniel, long-haired dachshund, Scottish terrier, and old English sheepdogs.[22]

The age of dogs with IMT has been reported to range from 7 months to 14 years, with 6-7 years a consistently reported mean.[22,25,161,239,343,344] Lewis[160] similarly found an age range from 1 to 15 years (mean 6.4).[160] The lack of an age predilection compared to total hospital admissions has been reported.[343] While a gender bias has not been apparent in some reports,[247,344] the overall number of females reported with IMT is about twice the number of males.[22,25,40,161,239,247,343,344] Lewis similarly found that 62% of the dogs with IMT were female while the non-IMT thrombocytopenic group was mostly (67%) male. A similar increased incidence in females also occurs with chronic but not acute IMT in human patients.[204] A female gender bias is not apparent for dogs with Evans' syndrome.[40,247]

d) patient history at presentation

Dogs with IMT have had clinical signs noticed 1 day to 6 months prior to admission.[343] When noted, most preadmission signs occurred within 3 days of presentation.[239] The most common presenting complaints for dogs with primary or secondary IMT are reportedly anorexia, epistaxis, gastrointestinal hemorrhage, bruises,

lethargy, weakness, oral bleeding, vaginal bleeding, ocular bleeding, stiffness, and hematuria.[40,343] Bleeding may be noted after mild trauma such as grooming.[2,345]

e) physical findings

The physical findings and clinical signs associated with IMT include those due to thrombocytopenia plus those referable to any underlying or concurrent disease process. The hemorrhagic signs related to thrombocytopenia predominate and include: cutaneous ecchymoses and petechiae, especially on the ventral abdomen and inner limbs; mucosal petechiae affecting oral, ocular, nasal, and genital mucosal membranes; ocular hemorrhage including hyphema and retinal hemorrhage; melena, hematochezia, and hematemesis; epistaxis; and hematuria.[2,40,161,239,246,343] Hemorrhage may involve any system including cerebral bleeding with sudden death.[346] Pallor may accompany substantial hemorrhage or occur in association with concurrent immune-mediated hemolytic anemia.[40] Fever[161,239,343] has been reported in up to 2/3 of dogs with primary or secondary IMT,[239] but in less than 10% of dogs thought to have primary IMT.[343]

Other commonly reported physical findings include lethargy/weakness, collapse, and dyspnea in dogs with primary IMT,[343] and systolic heart murmurs, splenomegaly, pale mucous membranes, shock, abdominal pain, lymphadenopathy,[40] infection, masses, and neurologic signs associated with hemorrhage[239] in dogs with either primary or secondary IMT. Some of these findings may have been coincidental, while others were likely related to the underlying disease with which IMT was associated. In some studies, splenomegaly in dogs has been used as it has in human patients[204] to exclude the

diagnosis of primary IMT.[343] Consequently, the incidence of splenomegaly in primary canine IMT is impossible to evaluate from reported studies.

f) laboratory findings

The primary laboratory abnormality in IMT is obviously thrombocytopenia. Thrombocytopenia is generally severe in reported cases of IMT, though one should consider the possibility that mild and moderate thrombocytopenia may occur but go unrecognized due to a lack of clinical signs. Moreover, when mild and moderate thrombocytopenias are encountered, they may be attributed to other causes because they do not fit the expected pattern of IMT. In any case, reported dogs have usually had fewer than 50,000 platelets/μl,[25,40,347] and frequently fewer than 10,000/μl.[343] As a group, these platelet concentrations have been found to be significantly lower than those of dogs with thrombocytopenias thought to be of nonimmune origin.[25,347] In contrast, Lewis[160] found that the platelet concentrations of dogs with IMT ranged from 4,000 to 555,000/μl (mean 91,000/μl) which was reportedly not different from dogs in the non-IMT group. However, 6 of the 34 dogs with IMT had responded to glucocorticoid therapy at the time of evaluation.

Microthrombocytes and megathrombocytes may be present on blood smears.[2,25] Decreased mean platelet volumes (MPVs)[220,347,348] and increased MPVs[26] have respectively been associated with immune-mediated platelet destruction and megakaryocytopoiesis in dogs. Decreased MPVs were found to be specific for IMT in one study,[347] though several concerns about this study will be discussed below. In summary, decreased,

normal, or increased MPVs and small, normal or large platelets may be present in dogs with IMT.

Clinical hemorrhage in dogs with IMT has been reported to be a consistent finding with platelet concentrations less than 50,000/μl,[239] though most reports associate bleeding with concentrations less than 30,000/μl.[343] In the latter study of primary IMT, 80% of the 54 dogs had fewer than 10,000 platelets/μl during bleeding episodes. Varying bleeding tendencies among dogs with similar platelet concentrations has been noted[349] and may relate to variations in vascular stability, trauma, the rapidity with which thrombocytopenia develops,[350] or other unknown factors. As discussed previously, the antigenic specificities of PAIg and the age and metabolic activity of the circulating platelets may also affect the occurrence of clinical hemorrhage.

Dogs with IMT may have a neutrophilia with, or more often without, a left shift, as well as a monocytosis.[2,25,40] However, this is inconsistent and leukopenia and neutropenia have also been reported.[2,25] Anemia commonly accompanies IMT,[2,25,40,343] occurring in roughly half of all reported cases. Anemia was documented in 83% of 45 dogs thought to have primary IMT, and it was usually regenerative and associated with hemorrhage, especially melena.[343] Decreased total protein[2] and laboratory evidence of iron deficiency[343] may be present in some dogs. Anemia may also occur because of concurrent immune-mediated hemolytic anemia, but it most commonly appears to be secondary to blood loss which is presumably related to decreased platelet concentrations.[40,161,239,247,351]

Bone marrow aspirations have not been associated with excessive or uncontrollable hemorrhage in dogs with IMT.[343] Megakaryocytes are usually in normal or increased

numbers, but they may be reduced in number or even absent.[246,343,352] The latter situations may be similar to the nonregenerative form of canine immune-mediated hemolytic anemia in which there appears to be a maturation arrest.[353] The prognosis appears poorer for dogs with marked megakaryocytic hypoplasia.[343,352] An increase in immature megakaryocytes is common, the ratio of mature to immature megakaryocytes changing from a normal of 2.35-5.25:1 to 0.17-0.85:1.[246,343,352] Morphologic changes reported in megakaryocytes from dogs with IMT include cytoplasmic vacuolation and foaminess, basophilia, and decreased granularity.[246,352] Bone marrow evaluation in dogs with isolated thrombocytopenia has been considered of limited help by some because megakaryocyte numbers are not consistently elevated in canine IMT.[22]

Recently, the potential for serum and IgG from dogs with IMT to inhibit platelet function was assessed using platelet aggregometry.[354] This indirect approach was chosen because patients were generally too thrombocytopenic for direct aggregation studies. If possible, direct studies would more clearly indicate the influence of PAIg on platelet function. Indirect studies may show effects due to alloantibodies or sample handling artifacts. In this study, some of the serum and purified IgG samples mildly inhibited the extent and rate of platelet aggregation, but no correlation could be made between the apparent platelet dysfunction and the severity of clinical bleeding. Moreover, the results are questionable for several reasons: 1) a collagen concentration that should induce platelet aggregation did not do so without addition of epinephrine, indicating a problem with the system (perhaps the ACD anticoagulant or the source of collagen); 2) control plasma was apparently used in place of serum, so the potential effects of serum factors were not controlled for; 3) sera were apparently not treated to remove residual thrombin;

4) the number of normal controls did not appear adequate to provide an adequate reference range, so the mild depression in aggregation may not have been significant; 5) serum and its corresponding IgG did not have the same effect in 30% of the possible comparisons; and 6) many samples were tested just once.

While some antiplatelet antibodies in canine IMT may bind to functional platelet epitopes and contribute to hemorrhage, they may also bind to endothelial cells and decrease vascular integrity. Shared antigenicity between canine endothelial cells and platelets likely exists. Dogs immunized with allogeneic aortic endothelial cells had greatly reduced allogeneic platelet survivals but normal autologous platelet survivals compared to control dogs.[355] Similarly, dogs sensitized to allogeneic platelets had accelerated vascular rejection of renal transplants, possibly related to shared antigenicity between platelets and endothelial cells.[356]

Dogs with idiopathic amegakaryocytic thrombocytopenia have been reported and may have had immune-mediated disease.[94,341] Canine platelets and megakaryocytes have been shown to share reactive sites including epitopes on GPIIIa,[331,357] though the techniques employed may have allowed detection of cytosolic epitopes. Dogs injected with rabbit anti-[canine platelet] antiserum developed marked thrombocytopenia associated with megakaryocyte immaturity and morphologic abnormalities described previously.[331] Similar morphologic changes including cytoplasmic foaminess, vacuolation, reduced cytoplasmic granularity, and karyolysis have been described in dogs with naturally occurring IMT.[246] Together, the findings suggest that impaired thrombopoiesis may occur despite stimulated megakaryocytopoiesis in at least some dogs with IMT.

g) therapy

In treating dogs with IMT, aspirin and other antiplatelet drugs should be avoided, any medications considered capable of inducing thrombocytopenia should be discontinued, and trauma should be minimized.[27] Underlying problems should be treated appropriately, but otherwise therapy is aimed at elevating platelet concentrations sufficiently to protect against clinical hemorrhage. While platelet concentrations in the normal range are optimal, a concentration of 50-100,000/μl is probably adequate in the absence of platelet dysfunction.[22,23,27,247,351] Efforts to reach values in the normal range can be frustrating for both the client and the veterinarian, and side effects of therapy can be a detriment to the patient. Occasional dogs may even do well clinically despite low platelet concentrations and no medical management.[247]

While immunosuppressive doses of corticosteroids are a routine part of therapy for IMT, additional treatments reflect clinical bias and are not based on randomized, controlled, clinical trials. Many approaches have been taken, and the common immunosuppressive options for immune-mediated diseases of dogs have recently been reviewed with respect to the pathophysiology of the disorders.[358] Various combinations of azathioprine, cyclophosphamide, and vincristine have been used with inconsistent success.[27,40,343] Danazol has reportedly been successful in treating selected dogs with refractory IMT.[234,359] Cyclosporine may also be effective in some dogs with chronic, refractory IMT.[360] Most recently, human gamma globulin (IVIgG) has been reported to be effective in treating dogs with IMT.[145] The clinical utility of this therapy awaits further studies.

The use of vincristine has been questioned due to reports that it may affect platelet function. Vincristine reportedly causes impaired ex vivo aggregation responses with platelets of treated human patients,[361] but it did not significantly impair function when given at recommended doses to normal dogs.[362,363] It has been used successfully to treat dogs with clinical IMT,[364] sometimes given in the form of vincristine-loaded platelets.[348,351,365] Since therapeutic concentrations do not appear to cause platelet dysfunction in dogs, concern over induction of such dysfunction is not justified.[363] However, studies in dogs with IMT are needed to test vincristine's therapeutic efficacy and to evaluate the severity of its side effects.

The value of splenectomy for canine IMT has frequently been discussed, but no large-scale investigation of its clinical utility has been undertaken. Post-splenectomy infection with *Hemobartonella canis* is a recognized complication, though the incidence appears to be low.[90,127,366] Splenectomy of dogs that have been sensitized to allogeneic platelets has inconsistently decreased the removal of transfused allogeneic platelets as measured by platelet survival studies.[279] Similarly, in IMT, splenectomy has sometimes been associated with clinical improvement[247,344,367,368] and sometimes has appeared to be unhelpful.[22,40,343,351] It remains to be seen if response to splenectomy can be predicted.

Transfusion has been suggested for presurgical boosting of the platelet concentration when dogs with IMT require surgery.[27] It has also been used to control severe hemorrhage.[239] However, it is not generally indicated because transfused platelets will be rapidly destroyed by circulating antiplatelet antibodies just as allogeneic platelets are rapidly cleared by alloantibodies in refractory dogs.[369]

h) outcome

Overall, as many as half the dogs with primary or secondary IMT may experience a single bout of thrombocytopenia followed by recovery.[40,239,344,350] Recovery may occur within a week,[350] though others have found that 2-35 days (mean of 8) were necessary for platelet concentrations to exceed 100,000/μl.[40] Other dogs respond initially but relapse, sometimes requiring continued therapy.[40,239,344,350,370] Relapses have reportedly been precipitated by the severe cold of winter or the heat of summer.[22] Live virus vaccination, infection, estrus, and pregnancy have also been incriminated, and ovariohysterectomy has been recommended.[27,136] However, these observations have not been substantiated. Approximately 20% of the dogs die without responding to therapy, often because of hemorrhage.[40,239,350]

Of 54 dogs diagnosed as having primary IMT based on thrombocytopenia, normal PT and PTT during bleeding episodes, no palpable splenomegaly, and no other diagnosis, the outcomes were as follows: 14/54 died naturally or from euthanasia during the first bout of thrombocytopenia; 17/54 had acute IMT and recovered after 1 bout; 8/54 had acute, recurrent IMT; and 15/54 had chronic IMT with recurrent episodes for up to 8 years.[343] Which of these outcomes a particular dog will have is currently unpredictable.[343,344] The major cause of death in dogs with chronic IMT has been considered to be discontinuation of treatment due to the cumulative expense of monitoring and treating dogs with an apparently incurable condition.[343]

It appears then, that dogs suffer from acute and chronic forms of primary IMT analogous to the human situation. Attempts to clearly establish any potential differences between these groups have not been reported. The acute form has been reported to occur

generally in younger animals 1-2 months after a viral infection or vaccination,[27] but the evidence to support this is weak or lacking. Prognosis has been considered good,[27] but if categorization as acute has been based on response to therapy, prognosis is by definition good. In the chronic form, onset is reportedly insidious and recovery is not spontaneous due to the continued production of PAIg.[27] Again, the outcome defines the category, but the outcome cannot yet be predicted by the presentation. Further studies using a direct assay for PAIg are required to better define types and potentially subtypes of canine IMT.

e. conditions for which the mechanism of thrombocytopenia is largely multifactorial or not clearly established

(1) infections

Some infectious diseases commonly associated with thrombocytopenia have been discussed in previous sections corresponding to the apparent primary mechanism by which thrombocytopenia appears to occur. For several canine infectious diseases, however, the pathogenesis of thrombocytopenia is either too complex or too poorly understood to categorize. These diseases are discussed below.

To reiterate previous comments, thrombocytopenia is commonly associated with canine infections, including those caused by viruses, bacteria (especially the rickettsials), protozoa, fungi, and nematodes.[25,116] While thrombocytopenia may be sporadically associated with secondary DIC in many critically ill dogs with many different infections, thrombocytopenia is part of the expected clinical picture with some infections. The pathogeneses of these infectious thrombocytopenias may be complicated, involving

various combinations of suppressed platelet production, altered platelet distribution, increased consumption, or immune-mediated and nonimmune destruction.

(a) rickettsials

As a group, rickettsial diseases are a common infectious cause of thrombocytopenia in endemic areas. With widespread travel being commonplace, these diseases must also be considered in regions less populated by their vector ticks.

i) canine monocytic ehrlichiosis

Canine monocytic ehrlichiosis (tropical canine pancytopenia[371] is caused by the obligate intracellular rickettsial *Ehrlichia canis* which is transmitted by its vector, the brown dog tick (*Rhipicephalus sanguineus*). The disease has three phases:[184] an acute phase 1-3 weeks after infection which lasts 2-4 weeks and is associated with myeloid and megakaryocytic hyperplasia;[112,372,373] a subclinical phase occurring 6-9 weeks postinoculation; and a chronic phase associated with unexplained bone marrow hypoplasia.[112,113] An inadequate cell-mediated immune response has been suggested to allow progression to the chronic phase.[374] Thrombocytopenia, the most consistent hematologic abnormality, occurs in each phase, and pancytopenia, occurring in fewer than 25% of reported dogs, characterizes the chronic phase.[184] However, in one review, most infected dogs (241/327) were not thrombocytopenic.[375] Granular lymphocytosis,[376] hyperglobulinemia, and neutropenia are also common in subclinical and chronic phases.[377]

Thrombocytopenic hemorrhage may occur, but it usually does not occur in the acute phase, even with significant thrombocytopenia.[184,375] Hemorrhage such as epistaxis

may also occur with normal or mildly decreased platelet concentrations, presumably because of platelet dysfunction.[82,378-380] Platelet dysfunction in those dogs with a marked gammopathy likely relates to hyperviscosity syndrome.[82,378,379]

Platelet dysfunction has been noted in experimentally infected dogs whose platelets had markedly decreased adhesiveness to glass beads.[381] Sera from other experimentally-infected dogs caused less PF3 release in a PF3 assay than did control sera.[382] Something in the serum seemed to inhibit rather than to stimulate platelets. In other studies, dogs infected with *E. canis* developed an uncharacterized serum factor that inhibited platelet migration and, compared to preinfection sera, caused platelets to be more rounded with reduced pseudopod formation.[383] The serum factor had no direct relationship to detectable antibody titers to the organism, and its inhibitory activity could be removed by prior incubation with platelets. The platelet migration inhibition appeared as thrombocytopenia developed, and remained during the chronic phase of the disease. The nature of this factor has not been determined.

The acute-phase thrombocytopenia has been considered to be due to a combination of consumption, sequestration, and antibody-mediated destruction,[184] while decreased production is thought to be the critical factor in chronic ehrlichiosis.[116] This interpretation has come largely from kinetic studies in experimentally infected dogs using ^{32}P-labeled autologous platelets,[384] ^{75}Se-selenomethionine and ^{51}chromium-labeled autologous platelets,[385] or ^{51}chromium-labeled homologous platelets.[382] Increased platelet destruction appears to occur within a few days after experimental infection, suggesting that the thrombocytopenia of acute ehrlichiosis is not entirely antibody-mediated. The presence of *E. canis* organisms in endothelial cells of experimentally infected dogs[386] suggests that

endothelial damage may contribute to consumptive thrombocytopenia. Concurrent with accelerated platelet destruction in acute ehrlichiosis, megakaryocyte production and platelet release appear to occur at an accelerated but inadequate rate to compensate for destruction. In the chronic phase, platelet survivals are moderately decreased, but not enough to suggest that destructive processes are the principal cause of thrombocytopenia.

Immune-mediated platelet destruction has been proposed to contribute to thrombocytopenia in dogs with canine monocytic ehrlichiosis. While negative PF3 results have been reported,[382] positive PF3 tests and increased antinuclear antibodies have also been reported in association with chronic, naturally-occurring ehrlichiosis.[380] A dog with clinical IMT and concurrent ehrlichiosis and babesiosis was positive for PAIg and PBIg,[160] and 3 other dogs with diagnoses of IMT by PF3 or megakaryocyte immunofluorescence tests had ehrlichiosis.[161] Positive Coombs' tests have been reported for a few infected dogs,[372,373,378,387] some being positive for IgG, IgM, and/or C3.[372] The significance of this is unknown, but it may relate to hyperglobulinemia or immune complex disease.[387] An immune complex mechanism is intriguing in light of the fact that proteinuria and renal lesions suggestive of immune-mediated renal damage have been reported to occur in dogs made thrombocytopenic by experimentally-induced acute infections.[388]

In another study of experimentally infected dogs, complement was consumed in vivo in infected dogs, and the drop in complement activity followed a few days behind the fall in platelet concentrations.[381] As this suggested a possible role for complement in causing the thrombocytopenia, dogs were treated with cobra venom factor (CVF) to reduce mean hemolytic complement activity before infection. Thrombocytopenia was

moderated in the CVF-treated dogs, but since a marked increase in platelet concentration occurred in the only CVF control dog, the moderated platelet concentrations may have been a direct effect of CVF. These studies neither demonstrate nor preclude a role for complement in the thrombocytopenia of canine monocytic ehrlichiosis.

Splenic sequestration may contribute to thrombocytopenia in some dogs, as splenomegaly has been present in natural[375,389] and induced infections.[112] However, only 20/345 (6%) dogs with ehrlichiosis that were evaluated for splenomegaly had it.[375]

In summary, the pathogenesis of thrombocytopenia in canine monocytic ehrlichiosis is not yet clear. Because of the possibility that thrombocytopenia is at least partially immune-mediated, short-term glucocorticoid therapy has been suggested when life-threatening thrombocytopenia is present.[375] Some clinicians have routinely treated dogs with tetracycline and glucocorticoids while awaiting serologic test results, because IMT and canine ehrlichiosis can appear very similar. However, dogs with ehrlichiosis usually have a fever and other constitutional signs,[375] while dogs with primary IMT do not.

ii) canine granulocytic ehrlichiosis

Thrombocytopenia appears to be the most common hematologic abnormality in canine granulocytic ehrlichiosis caused by *Ehrlichii ewingii*[390] in southern states[391] and apparently a different species in the upper midwest.[392] Clinically, granulocytic ehrlichiosis is associated with acute polyarthritis that is responsive to tetracycline.[393,394] Ehrlichial morulae are present in neutrophils and eosinophils, possibly monocytes, but not lymphocytes, basophils, or platelets.[391] Infected granulocytes may also be detected

in synovial fluid samples.[394] Platelet concentrations in 37 naturally infected dogs averaged 111,417 with a range of 34,000-196,000/μl, and clinical hemorrhage was not associated with the disease.[391,394]

In studies of experimentally-induced canine granulocytic ehrlichiosis, inoculated dogs have developed mild to marked (6,000/μl[395]) thrombocytopenia concurrently with neutropenia, lymphocytosis, pyrexia, morulae in neutrophils and eosinophils, and positive *E. canis* titers (about day 18-24).[396] Clinical polyarthritis has not generally developed, only 1 dog having had mildly swollen carpi.[395] It is therefore not yet clear if the organism causes the clinical polyarthritis with which it has been associated. Studies of the pathogenesis of thrombocytopenia have not been reported.

iii) infectious canine cyclic thrombocytopenia

In canine cyclic thrombocytopenia caused by *Ehrlichia platys*, organisms infect platelets and reside as morulae within membrane-lined vacuoles.[397] The mechanism by which platelets become infected is not known, but megakaryocyte infections have not been found.[397,398] Thrombocytopenia develops rapidly with the appearance of the organisms, and resolves within 3-4 days after their disappearance. It continues to occur every 1 to 2 weeks in experimental infections, but the first parasitemia is associated with significantly more infected platelets than are subsequent ones. This has led to the suspicion that direct effects of the organism may be responsible for the initial thrombocytopenia, and immune-mediated mechanisms may modulate thrombocytopenia in later phases.

In experimentally-induced disease, bone marrow cellularity was increased[399] and the density of megakaryocytes was about twice that of uninfected controls,[398] suggesting that decreased platelet survival and not decreased production causes the thrombocytopenia. Platelet aggregation was assessed by optical aggregometry of PRP from infected and control dogs. With acute infection, 5 outbred dogs but not 5 Beagles had significantly reduced mean maximal aggregation responses to very low (0.1 Mm) concentrations of ADP. It was suggested that in vivo platelet stimulation may make them refractory to ex vivo aggregation, the implication being that the organism may induce a hyperaggregable state in vivo leading to accelerated platelet consumption.

While vasculitis appears to contribute to thrombocytopenia in other rickettsial infections, vascular lesions have not been detected in experimental infections with *Ehrlichia platys*.[399] Immune-mediated destruction has been considered but is not supported by the cyclic nature of the thrombocytopenia in the face of rising titers, or by the presence of thrombocytopenia in some dogs with no detectable serum titers to *E. platys*.[399] However, the indirect immunofluorescence assay for titers[400] detects only IgG. The thrombocytopenia could still be mediated by antibodies reactive to combined platelet/organism epitopes or platelet epitopes induced by the infection. The role of immune complexes has similarly not been investigated.

In the original description of this disease, clinical illness did not accompany infection.[397] Since then, a febrile dog with uveitis and thrombocytopenia was found to be infected with *E. platys*, though the clinical signs may not have been caused by the organism.[401] In a Michigan case,[402] clinical hemorrhage occurred in a dog with cyclic thrombocytopenia that responded to tetracycline. Morulae were not detected, but the dog

had a supportive (not strong) titer for *E. platys*. However, other ehrlichial titers were not reported.

In Greece, 15 dogs naturally infected with a Greek strain of *E. platys* had signs including anorexia, depression, weight loss, intermittent epistaxis, pale mucous membranes, and fever.[403] Fourteen of the dogs were thrombocytopenic, and each responded well to tetracycline therapy. While concomitant infections may have existed, experimental inoculations of normal dogs with blood from one of the dogs with no evidence of a combined infection also produced severe thrombocytopenia associated with fever, depression, anorexia, and petechial and ecchymotic hemorrhages. It appears then, that some strains of *E. platys* may induce clinical disease. However, if clinical signs accompany *E. platys* infections, other disease processes should be thoroughly sought.

iv) hemobartonellosis

Canine hemobartonellosis is caused by the epicellular rickettsial *Hemobartonella canis*. While thrombocytopenia is not characteristic of the disease,[2] it was associated with canine hemobartonellosis in one splenectomized dog.[366] The thrombocytopenia in this dog resolved as the organisms disappeared from the peripheral blood, though this may have been coincidental and not causative.

(b) protozoa

i) babesiosis

Thrombocytopenia may occur in canine babesiosis caused by tick-borne or transfused *Babesia canis* or *Babesia gibsoni*.[404,405] Thrombocytopenia may be moderate

to severe, and sometimes complicated or caused by DIC.[406] The splenohepatomegaly of babesial infections may cause platelet sequestration and contribute to the thrombocytopenia.[389,406,407] Immune-mediated mechanisms may also contribute since 31 of 37 infected dogs in one study were Coombs' positive,[408] though none of the eleven in another report were.[407] The rapid return of normal platelet concentrations after therapy argues against a deficiency of megakaryocytes,[405] though thrombopoiesis may be inhibited. Hemolysis may also be contributory. The principal cause of thrombocytopenia in canine babesiosis remains to be discovered.

ii) leishmaniasis

Clinical canine leishmaniasis[114,159] caused by diphasic protozoa of the genus *Leishmania* is associated geographically with its primary sandfly vectors of the genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World.[409] In 80 untreated dogs with natural infections of leishmaniasis, the platelet concentrations of 50 were measured and 25 dogs were thrombocytopenic.[159] Some of the dogs were markedly thrombocytopenic. Thrombocytopenia also occurs in experimental infections,[410] and it may be caused by decreased production, splenic sequestration, and immunodestruction. However, the operative pathophysiologic mechanisms have not been conclusively identified.

As circulating immune complexes (CIC) have been related to the thrombocytopenia seen in people with leishmaniasis,[411] they may play an important role in the thrombocytopenia of dogs, either directly or secondary to immune-complex vasculitis.[159] In natural canine infections, CIC were detected by nephelometry in 27 of 27 samples.[159]

Of 74 patients tested, 84% had positive Coombs' tests, though they were usually weak. The presentation of dogs with visceral leishmaniasis is reportedly sometimes very difficult to differentiate from SLE, especially since LE cell (3/23) and ANA tests (14/45) may be positive in infected dogs.[159] While these findings indicate the potential for IMT, such a cause remains to be established. As for sequestration, it may contribute in some cases, since splenomegaly occurs in about 1/3 of the cases.[159] Decreased production may also occur when marrows are packed with leishmania-laden macrophages.

(c) fungal

Moderate to marked thrombocytopenia commonly accompanies canine histoplasmosis caused by *Histoplasma capsulatum*.[116,412,413] In some cases it has been associated with gastrointestinal hemorrhage, and increases in circulating megathrombocytes have been interpreted as an indication of effective thrombopoiesis.[412] The pathogenesis of this thrombocytopenia is unknown.

In people, thrombocytopenia also frequently occurs with acute histoplasmosis, and it has been associated with increased PAIgG in a patient with primary pulmonary histoplasmosis.[414,415] When *Histoplasma capsulatum* organisms were incubated with human platelet-rich plasma, aggregation and release occurred.[414] The platelet release reaction required the presence of the yeast and IgG, while aggregation had an additional requirement for fibrinogen. Unlike the interaction of platelets with the yeast cell wall extract zymosan, complement was not required. Whether IgG interacts with the yeast in a specific or nonspecific manner is unknown, but it was suggested that IgG and the yeast may form an immune complex that interact with the platelet through Fcγ receptors.

Assays for PAIg have not been reported in dogs with histoplasmosis. Furthermore, canine platelets have not yet been evaluated for the presence or absence of an Fc receptor. Such studies are indicated to understand the thrombocytopenia of canine histoplasmosis. Other fungal diseases such as disseminated candidiasis caused by *Candida albicans* or *Candida parapsilosis* have also been associated with thrombocytopenia in dogs.[116]

(d) other

An incompletely characterized manganese-dependent reverse transcriptase producing infectious agent has recently been suspected as an agent causing thrombocytopenia in a dog.[416] Polymerase chain reaction failed to detect *Ehrlichia canis* DNA in samples and cultures from the affected dog, and an inoculated control dog developed thrombocytopenia but did not seroconvert to *E. canis*.

(2) neoplasia

The pathogenesis of thrombocytopenia associated with neoplasia may relate to any combination of the following: decreased production from myelophthisis, myelodysplasia, tumor secretion of estrogen, or chemotherapy; increased consumption by DIC, microangiopathies, or hemorrhage; increased destruction by immune mechanisms or secondary to concurrent infections from immunosuppression; or by sequestration in spleens enlarged by tumor infiltration or in other large vascular organs or neoplasms.[28,248,417] Some of these mechanisms have been discussed in the preceding sections. The frequent association of thrombocytopenia with neoplasia has recently been

emphasized by a retrospective study which documented thrombocytopenia ($<200,000/\mu l$) in 10% of 2,059 dogs with neoplasia, and in 12% of dogs with malignant neoplasms.[248] Moreover, 13% of 987 thrombocytopenic dogs in a previous review had neoplasia.[25]

In the aforementioned review of 2,059 dogs with neoplasia, dogs with hemangiosarcomas (47%), lymphoid neoplasia (37%), hemic neoplasia (24%), and carcinomas were at significant risk of developing thrombocytopenia as compared to dogs with other neoplasms.[248] Thrombocytopenia was most marked with brain tumors, hemangiosarcomas, melanomas (due to cytotoxic therapy), lymphomas, and pulmonary carcinomas. Dogs with infections plus neoplasia had more severe thrombocytopenia than dogs with neoplasia alone, but fewer than 25% of the dogs had platelet concentrations less than $100,000/\mu l$. Golden Retrievers with neoplasia were at an increased risk of developing thrombocytopenia compared to other breeds.[248]

In another review of dogs with untreated cancer, 36% of 100 dogs with untreated cancer and no evidence of hemorrhage were thrombocytopenic.[418] In this study, thrombocytopenia was commonly associated with lymphoma, multiple myeloma, and leukemias, including all 7 dogs with myelogenous leukemia or multiple myeloma. Twelve of 59 (20%) dogs with solid tumors were also thrombocytopenic. In another study, lymphoma was associated with platelet concentrations less than $200,000/\mu l$ in 31 of 69 (49%) affected dogs, and the cytopenias were considered to relate to myelophthisis.[81]

Myelophthisis has also been the presumed but unproven principal cause of thrombocytopenia in dogs with multiple myeloma, acute leukemia, and chronic lymphocytic leukemia. In a review of dogs with multiple myeloma, 17% of 60 dogs had

platelet concentrations less than 100,000/μl with 33% less than 200,000/μl.[82] Of 19 dogs with acute leukemia, thrombocytopenia occurred in 18, sometimes alone (10%), sometimes with anemia (37%), and sometimes with pancytopenia (48%).[77] Only in cases of acute lymphocytic leukemia was thrombocytopenia unaccompanied by other cytopenias. In a study of 22 dogs with chronic lymphocytic leukemia, 10 (45%) were mildly to moderately thrombocytopenic (110,000 - 193,000/μl).[79] The relative contribution to thrombocytopenia of such factors as immunologic mechanisms, myelophthisis, and the splenomegaly or hepatomegaly that was frequently present in these dogs is not known, though myelophthisis is presumed to play an important role.

Dogs with a variety of multicentric or metastatic neoplasms often have decreased platelet concentrations associated with shortened platelet survivals and an increased rate of platelet destruction.[419] Many nonthrombocytopenic dogs with malignancies may also have decreased platelet survivals, especially those dogs with metastatic carcinomas or lymphomas.[78] The improvement of platelet survivals in thrombocytopenic dogs with malignancies by treatment with a combination of phosphodiesterase and a cyclooxygenase inhibitor is consistent with the hypothesis that increased platelet consumption is important in the thrombocytopenia of many neoplastic conditions.[419]

Fibrinogen concentrations and survivals may be increased, decreased, or normal in dogs with neoplasia, whether or not platelet survivals are decreased.[78,248,419] Decreased fibrinogen survival is consistent with activation of the coagulation cascade as occurs in DIC. However, most evaluated dogs with neoplasia and decreased platelet survivals have had elevated fibrinogen concentrations and fibrinogen survivals that were not

decreased.[78,419] This suggests platelet consumptive/destructive mechanisms other than DIC in the thrombocytopenia associated with many neoplasms in dogs.

In one study, however, decreased fibrinogen concentrations and increased APTT were present in about 50% of thrombocytopenic dogs with neoplasia.[248] DIC was diagnosed in 33% of these dogs, and in 90% of the dogs with hemangiosarcoma.[248] Because DIC is commonly associated with hemangiosarcoma, dogs with suspected hemangiosarcoma were more likely than other dogs with cancer to undergo coagulation screening tests. The results were therefore likely biased toward dogs with hemangiosarcoma.

Thrombocytopenia occurs in many dogs with hemangiosarcomas, many but not all of which have laboratory evidence of DIC.[153] Some dogs with apparently just cutaneous hemangiosarcomas also have thrombocytopenia with evidence of DIC, a situation similar in many respects to human patients with Kassabach-Merritt syndrome.[420] Contributory factors other than DIC are not defined, but may involve sequestration in the vascular neoplasms or interactions with the proliferating neoplastic endothelial cells.

DIC may also be a regular contributor to the thrombocytopenia of canine systemic mastocytosis. In a study of 16 affected dogs, 3 of 5 tested were thrombocytopenic, and the thrombocytopenia was sometimes severe and associated with erythrocyte fragmentation and prolonged PT, PTT, or increased FDPs.[421]

Malignant histiocytosis has been associated with thrombocytopenia of unproven pathogenesis,[422] possibly related to the phagocytic activity of the atypical histiocytes.

C. Diagnostic Considerations for Thrombocytopenia

Once the potential causes of thrombocytopenia are known, determining the origin of thrombocytopenia in affected dogs begins with a thorough history.

1. history

A history should include knowledge of drug exposure, recent vaccinations, recent trips, recent contact with other dogs, previous transfusions, and previous and current medical conditions. The age of onset of the bleeding condition is important, as is the frequency, type, and any association with physical trauma. Owner observations of any bleeding including hematuria, melena, epistaxis, or petechiae should be elicited. Petechiae may be mistaken for a "rash" and may develop after such minimal trauma as grooming.[345] A history of tick exposure may suggest an increased likelihood of a tick-borne infectious disease such as RMSF, babesiosis, or ehrlichiosis.

2. physical exam

As for any patient, a thorough physical exam is mandatory. Petechiae and ecchymoses are the hallmark of thrombocytopenic hemorrhage, and they may be obvious or subtle. Thrombocytopenia may also be manifested by epistaxis, melena, hemorrhagic diarrhea, retinal hemorrhage, hematuria, prolonged estral bleeding, or bleeding after whelping, surgery, or venipuncture. These forms of hemorrhage should be carefully sought, including an ophthalmic exam, and differentiated from the hematomas, hemarthroses, and bleeding into other body cavities typical of coagulation protein defects.

Epistaxis, hematuria, gastrointestinal tract bleeding, and reproductive tract bleeding may occur with either type of disorder.

A physical examination should systematically assess all organ systems, but attention to the following may be especially useful in determining the cause of known or suspected thrombocytopenia: attitude, condition, fever, pallor, lymphadenomegaly, splenomegaly, testicular masses, other masses, joint disease, dermatopathies, and the presence of ticks. Besides bleeding and perhaps lethargy and pallor, dogs with primary IMT may have no other abnormal physical findings. Infections may be suggested by a depressed attitude, fever, lymphadenomegaly, ticks, joint disease, or peripheral cutaneous necrosis. Neoplasia may be suggested by the presence of lymphadenomegaly, splenomegaly, other masses, or cachexia. Systemic immune-mediated disease may be suggested by polyarthritis or certain forms of dermatitis. The presence of splenomegaly suggests that thrombocytopenia is a secondary process.[27] These and other findings each should lead to an appropriate diagnostic plan.

3. complete blood count (CBC)

A CBC is typically done in the process of confirming thrombocytopenia suspected from clinically detected mucosal hemorrhage. Unsuspected thrombocytopenia may be discovered when a CBC is done for other reasons. Fresh blood smears should be evaluated, especially in the absence of clinical hemorrhage, to confirm automated results and exclude the possibility of pseudothrombocytopenia. Careful venipuncture will help to prevent platelet activation and artifactually low platelet concentrations.

While many related or unrelated abnormalities may be present, the following findings are especially significant in establishing the cause of thrombocytopenia: bicytopenia, pancytopenia, leukemia, an inflammatory leukogram, granular lymphocytosis, intracellular organisms such as fungi, ehrlichiae, and other bacteria, distemper inclusions, extracellular parasites such as the microfilaria of *Dirofilaria immitis*, and erythrocyte forms such as spherocytes, fragments, and dacryocytes suggestive of immune-mediated hemolytic anemia, DIC, and myelofibrosis, respectively. If thrombocytopenia does not accompany hemorrhage typical of thrombocytopenia, testing should be directed toward the assessment of platelet function and vascular integrity.

4. chemistry profile

A routine chemistry profile may provide evidence for underlying conditions including renal disease, hepatic disease, and pancreatitis. Hypergammaglobulinemia may be seen with some lymphoproliferative disorders and chronic infections. Serum protein electrophoresis can provide more specific information regarding protein abnormalities, but it is important to recognize that monoclonal gammopathies may occur with infectious conditions such as canine monocytic ehrlichiosis in addition to neoplastic conditions. Hypercalcemia may indicate the need to search for otherwise occult neoplasia such as lymphoma.

5. bone marrow

Examination of bone marrow aspirates and core biopsies can be diagnostic. Core biopsies allow the best assessment of marrow cellularity and megakaryocyte density,

while aspirates allow careful examination of cytomorphology. Necrosis is best appreciated histopathologically. Marrow evaluation is indicated when multiple cytopenias are present or when there is a suspicion of leukemia, multiple myeloma, or some other myeloproliferative disorder. Other disorders associated with production failure may also be identified, including myelofibrosis, metastatic cancer, and granulomatous infections. If hypoplasia or aplasia is suspected, a core biopsy should always be collected.

Megakaryocytes should be evaluated for number and morphology. The number of megakaryocytes in the bone marrow has commonly been interpreted as an assessment of thrombopoiesis, the presence of many megakaryocytes suggesting that thrombocytopenia is due to decreased platelet survival. However, the number of megakaryocytes is a better indicator of megakaryocytopoiesis, and decreased thrombopoiesis may still be possible with an apparent adequate number of megakaryocytes. Megakaryocytic hypoplasia or aplasia suggest there may be a relatively delayed response to successful therapy. Megakaryocytic hyperplasia with increased immature forms and few mature cells actively shedding platelets suggests responsive megakaryocytopoiesis, though thrombopoiesis may be impaired. Morphologic changes may be indicative of cell damage and impaired thrombopoiesis. Megakaryocytic hyperplasia including many mature cells suggests responsive thrombopoiesis due to pathologically decreased platelet survival. Thrombocytopenia should not be considered a contraindication for bone marrow sampling.[119]

6. coagulation profile

Coagulation profiles are very important in detecting consumptive thrombocytopenias associated with DIC.[157] Several permutations of the profile results, including significant elevations of prothrombin times and/or partial thromboplastin times in conjunction with decreasing platelet concentrations or thrombocytopenia and decreasing fibrinogen concentrations or hypofibrinogenemia, are indications of an ongoing consumptive coagulopathy. Measurement of circulating concentrations of antithrombin III, fibrin/fibrinogen degradation products, plasminogen, and factor V can also be useful, as can inspection of blood films for erythrocyte fragmentation. The absence of abnormalities other than decreased platelet concentrations suggests the absence of such conditions.

7. survey radiographs

Survey radiographs and ultrasonography act as extensions of the physical examination. Occult neoplasia may be detected by such procedures.

8. blood cultures

When physical and laboratory findings are suggestive of possible septicemia, blood cultures may be used in an attempt to confirm the suspicion.

9. serology

Serology may be used to detect exposure to other infectious agents, most notably the rickettsials. The indirect fluorescent antibody assay is sensitive and specific for

Ehrlichia canis. A positive titer is indicative of infection since most dogs become seronegative within 6-9 months after successful therapy.[184] The serologic diagnosis of *Ehrlichia platys* infections is based on a specific indirect immunofluorescence assay for *E. platys* antibodies in serum samples. Diagnosis of RMSF requires direct immunofluorescent testing for *Rickettsia rickettsii* antigen in tissue biopsy or necropsy specimens, serologic testing, or rickettsial culture. The indirect fluorescent antibody test is diagnostic when convalescent samples have at least four times the antibody titers of acute samples.[423] The diagnosis of granulocytic ehrlichiosis currently requires the detection of morulae in granulocytes and seropositivity to the cross-reacting *E. canis*.[184] If other serology or virology is appropriate, it will be indicated by other specific findings.

10. immunologic screening tests

If systemic immune-mediated disease is suspected, tests for antinuclear antibodies, rheumatoid factor, LE cells, and erythrocyte-associated immunoglobulin may be indicated.

11. tests for PAIg and PBIg

Lastly, where available, assays can be used to detect platelet-associated or specific membrane glycoprotein-associated immunoglobulins. Routine assays do not detect antiplatelet antibody; rather, they detect any immunoglobulin that is associated with a patient's platelets or that is in the serum/plasma of a patient and will associate with normal test platelets. A positive result is not in and of itself diagnostic of an autoimmune

condition, because the assays generally do not determine the orientation or specificity of the detected immunoglobulin.

Immunoglobulins may be associated with the platelet surface in several different ways. Fab-mediated binding refers to the specific binding of an antibody by its antigen-recognizing end to its reactive epitope. This occurs in the case of platelet reactive autoantibodies and alloantibodies. Some of the drug-induced antibodies, such as those induced by quinine and quinidine, have also been shown to bind specifically by their Fab ends to specific platelet epitopes.[424,425] Fab-mediated binding can also occur to an antigenic determinant adsorbed onto the platelet surface, as has been shown to occur in human malarial infections.[259] Such antibodies are PAIgs but not antiplatelet antibodies. The frequency with which these PAIgs are present in infectious and noninfectious diseases is unknown. In each case, the Fc ends of the immunoglobulins are directed more or less outward such that they are available to Fc receptors on phagocytic cells. In this way, Fab-mediated binding may result in Fc-mediated phagocytosis.

The surface of human platelets have Fc receptors for IgG.[426] Fc receptors occur as one of several different phenotypes,[427-429] each of which can bind the Fc end of IgG molecules. The binding specificity for different IgG subtypes varies with the phenotype, but each platelet has about 1,000 to 2,000 FcγRII.[430,431] This number stays relatively stable in health, but may increase with disease or platelet activation.[432,539] Immunoglobulins can bind to the FcγRII by their Fc portions, thus becoming PAIg. This immunologically nonspecific binding can occur with immune complexes, IgG dimers, IgG trimers, and larger aggregates of IgG.[426]

The best described Fc-mediated binding of immunoglobulins to platelet FcγRII occurs in immunologic heparin-induced thrombocytopenia. In this drug-induced disorder, antibodies bind to platelet factor 4 and heparin to form complexes which bind to platelet FcγRII by the Fc ends of the immunoglobulin molecules.[433] The Fc ends of other IgG molecules bound to the complex are presumably exposed such that the Fc receptors of phagocytic cells can mediate platelet destruction. Moreover, intraplatelet and interplatelet bridging by these complexes can cause platelet activation which can presumably contribute to increased platelet consumption and thrombocytopenia[434] Similarly, the Fc-mediated binding of aggregated IgG to platelets in vitro causes platelet aggregation.[426]

Data suggest that circulating immune complexes (CICs) can accelerate platelet destruction, perhaps by an Fc-mediated mechanism. Concentrations of CIC in human leukemia patients have been shown to correlate directly with the removal rate of transfused platelets and indirectly with the increment in platelet concentration after transfusion.[435,436] Platelet transfusions decreased the concentrations of CIC (Raji cell).[435] This is indirect evidence to suggest that immune complexes may bind to platelets, perhaps by complement or Fc receptors, and accelerate their clearance. The significance of these findings for dogs remains to be shown. It is also not yet clear to what extent such immunoglobulins may be detected by assays for PAIg.

Canine platelets have not been assessed for the presence or absence of Fc receptors for IgG.[21] Therefore, it is not known if Fc-mediated binding of antibodies and immune complexes can occur and cause thrombocytopenia in dogs. It is known, however, that not all species have platelet Fcγ receptors. The rabbit, for instance, does not.[437] Unless immune complexes can bind to the platelet other than by Fc receptors, a

lack of Fc receptors would imply that thrombocytopenia should not occur in dogs due to circulating immune complexes from neoplasia, infectious diseases, drugs such as heparin, systemic autoimmune diseases, or from the formation of antigen-antibody complexes after vaccinations. Similarly, aggregated IgG would not be expected to aggregate canine platelets in vitro.

It is possible, however, that immune complexes can bind to and activate canine platelets other than by Fc receptors. Specifically, they may bind via a complement protein bridge in a manner known as immune adherence.[438,439] Several receptors for complement proteins have been identified on human platelets, but their significance is not fully appreciated.[440] Non-primate mammals have been considered to have platelets capable of immune adherence,[439,441-443] but relatively little is known about the immune adherence potential of canine platelets. Complement receptors have not been specifically identified on canine platelets.

The last way that immunoglobulins can be associated with the platelet surface fits neither of the above categories. Aggregates of IgG have been shown to bind to platelet GPIIb/IIIa in a non Fab and a non Fc-mediated manner, the nature of which was not determined.[444] It is possible that some immunoglobulins contain the RGD sequence that allows them to bind to this integrin.[445] Further studies are necessary before the importance and frequency of such binding are determined. Whether or not other immunologically nonspecific mechanisms of binding exist also remains to be seen.

The presence of PAIg, whatever its orientation, is evidence that there is an immune component to the thrombocytopenia, and in the absence of other detectable abnormalities requiring therapy, positive results can support a clinician's decision to

institute immunosuppressive therapy. Even after three decades of assay development in human medicine, the diagnosis of primary IMT is primarily one of exclusion. Assay results may support clinical suspicions, but diagnostic and therapeutic plans may be affected little by the results.[285] Assays are very important, however, in diagnosing drug-induced IMT, posttransfusion purpura, neonatal alloimmune thrombocytopenia, and refractoriness to platelet transfusions in which case potential donors can also be screened for platelet compatibility.[214] They are also an important research tool to better understand the contributions of immune-mediated platelet destruction to thrombocytopenic patients.

III. Detection and Significance of PAIg and PBIg

A. Significance of PAIg

The studies Harrington reported in 1951 established that the plasma or whole blood of some human patients with idiopathic thrombocytopenia could induce acute thrombocytopenia, sometimes accompanied by purpura, when infused into normal recipients or patients with inoperable cancer.[446] Control infusions did not induce thrombocytopenia. The thrombocytopenic factor was found in the globulin fraction of the one plasma sample fractionated. Shulman[447] later repeated these studies and also induced thrombocytopenia by infusing acute plasma from an affected patient into the same patient in remission, thus demonstrating autoreactivity. Moreover, he showed that the thrombocytopenic plasma activity resided in 7S immunoglobulin fractions, it affected autologous and homologous platelets, it had at least some species specificity, and it produced effects in vivo that were similar to those produced by known antiplatelet

antibodies.[447] These findings strongly incriminated immunoglobulin as having a key role in the pathogenesis of human idiopathic thrombocytopenia. While it was not known if the antibody was directed toward autologous platelet antigens, antigens adsorbed onto platelets, or antibody complexed with antigen that became nonspecifically adsorbed to platelets, the search for ways to detect PAIg and PBIg began.

Since then, evidence has mounted to indicate that IMT usually results from the unexplained production of platelet-reactive autoantibodies that mediate platelet destruction by the mononuclear phagocyte system (MPS). Similar to the plasma transfusion experiments of Harrington[446] and Shulman[447] are the natural transfer studies of Mother Nature in which fetuses of mothers with thrombocytopenia and increased PAIgG also develop thrombocytopenia associated with increased PAIgG.[204,448] This is consistent with placental transfer of the pathogenic immunoglobulins.

Accidental transfer experiments have also been instructive. A liver transplantation recipient developed thrombocytopenia and elevated PAIg after receiving the liver of a donor with thrombocytopenia and elevated PAIg. This is further support of a transmittable, immune-mediated pathogenesis.[449] Another patient developed thrombocytopenia associated with elevated PAIgM after an allogeneic bone marrow graft from a donor who was later shown to have elevated PAIgM with the same spectrum of reactivity.[450,451] The findings suggest that autoimmune thrombocytopenia can be transferred by cells capable of proliferating in a bone marrow graft.

There is other evidence linking PAIg to cells of the immune system. Normal human lymphocytes have the capacity to produce antibodies reactive with normal platelet glycoproteins, DNA, and phospholipids,[452,453] but the stimulus for their production or

overproduction is not known. As compared to T-helper lymphocytes from healthy people, those of patients with primary IMT proliferate more and secrete significantly more IL-2 when stimulated by normal platelets.[454] This may be evidence of an immunoregulatory abnormality that enhances autoantibody production. Compared to controls, an increased percentage of B lymphocytes expressing the pan-T cell marker CD5 has been found in the blood and spleens of patients with diagnoses of IMT.[455] These cells were capable of producing platelet-reactive antibodies in vitro, suggesting that they had an in vivo role in the pathogenesis of IMT.

In other studies, monocytes and lymphocytes isolated from patients with diagnoses of IMT adhered much more to GPIIb/IIIa than did cells from controls.[456] The adherence to GPIIb/IIIa was shown to be $F(ab')_2$-mediated, and the antibodies were fixed to the mononuclear cells in an Fc-dependent manner. These findings support the concept that antibody-armed leukocytes participate in the pathogenesis of IMT. While some PBIgs from people with diagnoses of IMT have been shown to bind specifically by their Fab portions to platelets and isolated platelet membrane glycoproteins, more studies are needed to document the presumption that this is indeed the rule.[444]

Clinical studies have provided further evidence that PAIg is pathogenic and related to active disease in human IMT patients. When PAIg was assessed in patients with active disease and in nontreated patients in remission, 39/53 (74%) patients with active disease had directly detectable PAIg, while only 2/26 (8%) in remission did.[297] Furthermore, of 8 patients with active disease whose platelet concentrations responded to prednisone therapy, PAIg decreased significantly in 2 and became undetectable in 6. When 2 of the responding patients relapsed, PAIg reappeared. Detectable PAIg

remained unchanged, however, in 4 patients whose platelet concentrations did not respond to prednisone. While these data suggest a causative role for PAIg in the development of thrombocytopenia, they do not prove it. PAIg could simply be a secondary reflection of thrombocytopenia, rising as platelet concentrations fall and falling as platelet concentrations rise. However, the composite findings do not support this hypothesis.

To investigate if increased PAIgG could be the result of thrombocytopenia, rather than its cause, surface PAIgG was measured on washed platelets from rabbits made moderately thrombocytopenic in three ways: 1) IMT was induced by the injection of guinea pig anti-[rabbit platelet] serum; 2) nonimmune nonregenerative thrombocytopenia was induced by irradiation; and 3) nonimmune consumptive thrombocytopenia was induced by injection of ADP.[457] PAIgG was increased above controls in all of the rabbits with IMT but in none of the rabbits with nonimmune consumptive or nonregenerative thrombocytopenia. This evidence suggests that PAIgG does not increase just as a result of thrombocytopenia. Rather, its presence is evidence of an immune-mediated process. Unfortunately, this study did not exclude the possibility that elevations in PAIgG could occur in response to more marked thrombocytopenias of nonimmune pathogenesis.

The detection of platelet-reactive immunoglobulins in the plasma or serum of apparently normal people has also brought doubt to the significance of PBIgs.[299] Platelet survival studies done in some patients with elevated PAIg have been normal,[120] indicating that the elevated PAIg was not associated with an increased rate of destruction and suggesting that PAIg may not be of pathogenic significance. However, elevated PAIg will lead to decreased platelet survival only if the MPS is functioning adequately and

removing the antibody-coated platelets. Kelton[458] showed that 5 patients with elevated PAIg but normal or near-normal platelet concentrations had normal platelet survivals but impaired MPS activity as assessed by measuring the clearance of autologous chromium-labeled erythrocytes sensitized with alloantibody. In contrast, thrombocytopenic patients with elevated PAIg had normal MPS activity and shortened platelet survivals. These studies indicate that elevated PAIg in a nonthrombocytopenic patient should not be considered a nonspecific finding; thrombocytopenia may be prevented by low MPS activity or compensated platelet production.

The importance of the MPS was also suggested by studies that showed the highest M-CSF concentrations in patients with severe and steroid-refractory IMT.[459] M-CSF promotes proliferation, differentiation, and maturation of monocytes, as well as enhances antibody-dependent cellular cytotoxicity and the expression of low affinity IgG receptors. Higher concentrations of M-CSF may lead to enhanced macrophage activity and platelet destruction and be causally related to the poor response to treatment of IMT patients with high M-CSF concentrations.

In vitro studies with human[460] and canine platelets have also suggested a role for neutrophils in the removal of antibody-coated platelets from circulation.[461] Canine platelets undergo phagocytosis by autologous neutrophils when co-incubated in vitro with rabbit anti-[canine platelet] serum, and the phagocytic activity is detectable by electron microscopy or by measuring neutrophil reduction of nitroblue tetrazolium (NBT). Sera from dogs with suspected IMT and positive PF3 assay results also supported increased NBT reduction, while normal sera and sera from PF3 negative dogs suspected of having

IMT did not. The in vivo significance of these findings with respect to IMT remains to be determined.

In summary, the bulk of the evidence suggests that PAIg does mediate platelet destruction in IMT. Reliable measurement of PAIg should therefore be of diagnostic significance. However, increased PAIg may not cause thrombocytopenia if destructive mechanisms are impaired, because the net circulating platelet concentration depends on the balance between platelet production and platelet removal. All factors in this equation must be considered to understand the pathogenesis of IMT and its response to therapy. It is also possible that the cellular arm of the immune system can play a variable but important role in IMT,[462-464] conceivably being solely responsible for mediating some immunological thrombocytopenias. In such cases, PAIg would not be elevated.

B. Assays for Human PAIg and PBIg

1. types

There are two general classes of assays for investigating antibody-mediated platelet destruction: 1) direct assays which attempt to detect patient immunoglobulin already bound to patient platelets (PAIg), and 2) indirect assays which assess patient serum or plasma for evidence of PBIg, the assumption being that if positive, the patient's own platelets were presumably affected in vivo. Numerous assays have been developed over the last three decades,[214,465] and they have generally improved with advances in knowledge and technology. However, even with today's most widespread assays, the interpretation of results can be problematic.

The early assays were indirect functional assays. Test and control plasma, sera, or variously purified immunoglobulin fractions were tested for their capacities to elicit activation of normal platelets.[465] Activation was assessed by platelet aggregometry, detection of released platelet products such as serotonin, ATP, and ^{51}chromium, or by a shortening of the coagulation time in the PF3 assay. These functional assays were insensitive and nonspecific, and they are generally considered obsolete.[214]

The ability of some antibodies to fix complement led to the development of an indirect quantitative platelet complement fixation test.[214,466] This assay is limited to complement-fixing antibodies and is consequently insensitive.[214] An important advance came with the development of a complement lysis inhibition assay.[467] In this assay, the amount of immunoglobulins bound by platelets is quantified by the consumption of a fixed amount of hemolytically active antihuman IgG which is detected by the degree of inhibition of complement-mediated lysis of sheep erythrocytes added subsequently. This method allowed for direct and indirect determinations and quantitation without depending on the complement-fixing activity of the PAIg or PBIg.[445] While the assay was widely used, quantitation was inaccurate due to the inadvertent release of internal IgG.[445] The assay has mostly been replaced by more direct testing techniques.[214]

Modern assays are generally based on immunoglobulin detection through the use of labeled antiglobulin reagents.[465] These assays can indirectly test for PBIg by incubating normal allogeneic platelets with serum, plasma, immunoglobulin fractions of serum or plasma, or platelet eluates. They can also be direct assays where patient platelets are washed and assessed directly for bound immunoglobulin. Similarly, platelet-associated complement components may be detected with labeled anti-complement

reagents. Of significance for direct assays is the finding that small platelet fragments induced by repetitive freezing and thawing of platelets are lost during routine washing steps for platelet antibody assays.[468] Therefore, if the fragments and microvesicles present in patients with IMT[469,470] are similar, they will not contribute to the assessment of PAIg.

The antiglobulin reagents commonly used are polyclonal antiglobulins, monoclonal antiglobulins, $F(ab')_2$ antiglobulin fragments,[471,472] lectins, and bacterial Fc receptors such as SpA.[473,474] The type of labeling of the reagents is dictated by the general type of assay to be used: 1) radioactive assays employ radiolabeling, usually with ^{125}iodine; 2) immunofluorescence assays employ fluorescence labeling, usually with fluorescein isothiocyanate; and 3) enzyme-linked immunosorbent assays (ELISAs) employ enzyme conjugation, usually with peroxidase or alkaline phosphatase. After incubation, the separation of bound from unbound reagents is done with washing or with centrifugation through Percoll, oil, or sucrose.[214] In most assays, the reactivity of the antiglobulin reagent is measured directly, while in competitive assays, consumption of the antiglobulin reagent is measured by decrements in its fluid phase concentration after it binds to PAIg. Quantitation of the number of molecules of immunoglobulin per platelet has been possible with each general method,[214,465] but none has gained universal acceptance as the best. Each reagent, label, and technique has its own strengths and weaknesses.

Radioactive assays offer a high degree of reproducibility and the potential for precise quantitation.[292,475,476] Their only real disadvantage is simply that they require use and disposal of radioactive materials. ELISA techniques are radioactive-free and easily applied to clinical laboratories. While some investigators have considered them as

sensitive and quantifiable as radioactive antiglobulin tests,[214] others have found them less reproducible and reliable.[465] Immunofluorescence assays have been widely used since von dem Borne found that platelets fixed in 1% paraformaldehyde for 2-5 minutes at room temperature lacked the nonspecific fluorescence of unfixed platelets which had hampered previous attempts at fluorescence assays.[477] The major disadvantages of these assays include the subjective assessment of fluorescence and the need for immediate evaluation in the dark.[214] However, these disadvantages have been minimized with the more recent adaptation of immunofluorescence assays to flow cytometric technology.

Flow cytometry, where available, can reduce the subjectivity inherent in most immunofluorescence assays, and it is more sensitive than microscopic assays.[478] Smaller blood volumes are required, and other cell types can be simultaneously evaluated in cases of multiple cytopenias.[478] Attempts to make flow methods quantitative have been reported, but the estimate of the number of IgG molecules per normal platelet was higher than the figures under general acceptance at this time.[479]

The most recently developed assays are based on reactivity toward specific platelet glycoproteins rather than on general reactivity toward whole platelets. These assays help reduce nonspecific binding while identifying specific target glycoproteins or even the specific reactive portions of target glycoproteins. In antigen capture assays, murine monoclonal antibodies are used to capture their reactive platelet glycoproteins. In one method, the monoclonal antibodies are fixed to such surfaces as polystyrene beads or microtitration plate wells[301] before incubation with solubilized platelets. After attachment of the specific antigens, test serum or plasma is incubated, and bound antibodies are detected with antiglobulin reagents after washing.[301]

In an improved method, platelets are incubated with test serum or plasma before solubilization, thus limiting the reactivity to exosolic sites, preserving epitopes that may be lost by solubilization, and reducing nonspecific binding to the solid phase.[480,481] In the direct form of the assay, test platelets are simply solubilized after washing. Antigen-antibody complexes can then be captured by anti-[human IgG] antibodies fixed to wells or beads (immunobead assay), and the bound antigens can be detected by specific monoclonal antibodies to a panel of platelet glycoproteins.[480] Alternatively, the complexes can be captured by the monoclonal antibodies to platelet glycoproteins, and the bound immunoglobulin can be detected by antiglobulin reagents.

In another modification,[481] platelets are exposed to the test serum and specific monoclonal anti-glycoprotein antibodies simultaneously, and the platelets are then washed and solubilized. The trimolecular complexes of glycoprotein with bound anti-glycoprotein antibodies and bound human immunoglobulin (if positive) are captured by anti-[murine Ig] antibodies and the human immunoglobulin is detected by an ELISA. In the direct form of this assay, washed test platelets are incubated with specific anti-glycoprotein monoclonal antibodies, and the assay proceeds as for the indirect form. This assay is commonly referred to as a MAIPA (Monoclonal Antibody-specific Immobilization of Platelet Antigens). Recently, an indirect assay using isolated or captured proteins more sensitively detected reactive antibodies than did an indirect flow cytometric immunofluorescence technique.[299]

Similar to these antigen capture assays are methods which make use of purified platelet proteins and synthetic peptides corresponding to portions of sequenced platelet glycoproteins.[299] Serum, plasma, or platelet eluates can be assessed for antibodies

reactive to the specific proteins, and adsorption studies can be done with the peptides to further define the locations of the reactive epitopes. Assays with captured antigens and purified proteins identify target glycoproteins much more rapidly than do the traditional techniques, namely crossed immunoelectrophoresis,[482] immunoblotting and immunoprecipitation. Moreover, immunoblotting has frequently been unsuccessful, probably due to SDS denaturation of some of the target epitopes.[214,483] The presence of bands after blotting with control sera has also caused problems in interpretation. A large percentage of normal human sera contain IgG, IgA, and IgM immunoglobulins reactive to vinculin and talin, proteins present within platelets as well as many other cells.[484] The 90 kD band of IIIa could be easily confused with the 95 kD fragment of vinculin, and the 110 kD band of reduced IIIa could be confused with 120 kD band for intact vinculin. Again, this shows the importance of using procedures that assess surface rather than internal binding epitopes.

As for immunoprecipitations, radioactive methods have proven utility for identifying the surface epitopes reactive with some, but not all, antibodies in IMT.[301,302,483] Relatively quick and easy nonradioactive methods have also been reported.[485] Radiolabeling with lactoperoxidase, which incorporates the label primarily into tyrosine residues, works best for GPIIb and GPIIIa. A more uniform labeling of surface sialoglycoproteins can be obtained with biotin by using a periodate method, and the biotin can be detected by avidin-peroxidase using luminol as the substrate.[485] With this method, immunoblotting and immunoprecipitation studies of GPIb and GPIIb/IIIa were done successfully, and reportedly with the same sensitivity as with use of radioisotopic methods.

Lastly, target glycoproteins have been identified by competitive platelet suspension immunofluorescence assays based on the ability of the antibody being detected to block platelet binding of antibodies with known specificity.[486] To be successful, this method requires a large panel of defined monoclonal antibodies. Of all the aforementioned options for identifying the specific target of platelet-reactive antibodies, the best method probably varies with the target.[487]

While most of the assays developed for PAIg or PBIg fit into one of the preceding types, other assays have included an indirect slide immunoperoxidase technique,[488] a radial immunoprecipitation assay,[489] an electroimmunoassay,[490] and a direct latex particle assay for which beads coated with anti-[IgG(Fc)] are incubated with test platelets, the combination of which, upon centrifugation, forms recognizably different pellets when PAIg is present.[491] These assays do not have widespread use.

2. technique considerations

When evaluating results of IMT studies, it is important to consider the assay technique employed and its potential for producing misleading results. Misleading results are more likely to occur with indirect assays, where "In many studies of serum and plasma, no special precautions were taken to discriminate among autoreactive antibodies, alloantibodies, circulating immune complexes, and aggregates of normal IgG present in stored sera".[204] However, direct assay protocols must also be carefully evaluated. Important aspects of assay protocols that must be considered when interpreting results are addressed in the following section. These points should similarly be considered in assay development.

a. low titers

Samples from patients with chronic IMT have more commonly been positive with direct assays for PAIg than with indirect assays for PBIg.[480] The decreased sensitivity of indirect assays has often been considered to be due to titers of PBIg that are low because antibodies have been removed from the circulation by binding to platelets. This may be true, but it is unproven. It is also possible that the conditions of assays somehow hamper detection by indirect methods. The frequency of positivity was recently higher by testing for multiple classes of antibody with an extended panel of platelet proteins.[299] However, low affinity antibodies may still go undetected with direct or indirect assays.[492]

b. alloantibodies vs. autoantibodies

The reverse problem also arises, in that indirect assays may be positive for platelet-bindable alloantibodies when direct assays are negative. Such alloantibodies will not be present on the patient's own platelets and are not pathogenic, but they may react with the allogeneic test platelets from normal donors. Platelet-reactive alloantibodies arise from previous transfusions or pregnancies, and they can also be related to blood type and be directed toward erythrocyte ABH antigens on platelets.[493,494] Since exposure to the ABH antigens can occur without transfusion,[495] alloantibodies reactive with platelet ABH may occur naturally. For this reason, human indirect assays routinely use platelets from blood group O donors. These platelets lack the A and B antigens and will be unreactive to blood group-related alloantibodies. Alloantibodies to HLA class I antigens may still be detected, however, and these are again not indicative of patient PAIg. Rather, they are indicative of previous immunization with a different HLA allotype, most

likely from transfusion. In studies of canine platelet refractoriness, 5 dogs that had never been pregnant were refractory to their first donor but not to all donors, suggesting the presence of preexisting specific alloantibodies not related to transfusion.[369] Therefore, naturally occurring platelet alloantibodies must be considered potential confounding factors in the interpretation of indirect assays for canine PBIg.

c. serum

Serum, as opposed to plasma, has frequently been used as the sample for indirect assays. Serum contains residual thrombin generated during clotting, and thrombin can activate platelets allowing them to express new reactive sites. As a rather promiscuous enzyme, it also has the capacity to enzymatically alter surface antigens on test platelets, perhaps reducing or inducing reactivity. To avoid residual thrombin, serum samples have sometimes been heated to 56° for 30-60 minutes to inactivate the thrombin in addition to complement. While heating may inactivate thrombin if done long enough, it also induces formation of immunoglobulin aggregates. One must be concerned with the possibility that such aggregates may bind to platelet FcγRII and induce positive results. While Fc-mediated binding may be of low affinity, the absence of such binding should ideally be demonstrated for any assay where samples are heated. When assays use fixed platelets as the test platelets, thrombin induced activation is not a problem. Thrombin may still be able to enzymatically alter the platelet surface, however.

d. anticoagulant

The calcium-dependent heterodimer GPIIb/IIIa is a common target of antibodies in human IMT.[299] It undergoes irreversible dissociation and conformational changes when exposed to EDTA in an alkaline medium for 15 minutes at 37°C, but not at room temperature.[496] The dissociation of GPIIb/IIIa results in ultrastructural platelet changes characterized by a collapse of the surface-connected open canalicular system.[497,498] When this occurs, reactivity with GPIIb/IIIa complex-dependent antibodies and fibrinogen is markedly reduced,[496,498] and some autoepitopes on GPIIb/IIIa are lost upon chelation such that autoantibodies will bind weakly or not at all.[298,499] These are presumably topographic (configuration-dependent) epitopes.

Indirect assays that use EDTA for anticoagulation of donor assay platelets or during washing or incubation steps have failed to detect some complex-dependent antibodies, whereas use of citrate has preserved the reactivity.[499] Presumably, enough conformational change occurs at room temperature to alter epitopes, even if complete dissociation does not occur. If platelets are exposed to EDTA in an indirect assay system, the reactivity of complex-dependent antibodies should be tested. Use of EDTA-anticoagulated plasma as the test sample should not be a problem if the final concentration of EDTA to which the donor assay platelets are exposed is low. Complex-dependent monoclonal antibodies preincubated with human platelets protect GPIIb/IIIa from EDTA-induced dissociation.[498] Therefore, direct tests for PAIg with EDTA-anticoagulated blood would probably be able to detect antibodies bound to complex-dependent epitopes on GPIIb/IIIa. It is not yet known whether EDTA has similar effects on canine platelets,

but the possibility must be considered. Since similar treatment of rat platelets does not dissociate GPIIb/IIIa, EDTA may not pose this problem in dogs.[500]

While the preceeding discussion has related to the potential for EDTA to lead to false negative assay results, it can also cause false positive results. EDTA-induced immunoglobulin binding to platelets is well known in the context of pseudothrombocytopenia,[13] but it may be important for platelet antibody assays as well. When canine[21] platelets are tested immediately or at 24 hr, PAIgG results are higher with platelets harvested from blood anticoagulated with EDTA than they are with citrated samples. In a human study, solid but not liquid K_2EDTA caused an increase in PAIgG associated with IgG-rich particulate material larger than 33 fl and therefore too large to be platelet-derived.[467] These particulates could be separated from platelets by a Percoll density gradient such that PAIgG was not affected. However, use of solid K_2EDTA in assays where platelets are harvested by differential centrifugation should be avoided.

EDTA-related increases in background binding will decrease assay sensitivity and, if inconsistent in degree, lead to false positive results. For example, von dem Borne[501] reported that 10 of 91 ether eluates from EDTA-harvested, PAIg-positive platelets were EDTA-dependent. That is, they contained immunoglobulins that bound to normal platelets from EDTA-anticoagulated blood but not to platelets from citrated blood. Therefore, over 10% of the positive results for PAIg were false positives due to EDTA-induced immunoglobulin binding. It is significant that only one of these 10 patients had a diagnosis of IMT, and that patient was in remission. Similarly, EDTA-dependent binding of serum immunoglobulins occurred in 23 of 98 samples from patients whose EDTA-harvested platelets were positive in a direct platelet immunofluorescence test.[501]

The frequency of false positive results related to EDTA with other assay systems is not known, but the potential for a significant problem is likely.

As compared to citrate, some[190,502] but not others[503,504] have found that use of EDTA for anticoagulation of canine blood has been associated with higher mean platelet volumes using impedance methods, and much more platelet surface irregularity and pseudopod formation.[502] It is possible that the final concentration of EDTA and anticoagulant osmolality have varied among studies and that adverse effects occur only under specific conditions.[504] However, this reported effect of EDTA on platelet morphology may be related to the increased PAIg values seen on normal canine platelets exposed to EDTA as compared to citrate.[21]

Using an ELISA,[21] reported that acid citrate dextrose (ACD) is preferable to citrate and EDTA as an anticoagulant for detecting PAIg in dogs. To reach this conclusion, blood was collected from 3 normal dogs into ACD, citrate, and EDTA and placed on ice. Platelets harvested immediately, at 24 hr, and at 48 hr were assayed for PAIg with the described assay. Increasing storage time before platelet isolation was associated with significant increases in PAIg. Trends in the data suggested that the greatest increases occurred with EDTA-anticoagulated blood, and the least effect was seen with blood collected into ACD. Several speculative causes of the reported storage and anticoagulant effects were suggested. These included possible roles for lowered blood calcium, in vitro formation of immunoglobulin aggregates which might bind nonspecifically to a platelet Fc receptor, anticoagulant-induced neoantigens, or other membrane alterations induced by anticoagulants over time that allow nonimmunologic

binding of immunoglobulin to the platelet surface. The presence of surface perturbations induced in canine platelets by EDTA may have a role.[502]

The data do support the contention that ACD is preferred over citrate and EDTA in the system used, but it is not known if the same would be true with other assays, other techniques of platelet isolation, other storage conditions, and other immunoglobulin detectors. For example, since canine platelets undergo shape change at 4°C, especially in EDTA,[502] the storage temperature might have contributed to the effect; perhaps room temperature storage would be superior. It should also be emphasized that the differences between ACD and citrate were minimal, not statistically significant, and based on only 3 dogs. While the trends are clear with the techniques employed, the general statement that "the preferred anticoagulant for platelet-bound antibody measurement is acid citrate dextrose" may be an overstatement at this time.

e. fixation of platelets

In many indirect assays, normal donor assay platelets are fixed with para-formaldehyde (PFA). This is done in fluorescent assays to reduce nonspecific platelet autofluorescence.[477] However, such fixation is apparently not innocuous. von dem Borne[501] reported the presence of PFA-dependent antibodies in 12 of 98 sera from patients with positive direct platelet suspension immunofluorescence test results. That is, their serum immunoglobulins bound to PFA-fixed but not unfixed platelets. No PFA-dependent antibody binding was detected in platelet eluates. Using the same assay, PFA-dependent PBIg were also detected in the sera of 22/103 patients with chronic renal failure.[505] It was suggested that the aldehydes used in the dialysis equipment could have

sensitized these patients to PFA-dependent antibodies, though healthy controls were not tested to confirm the association with chronic renal failure and dialysis. It is clear, however, that PFA fixation of test platelets in indirect assays may induce a significant number of false positive results.

The use of fixation has been questioned even for flow cytometric fluorescence assays, because fixation reportedly alters the light scattering properties of platelets indicative of some structural alterations.[479] Platelet fixation in 0.5 and 1% formaldehyde has been shown to induce the expression of P selectin (CD62) and GP53 (CD63, a lysosomal granule protein) on normal platelets,[506] so other surface alterations are possible. This could conceivably include alterations leading to false negative results. However, lack of fixation in fluorescence assays may decrease the signal-to-noise ratio and reduce sensitivity.

Fixation of patient's test platelets is also sometimes done in direct assays. Therefore, the potential exists for fixation-induced false negative and false positive results for PAIg.

f. freezing platelets

Patient test platelets have been frozen so that samples could be assayed later in batches, and donor assay platelets have been frozen for convenience. Potential adverse effects of such freezing have been evaluated for human platelets in a platelet immunofluorescence assay.[507] While there was some loss in platelet yield, the platelet antigenicity was apparently minimally affected in an indirect immunofluorescence assay, and freezing

only mildly decreased the PAIg detectable on patient platelets in a direct immunofluorescence assay.

Platelet freezing has also been used for canine assay systems.[191,240,508] In an indirect ELISA, the binding of normal sera to previously frozen normal assay platelets over 13 weeks was quite consistent, but the binding of positive serum samples over time was not done to confirm continued reactivity[191] In an indirect flow cytometric platelet immunofluorescence assay, the use of frozen versus fresh platelets was evaluated by using serum from 3 healthy dogs and from 3 dogs with suspected IMT[240] There was reportedly no statistically significant difference between the fresh and frozen platelet data, though the data were not shown. The possibility that freezing alters the reactivity to some but not other antibodies must be considered. Studies of platelet survival in dogs using frozen platelet concentrates showed a marked decrease in survival with platelets frozen 1 year as compared to fresh platelets[67] It is possible that freeze-thaw steps alter platelet antigenicity as well as platelet survival.

g. positive conversion of stored samples

Indirect tests are often batched so that many samples can be run at one time, thus necessitating the freezing of serum or plasma samples until test day. There is evidence to suggest that IgG in stored sera (-20°C for 3 months) from patients with IMT may develop disulfide-bonded aggregates that bind to GPIIb/IIIa in a non-$F(ab')_2$, non-Fc manner.[444] In this study, serum samples converted from negative to positive with time, though only for patients with diagnoses of primary IMT. These findings question the meaning of some positive results with indirect assays, and suggest that samples for

indirect assays should not be frozen for prolonged periods at -20°C. More work is required know the impact of this report, but it indicates the need to further explore and define the binding orientation of PAIg in IMT.

h. cytosolic reactivity

Another problem with indirect assays relates primarily to the newer assays that use captured or purified platelet proteins rather than whole platelets. Some serum antibodies have been shown to react to internal cytosolic portions of platelets that are not normally exposed on the surface of intact platelets.[299,509,510] These cytosolic sites are exposed when surface proteins are extracted from the platelet membrane.

In one study,[299] antibodies reactive with cytosolic epitopes of at least one glycoprotein were present in 66% of 47 sera from patients with chronic IMT, though they were never present without accompanying antibodies reactive to exosolic epitopes. The specificity of the serum antibodies with exosolic reactivity appeared to be representative of the PAIg in these cases. Patients who had recovered from other platelet-destructive states such as PTP and drug purpura often (38% of 16 tested) had serum antibodies with cytosolic but not exosolic reactivity. Antibodies reactive to cytosolic epitopes are therefore thought to be produced in platelet destructive states when cytosolic material is made available for presentation to the immune system. Such antibodies are currently suspected of being nonpathogenic. Therefore, while indirect assays based on captured antigens or purified proteins reduce nonspecific immunoglobulin binding to the platelet surface and define the target of PBIg, positive reactions are only interpretable after exosolic reactivity has been confirmed.

Use of assays based on reactivity to whole platelets should be less affected by cytosolic reactivity, though partial reactivity to intact platelets may occur secondary to changes induced by centrifugation and washing.[509] Gel filtration may be a preferable method of obtaining washed platelets for indirect assays because cytosolic reactivity was not detectable in such systems.[299] However, more work is required to confirm that cytosolic-reactive antibodies are nonpathogenic, as it is conceivable that in vivo binding could occur with platelet surface perturbations. Also, since antibodies have been shown to enter T lymphocytes via Fc receptors and affect their function,[511] perhaps platelets can internalize antibodies by an Fc-mediated process that results in altered platelet function.

Another potential advantage to using whole platelets in indirect assays is that many glycoproteins, lipids, and carbohydrates are not included in assays based on captured or purified antigens, but all potential surface targets are tested with whole platelets. However, there is a greater potential for nonspecific immunoglobulin binding including that due to immune complexes and immunoglobulin aggregates binding to platelet Fcγ receptors. Since essentially all of the cross-linked IgG dimers bound to platelet FcγRII can be removed with a single wash of PBS, immune complexes may not be detected by assays employing several wash steps.[431] However, the behavior of larger immune complexes that may be present in disease states is unknown. The contribution of such FcγRII binding to PBIgG remains to be clearly established, but it appears to be minimal in sera from human IMT patients.[512]

i. antiglobulin reagents

Many human platelet immunoglobulin assays have actually been assays for IgG because the antiglobulin reagents have been IgG-specific. Pathogenic IgA and IgM antibodies have therefore been underdetected.[299] When SpA has been used to detect platelet antibody, IgG_3 antibodies have been missed unless SpA incubations were preceded by incubations with a polyclonal rabbit anti-IgG antibody.[513,514] In these ways, the choice of detector has an obvious effect on the sensitivity of the assay. In a recent study of 47 sera from patients with chronic IMT, immunobead techniques were used to identify IgG, IgA, and IgM reactivity to purified Gps IIb/IIIa and Ib/IX, and to captured glycoproteins IV, and Ia/IIa.[299] Specific GP-reactive antibodies to exosolic epitopes were detected in 38 (80%) of the sera, and were IgG in 71%, IgA in 58%, and IgM in 18% of the positive samples. Less successful rates for detection of serum PBIg in other studies may relate to the attempted detection of fewer immunoglobulin classes.

The use of polyclonal as opposed to monoclonal detectors may also have an effect on assay sensitivity. There is evidence that the hinge region of IgG allows enough flexibility for an antibody molecule to bind to a single platelet by both its Fab and Fc ends simultaneously.[515] Antibodies bound in this way may go undetected by monoclonal detectors that bind to the Fc region and require it to be exposed. Since there are an estimated 1000-2000 FcγRII per human platelet,[430,431] this number of PAIg could conceivably be masked unless detectors with non-Fc specificity are used. Therefore, the sensitivity of monoclonal Fc-reactive detectors may be lower than optimal.

j. controls

While proper positive and negative controls are imperative if assay results are to be completely reliable, their use is not always clearly outlined. Generally, however, normal platelets incubated with buffer serves as a negative control. Plasma/serum samples containing PBIg function as the positive controls. The control sera must be from the same species as the test samples. A control that is not typically included but may be important for indirect assays is a control antiglobulin reagent that does not bind specifically to the immunoglobulin of the species in question. Using a flow cytometric technique, Christopoulos[479] recently found that platelets from people with IMT, as compared to those from normal people, bound more nonspecific goat IgG as well as specific goat anti-human IgG(γ). This suggests an increased "stickiness" of IMT platelets that may not necessarily reflect the presence of as much PAIg as might be thought. Further investigations will be needed to clarify this potential problem.

Lastly, standardization such that comparisons can be made from lot to lot of antiglobulin reagents is often not discussed, but is very important, especially for quantitative assays. If lot-to-lot comparisons are to be made, the relative activity of each new batch of reagent must be tested against some standard, generally the positive control antibody, unless new reference ranges are established with each new batch.

k. erythrocyte and leukocyte contamination

False positive reactions for platelet antibodies could be induced by the presence of other cell types in the incubation mixtures. For direct assays, lymphocytes bearing immunoglobulins could theoretically be problematic, though 20% lymphocyte

contamination of test platelets did not significantly affect PAIg results in a study using a SpA-based radioimmunoassay.[513] Granulocytes or erythrocytes could also be the source of detected immunoglobulin in the event of concurrent immune-mediated neutropenia or immune-mediated hemolytic anemia. Contaminating erythrocytes have been lysed by ammonium oxalate, but whether or not all erythrocyte membranes get removed by centrifugation is not clear.

For indirect assays, leukocyte contamination of the platelets is the primary concern because leukocytes have numerous high affinity Fc receptors that can potentially bind serum or plasma immunoglobulin. This could raise the background or cutoff level for all samples tested with the leukocyte-contaminated batch of platelets as compared to tests with uncontaminated platelets.

1. total vs. surface immunoglobulin

Much of the difficulty related to the interpretation of platelet antibody assay results has stemmed from the fact that over 99% of the total IgG associated with human platelets is internal and located in α-granules.[516,517] Only a small percentage is surface bound. Megakaryocytes and platelets incorporate this internal IgG by fluid-phase endocytosis such that platelet IgG concentrations and isotype distributions correlate directly with those of the plasma. This is true in healthy people as well as in patients with abnormal plasma IgG concentrations. IgA, IgM, and albumin are also incorporated by fluid phase endocytosis and are present in platelets at concentrations proportional to plasma concentrations.[517] In contrast, the uptake of fibrinogen is mediated by GPIIb/IIIa and leads to higher platelet concentrations of fibrinogen than would be expected by

fluid-phase endocytosis.[518] Since endocytosis continues throughout the circulatory life of human platelets, internal IgG concentrations increase as platelets age.

Increased total PAIgG is also present in large platelets that contain more α-granules. Since the megathrombocytes and "stress" platelets[519] released during periods of intense thrombopoiesis are large, one would expect total platelet IgG concentrations, reported on a per platelet basis, to be elevated in any acute regenerative thrombocytopenia, whether immune or nonimmune. This is indeed the case in people. Similarly, both total PAIgG and surface PAIgG have been shown to be lower in patients with thrombocytopenia due to marrow failure and minimal thrombopoietic capacity. Thus total PAIg is not a good marker for immune destruction; rather, it appears to be more of a general marker of thrombopoiesis, much as reticulocytes are a marker of active erythropoiesis.[516]

Surface-bound PAIgG, however, as opposed to total PAIgG, has been minimally elevated in nonimmune conditions associated with peripheral platelet destruction, while markedly elevated in people with clinical IMT.[171,476,516] Direct flow cytometric platelet immunofluorescence evaluations of surface PAIgG on platelets of thrombocytopenic patients has shown that platelet populations of all evaluated sizes contributed the same percentage of total fluorescence as did the same size populations of platelets with normal PAIgG.[520] Therefore, elevated surface PAIgG in thrombocytopenic patients is not simply an artifact related to an increased percentage of microthrombocytes or megathrombocytes. It appears, then, that surface PAIgG is generally reflective of antiplatelet antibody, but minimal elevations may occur in other disorders usually considered to lack an immune pathogenesis. Elevations of surface PAIgG in these cases may reflect an

unsuspected immune component, an unsuspected technical problem, or nonpathogenic increases due to changes in the platelet surface induced by the underlying disease state.

The importance of this discussion with respect to assays for PAIg is that platelet surface immunoglobulin and not total platelet immunoglobulin must be assessed for meaningful results. Assays with steps that purposely (sonication, freeze-thaw, detergent treatment) or unintentionally[521] cause platelet lysis or secretion may allow detection of internal immunoglobulin if the fluid phase containing the internal platelet products is not separated from the platelets before the final assessment of PAIg.[522]

The same conclusions likely hold for dogs. Canine platelets also contain internal IgG which they accumulate in the circulation as they age,[523] and large platelets are produced in dogs with strong thrombopoietic stimuli.[134,524] Studies of canine cyclic hematopoiesis have shown that the cyclic increases in platelet concentration are directly associated with cyclic increases in mean platelet diameter and plasma thrombopoietin activity.[106] Dogs given IL-6 had as many as 20% of their platelets as large as erythrocytes, and platelets were more sensitive to platelet agonists; however, it is not clear if the large platelets were more functional because of their size alone or because of another effect of IL-6.[9] Together these findings suggest that in dogs, as in people, total PAIgG measured on a per platelet basis may also increase in response to any strong thrombopoietic stimulus in concert with an increased number of large platelets, and circulatory endocytosis may accentuate the increase over time. Measurements of total PAIg in dogs should therefore be interpreted cautiously, and assay development should focus on detection of surface PAIg.

The effect of young platelets on PAIgG has been minimally evaluated in 4 dogs, but the details of the assay for canine PAIg were not reported.[492] It is therefore not clear if the assay measured surface PAIg or total PAIg. The assay was probably either a ^{125}I-labeled antiglobulin technique or a complement lysis inhibition assay. Dogs were platelet pheresed, and their PAIgG concentrations were measured as platelet concentrations rose to normal. There was no consistent effect of young platelets on increasing PAIgG, suggesting that young platelets did not affect results with that assay. However, the platelet concentrations dropped maximally to just under 100,000/μl, and there were no data to indicate that pheresis induced an increase in MPV.

m. sample handling

Proper sample handling is typically not discussed in reports of platelet antibody assays, but there are many aspects of sample management that may have an effect on the outcome of results. Aspects to consider include sample age, sample storage temperature, assay temperature, and general handling trauma. Variations in these factors has not generally been evaluated in a critical manner, so the importance of uniform sample handling is not known. Clearly, uniform handling of control and test samples should be the rule to avoid any potential effects of inconsistent handling. It is conceivable that equivalent handling of normal control samples and patient samples does not always occur due to the ready availability of normal control subjects but the sporadic and sometimes untimely appearance of patients. The need for sample handling consistencies in dogs has been noted.[21]

C. Veterinary Assays for PAIg and PBIg

Assay development for canine IMT is in its infancy compared to assay development for human IMT. Better veterinary assays have been developed in recent years, but they have not yet been widely tested and few results have been published. Estimates of assay sensitivities and specificities have been regularly reported, but they must be interpreted cautiously. Such values are based on the ability to differentiate immune-mediated from nonimmune thrombocytopenic conditions. This differentiation cannot currently be done because there is no definitive diagnostic test ("gold standard") to confirm the presence or absence of immune-mediated platelet destruction. The contribution of immune-mediated platelet destruction to the thrombocytopenia of many diseases is therefore unknown. In lieu of such a definitive test, the diagnoses of thrombocytopenic conditions have generally been made clinically and are subject to error. As stated by Christopoulos, "It is logical to argue that the sensitivity and specificity of any new method for PSIgG [platelet surface IgG] detection should refer to its ability to reproducibility detect known amounts of IgG on the platelet surface rather than the compatibility of its results with the subjective clinical diagnosis of ITP which, after all, is a heterogeneous entity and a diagnosis of exclusion."[479]

Though some reports imply otherwise, current veterinary assays for PAIg should not be expected to differentiate primary autoimmune thrombocytopenia from other immune-mediated thrombocytopenic disorders. Rather, if the human thrombocytopenic conditions can serve as a guide, a more reasonable goal of these assays would be to differentiate primary and secondary immune-mediated thrombocytopenic conditions from thrombocytopenic conditions due to nonimmune mechanisms.

1. PF3 release assay

One of the earliest approaches to diagnosing IMT in dogs was through use of the indirect platelet factor-3 release (PF3) assay.[239] The samples used for the canine PF3 assays have been either dialyzed globulin fractions obtained by ammonium sulfate precipitation[239] or serum heated for 30 minutes at 56°C with[246] or without[525] subsequent barium sulfate adsorption to remove residual thrombin. Like the human assay after which it was modeled,[526] the theory behind the canine PF3 assay is that antiplatelet antibodies in a test patient's plasma or serum will bind to normal assay platelets and injure them so they express or release more phospholipid (called PF3) which then accelerates coagulation. Coagulation time is actually the test parameter measured, a positive sample being associated with a significantly shortened coagulation time compared to normal control samples.

Clearly, this is a very indirect test which does not specifically detect antibodies at all. Rather, it nonspecifically detects any serum or plasma factors capable of accelerating coagulation by any means. Besides autoantibodies, positive results may be due to alloantibodies or circulating immune complexes. Aggregated immunoglobulins formed through sample manipulations may also cause positive results, especially with serum and fractionated samples. When heated and unheated normal serum samples were tested in an indirect ELISA for titers of canine PBIg, significantly more PBIg was consistently detected in the heated samples, likely because of aggregated IgG.[237] Such aggregates generated in vitro by heating,[237] concentrating,[445] or frozen storage[204,444,445] may elaborate PF3 from assay platelets causing accelerated coagulation and false positive results.[239,527] Lipemic and hemolyzed samples have also been associated with false

positive results.[525] Negative results may occur whenever antibody binding does not sufficiently alter the platelet surface to expose PF3, or when titers are undetectably low.

In one system, the reported range and mean of coagulation times for 50 control sera tested at different times and with different PRP batches were 50-85.5 and 66.1 ± 8.7 seconds, respectively.[525] Two samples had times of 99.3 and 108.6 seconds and were not included. This suggests that the variability among normal test samples and donor platelets is considerable. Because of this variability which relates in part to donor platelet reactivity, test samples are generally tested in tandem with controls.[525] However, many controls may be required to provide a reasonable idea of normal variation with each run of the assay. PF3 results from normal dogs have generally been either superficially addressed or omitted from published reports, so it is difficult to tell how well controlled the assay has been in different hands.[191,239,246,343]

Removal of PF3 positivity by prior incubation of samples with a monospecific rabbit anti-[canine IgG] antibody has incriminated IgG as the factor responsible for PF3 positivity in some dogs with clinical IMT.[239] With normal control samples, the anti-[canine IgG] antibody shortened coagulation times, possibly due to the formation of immune complexes, instead of prolonging them as occurred in 6 of 8 samples from dogs with positive PF3 results. While such studies suggest immunoglobulin mediation of the PF3 positivity, they do not differentiate autoantibody from alloantibody, aggregated IgG, or immune complexes.

In a study of dogs with suspected IMT, platelet concentrations were less than 20,000/μl in 6 of 9 (67%) positive samples but in only 7 of 22 (34%) negative samples.[525] If the negative dogs in this study truly had IMT, the data indicate a greater

sensitivity of the assay in the presence of marked as compared to mild to moderate thrombocytopenia. Cumulatively, 72 of 168 reported dogs (43%) with this diagnosis had positive PF3 results.[161,191,239,246,247,343] This mediocre sensitivity for substantiating a clinical diagnosis of IMT, and the great potential for nonspecific positive results make the PF3 assay one that cannot be recommended.

2. megakaryocyte immunofluorescence

Another early veterinary assay was the direct megakaryocyte immunofluorescence assay (D-MIFA) which has been considered a test for antiplatelet antibodies.[246] The MIFA, in contrast to the PF3 assay which assesses platelet activation and coagulation, is actually an antibody detection technique. In this assay, fluorescein-conjugated antibodies to canine immunoglobulins are incubated with fixed bone marrow smears from thrombocytopenic or control patients. After washing, megakaryocytes are microscopically inspected for fluorescence. Positive megakaryocyte fluorescence is considered indicative of megakaryocyte-associated immunoglobulin which has in turn been considered diagnostic of antibody-mediated thrombocytopenia.

In dogs, Joshi and Jain[331] showed that injected rabbit anti-[canine platelet] IgG could be detected on megakaryocytes by a D-MIFA. This simultaneously demonstrated that a D-MIFA could detect megakaryocyte-bound IgG and that canine platelets and megakaryocytes share at least some antigenic sites. As discussed above, antibodies reactive with human platelets have been shown to have variable reactivity with human megakaryocytes; some react with essentially all megakaryocytes while other antibodies are unreactive with most megakaryocytes.[99] Some antibodies apparently recognize

megakaryocyte epitopes only after the megakaryocytes have reached an advanced stage of maturation. Because canine platelets and megakaryocytes may also not be antigenically identical, D-MIFA positivity may not always match PAIg positivity.

The first problem that may be encountered with D-MIFAs is inadequate samples. Some marrow samples are devoid of intact megakaryocytes or have too few to confidently evaluate.[161,241,246,525] With adequate samples, high background fluorescence has interfered with the differentiation of positive from negative,[241,528] though a first wash of marrow aspirates before incubating with antiserum helped decrease this background fluorescence.[528]

Even when samples are adequate and fluorescence is detected above background levels, the nature of the fluorescence may be in question. When dried marrow smears are used instead of cell suspensions, as has been the rule for veterinary assays, damaged cells may allow detection of intracellular immunoglobulins when in fact no relevant surface-bound immunoglobulin is present.[99] Megakaryocytes incorporate immunoglobulins into their α granules by endocytosis as discussed above for platelets.[517] The fluorescence of antibody-coated human megakaryocytes on such air-dried smears, as opposed to cells in suspension, routinely has a patchy cytoplasmic pattern while rim patterns characteristic of surface reactivity cannot be appreciated.[99] Megakaryocyte fluorescence may therefore be misleading on air-dried smears. Use of cell suspensions would eliminate the complication of detecting internal IgG exposed by smearing trauma.

Proper assay controls are another concern. It is not clear from some reports if a single negative control bone marrow sample is used repeatedly or if samples from different normal dogs are used.[246] It would be important to use many normal dogs to get

an appreciation of the variability in normal background fluorescence. In some cases, platelets present on stained preparations have served as positive controls. This is in essence using the test as its own control. It would be more appropriate to include in each run of the assay a positive control made by preincubating a normal bone marrow preparation with an antibody recognizing a megakaryocyte surface epitope. This and appropriate negative controls would confirm the expected specific and nonspecific reactivity of the fluorescein-labeled secondary antibody with each run of the assay.

Another possible problem with D-MIFAs depends on whether or not megakaryocytes have surface Fc receptors. At this time it is not known if megakaryocytes or canine platelets have Fc receptors.[21] If present, they could bind nonspecific immunoglobulin including any antibodies used as antiglobulin reagents.[324]

The cumulative sensitivity of D-MIFAs for substantiating clinical diagnoses of IMT in 44 reported dogs is about 50%.[161,241,246] While this sensitivity is similar to that of the PF3 assay, results of the 2 assays on the same dogs do not correlate perfectly. In one study, 6 of 9 D-MIFA-positive dogs were PF3-negative, suggesting increased sensitivity of the direct assay.[161,525] Similarly, in another study of dogs with solid tumors, negative PF3 results were present in 6 of 7 dogs with positive D-MIFA results.[253] In both studies, PF3 positivity was more likely with severe thrombocytopenia.

A D-MIFA has also been compared to an indirect platelet immunofluorescence assay for PBIg, and neither assay was consistently more sensitive.[241] Two of 5 dogs with positive D-MIFA fluorescence had negative results in the indirect fluorescence assay, and 5 of the 7 dogs with negative D-MIFA results were positive in the indirect assay.[241] Positive D-MIFA results with negative results in indirect assays suggest that D-MIFA

results are more sensitive, that they are associated with false positive results, or both. Negative D-MIFA results with positive results for circulating PBIg suggest that megakaryocytes may not always be involved antigenically in IMT, that the indirect assays produce false positive results, or both. Without a standard for diagnosing IMT, separating true positives and true negatives from false positives and false negatives is impossible.

Canine serum from 1 dog has also been assessed for megakaryocte-bindable immunoglobulin in an indirect MIFA, though the methods were not described and the result was negative.147(Murtaugh & Jacobs, 1985) The major problems associated with indirect MIFAs relate to adequate controls, and interpretation of the results is weakened by the same factors that affect all indirect assays as outlined previously.

3. ELISAs

Several enzyme-linked immunosorbent assays (ELISAs) have been developed to detect either PAIg on patients' platelets or PBIg in patients' plasma samples. In theory, ELISAs are attractive because of their relative simplicity and nonreliance on expensive instruments and radioactivity. In practice however, they have been associated with such problems as a relatively large amount of nonspecific background binding.[191,237]

a. with polyclonal anti-[canine IgG]

The first published ELISA for use in diagnosing IMT in dogs was an indirect assay for PBIg.[191] A horseradish peroxidase-conjugated, polyclonal anti-[canine IgG] adsorbed against pooled platelets from 4 healthy dogs was used to detect platelet-bound

IgG after incubation of test and control sera with normal donor platelets. The assay appeared to be an improvement over the PF3 assay since 15 of 17 dogs (88%) with clinical diagnoses of IMT had positive ELISA results while only 53% of the same dogs had positive PF3 results.

While an apparent improvement over the PF3 assay, this ELISA still suffered such problems inherent in indirect assays as potential positivity due to nonspecific immune complexes, immunoglobulin aggregates, and alloantibodies. Additional concerns with the assay relate to sample management, nonspecific background binding, platelet storage conditions, and the positive control. Test sera were heated to 56°C for 30 minutes to inactivate thrombin generated during clotting, and they were then frozen until use, thus creating the potential for in vitro immunoglobulin aggregate formation. Nonspecific aggregates could have increased the amount of PBIg,[237] thus decreasing the signal-to-noise ratio which was low compared to the capabilities of other assays. The mean and high absorbance of positive samples were only 2.2 and 4.5 times the mean of normal samples, respectively. Nonspecific adherence of immunoglobulins to the polystyrene wells used in the assay may also have contributed to the high background values.

The normal donor platelets for the assay were collected in EDTA and stored after washing at 4 or -70°C before use. Results from testing normal sera on the frozen platelets over 13 weeks were quite consistent, but there were no data to indicate that the refrigerated (4°C) platelets behaved similarly. Also, the binding of positive samples over time with the two batches of platelets was not done to confirm continued reactivity. The potential problems with EDTA have been discussed above.

As for controls, a positive control serum and negative control sera from at least 5 healthy control dogs were included with each run of the assay. However, the positive control serum was a rabbit anti-[canine platelet] serum that had to be detected with an anti-[rabbit IgG] antibody instead of the actual assay anti-[canine IgG] antibody. The activity of the assay's secondary antibody was therefore not evaluated by this so-called positive control serum.

Last are concerns relating to the long term use of this assay. Each batch of platelets had a different amount of background absorbance. The upper limits for a negative result with two batches were 0.385 and 0.463 absorbance units. Comparisons of results among batches would therefore require adjusting the data relative to a standard curve generated from a positive sample, but there was no discussion of such a normalizing positive control. Variation could also occur from lot to lot of the secondary antibody, again necessitating a method of assay normalization or use of a single lot. And again, trust in comparisons from one run to another would require data verifying that long term storage of the assay platelets had no effect on positive samples. Therefore, while the sensitivity of the ELISA technique appears to be greater than that of the PF3 assay, several questions remain to be answered before it can be accepted as a valid and useful assay.

A direct ELISA for PAIg was recently described in which 1% Triton X-100-solubilized, washed platelets served as the test samples.[508] Test samples were added to wells coated with anti-[canine IgG], and after incubation and washing steps, horseradish peroxidase-conjugated, polyclonal anti-[canine IgG] was added for detection of the bound IgG. Samples were evaluated from 50 normal dogs, 13 dogs with suspected IMT, 12

dogs with inflammatory/infectious conditions, 4 dogs with systemic immune-mediated disease, 14 dogs with cancer, and 11 dogs with miscellaneous conditions. The PAIg was reportedly significantly elevated in the IMT dogs compared to the other groups ($p=0.0015$), but the ranges and amount of overlap among groups were not reported in the abstract.

Two aspects of this abstract are surprising: 1) that enough platelets were reportedly harvested from thrombocytopenic dogs by centrifugation of just 5 ml of blood, and 2) that the use of solubilized platelets as test samples, which results in assaying whole platelet IgG (internal and surface-bound) instead of just surface-bound IgG, produced a significant difference from all other test groups. As discussed above, total platelet IgG is not a reliable indicator of surface-bound immunoglobulin and IMT.[445,517] In light of the likelihood that whole platelet IgG would be elevated in a significant number of dogs without immune-mediated platelet destruction, more thorough evaluation of the data is necessary to determine how much overlap occurred among groups and therefore how useful the assay really is on an individual basis.

b. with staphylococcal protein A

(1) staphylococcal protein A

Staphylococcal protein A (SpA) is a 42 kD bacterial type I Fc receptor secreted from, or found on the surface of, most strains of *Staphylococcus aureus*[514,529,530] As an Fc receptor, SpA binds with high affinity[531] to constant region determinants (CH2 and CH3)[532] on the Fc portion of heavy chains of IgG from many species including

dogs.[529,533-536] This property has made SpA a useful detector of antigen/antibody interactions in a wide variety of immunoassays.

The major limitations of SpA relate to either too broad or too narrow a spectrum of reactivity for the particular application of interest. Its breadth of reactivity is too great when immunoglobulin class-specific interactions are desired, because SpA reacts with some non-IgG immunoglobulins from several species.[536] Its reactivity is too narrow, however, to detect or purify all IgG subclasses in some species; for example, SpA does not bind well to human IgG_3 and murine IgG_1.[514] The binding spectrum of SpA to other mammalian immunoglobulins has been reviewed,[536] and there are contradictory findings with respect to dogs. Some investigators have reported that SpA binds to 87% of canine IgE, at least 90% of canine IgM and IgA, and essentially all of canine IgG.[533,534] Such a spectrum of reactivity would make SpA a very useful reagent for canine immunoassays because it would allow detection of most pathogenic immunoglobulins with a single detector. However, others[535] have reported that SpA binds to only 67 and 36% of canine IgG and IgM, respectively, and with no subclass specificity; if true, this would be expected to lead to a significant number of false negative findings in SpA-based immunoassays.

(2) assays

Using modifications of the Campbell ELISA,[191] McVey[237] investigated the use of a SpA-based indirect assay for PBIg in test sera. One of the major goals of the work was to assess the utility of an ELISA based on the use of extracted platelet antigens in place of whole platelets. To this end, sera were tested in 2 assays, one using whole

platelets and one using platelet antigen extracts as antigen sources. Platelet antigens were extracted by first incubating SpA-Sepharose with one serum sample that had been shown by the whole platelet ELISA to have a high titer of PBIg. After washing, the beads were incubated with a platelet lysate, presumably from the same pool of platelets used in the whole platelet assay such that comparisons in reactivity with the whole platelet ELISA would be meaningful. After washing, the bound platelet antigens and immunoglobulin were eluted from the SpA-Sepharose, concentrated by perevaporation, and adsorbed with fresh SpA-Sepharose beads to reduce the amount of residual immunoglobulin. For the ELISA, 10μg of this extracted platelet antigen were added to each microtitration well.

The assays were evaluated in 10 thrombocytopenic dogs, 5 normal dogs, and 7 dogs with other documented immune-mediated diseases. The selection criteria for the 10 thrombocytopenic dogs, their specific clinical diagnoses, and the basis for those diagnoses were not reported, but they presumably were all suspected of having IMT. Each had platelet concentrations less than 50,000/μl, and 5 were positive and 5 were negative in the PF3 assay. Conclusions were limited by the low sample numbers, but the ELISAs were positive in all 10 thrombocytopenic dogs (100%) and thus they appeared to be more sensitive than the PF3 assay. Normal dogs were negative in every assay, but dogs with nonimmune thrombocytopenias were not evaluated.

Data from the two ELISA assays were interpreted as indicating that there was generally greater reactivity with whole platelets than with extracted antigens, because the A_{405} values at 1:100 dilutions were much lower with the latter. However, even the serum used to extract the antigens in the first place reacted more in the whole platelet ELISA than in the extracted antigen ELISA, so it is likely that there was simply not an

equivalent amount of protein available in the two assays. There did not, in fact, appear to be any effort to equalize the amount of antigen in the 2 systems.

Assays based on defined, purified antigens can be very useful, but the antigens used in this assay were neither defined nor pure. By using whole platelet lysates instead of just platelet membrane components, the extracted antigens likely included cytosolic domains of questionable pathologic importance. Second, the use of only one positive serum sample to capture potentially important antigens is likely to leave many uncaptured, since one sample would not be expected to react with all the important platelet antigens. It is, in fact, surprising that with the use of this approach, every sample positive with the whole platelet ELISA was positive with the extracted antigen ELISA. This suggests common reactive antigens for all 10 sera, multiple reactivity of the extraction serum, very nonspecific extraction of platelet antigens, or other uncharacterized problems with the technique.

Interestingly, while all positive samples generally reacted less in the antigen capture assay than in the whole platelet assay, the differential in results between these assays was much greater for nonthrombocytopenic dogs with diagnoses of SLE, rheumatoid arthritis, and immune-mediated hemolytic anemia than for dogs with IMT. This suggests that the platelet binding sites involved in these conditions, whether specific or nonspecific, were not isolated by the extraction technique, and it supports the authors' conclusion that use of extracted antigens may enhance the specificity of immunoassays for canine PBIg.

Another interesting and important finding was that normal sera heated to 60°C for 30 minutes prior to assaying had significantly greater reactivity than unheated sera.

This suggests that nonspecific immunoglobulin aggregates may be detectable as PBIg in such a system. The use of heated serum in similar assays or in PF3 assays, as has often been routine, should therefore be questioned.

As described, there are several other potential problems with this assay. While the routine assay samples were apparently not heated, the handling of the serum samples was not characterized, and no mention was made of thrombin inactivation by heating, heparin, or hirudin. Therefore, results may have been affected by enzymatic degradation due to residual thrombin. If the samples were kept in frozen storage before use, as is probable, freeze-thaw artifact was also possible. A third potential problem relating to the serum samples was that they were incubated with platelets or platelet antigens at room temperature. Such an incubation temperature allows for the possible binding and detection of nonpathologic cold-reactive immunoglobulins which would produce false positive results. The last concern relates to the normal platelets used as antigenic sources for the assays. These platelets were harvested from blood anticoagulated with EDTA, thus raising the potential for false positive results due to EDTA-induced neoantigens and false negative results due to potential dissociation of the calcium-dependent GPIIb/IIIa complex.

The same investigator has reported quantitative results of an ELISA for PAIg in a single case report.[238] The assay was not described, but the results indicate that the quantitation was not realistic. For this dog, PAIgG was reportedly present at 25,000 fg/platelet, with a reference range provided of 5,000 to 15,000 fg/platelet. A value of 25,000 fg/platelet corresponds to about 1 X 10^8 molecules per platelet. In people, normal total PAIgG, internal and surface-associated, amounts to only 5 fg or about

20,000 molecules/platelet, and surface PAIgG, which is the IgG that should be measured, is less than 1% of the total.[445] While there could be species differences in the amount of IgG per platelet, these numbers are clearly unreasonably high.

Recently, an ELISA was developed for directly detecting canine PAIg by means of biotinylated SpA and alkaline phosphatase-conjugated avidin.[21,160] This is the first direct assay to be used on a large number of dogs with clinical IMT. In this assay, platelets were harvested by differential centrifugation from ACD-anticoagulated blood submitted on ice. Contaminating erythrocytes were removed by lysis with 1% ammonium oxalate, and then monolayers of 5 X 10^6 washed platelets were made in wells of microtitration plates and fixed with 1% paraformaldehyde. This was followed by sequential alternating incubations and washes with biotinylated SpA and then avidin-conjugated alkaline phosphatase. Absorbances were determined at 405 nm after the addition of substrate and termination of the reaction.

An indirect assay was also reported. It used thawed frozen serum diluted 1:5 with PBS. The serum was incubated with monolayers of homologous platelets from 3 clinically normal dogs for 1 hr at 37°C. The monolayers were then washed 6 times and assessed as for the direct assay. Use of these assays led to the following observations: 1) neither the amount of PAIg nor PBIg correlated with platelet concentrations or MPVs, and 2) there was no significant correlation between the amounts of PAIg and PBIg.

The intra-assay and interassay coefficients of variation (CVs) for the direct assay were calculated from 8 measurements and were 10 and 13% respectively. Four measurements were used for the indirect assay and the CVs were 7 and 10% respectively. Positive results were those that were greater than 2 standard deviations above the

mean of 20 clinically normal dogs. The direct assay reportedly had a sensitivity of 94% and a specificity of 62% for clinically diagnosed primary IMT[21] The 2 dogs with diagnoses of IMT but negative tests had both been treated with glucocorticoids and had platelet concentrations within the reference range. The authors actually state that these figures represent the sensitivity and specificity for idiopathic thrombocytopenic purpura which they define as an autoimmune disease. Data are lacking, however, to verify that all the antibodies detected were truly autoantibodies as opposed to other forms of PAIg. The moderate specificity indicates a high number of false positive reactions, an immunological component to thrombocytopenic conditions other than pure primary IMT, or both.

The indirect assay had a sensitivity of 34% and a specificity of 80%. The author says that this is lower than other reports for indirect assays that used polyclonal anti-[canine IgG] reagents, and that the lower sensitivity may have been due to the fact that SpA is reportedly[537] less sensitive than anti-IgG reagents. As discussed below, however, the conclusions in the referenced study may not be universally applicable.

As the first direct ELISA to be used for canine samples, this assay is a great improvement over previous ELISAs. The positive control for the assay, an alloantibody-containing serum from a multiply transfused dog, is a better control than those in prior reports. However, because it is serum and not plasma, the positive control is incubated with 20 U/ml heparin before use to inactivate thrombin.[21] Since the test samples are nonheparinized plasma and therefore not identical to the control, the control is adequate but not ideal.

Several other aspects of the assay are worthy of attention. First, 50 ml of blood were required for the direct assay when the dogs' platelet concentrations were less than 10,000/μl. This may not be tolerable in some dogs. Concerns over the use of 1% ammonium oxalate to lyse contaminating erythrocytes, and the fixation of platelets in 1% paraformaldehyde were addressed previously. In reference to SpA, the authors did not state how comparisons were made between lots or, alternatively, if a single lot was used for all the reported work. The range of SpA concentrations in the Sigma product used can be significant, so each new lot must be standardized such that lot-to-lot comparisons can be made. Despite these concerns, this direct assay for PAIg appears to be very promising for supporting a diagnosis of IMT in dogs. With the high sensitivity, a negative result is strong evidence against IMT.

4. platelet immunoradiometric assays

Though results of an undescribed technique for canine PAIg were briefly reported in 1982,[492] the first description of a direct assay for detecting canine PAIg was in reference to thrombocytopenia induced by gold compounds.[217] This assay was a solid phase immunoradiometric assay using ^{125}I-SpA. For this assay, after washed platelets were added to wells of microtitration plates, rabbit anti-[canine IgG] was added and incubated for 1 hr at room temperature. The wells were then rinsed 3 times and ^{125}I-SpA was added and incubated for another hour at room temperature. After 3 rinses, wells were cut from the plate and radioactivity was assessed in a gamma counter. Results were compared to normal control dogs but there was no positive control. The platelet wash

buffer contained 1% ammonium oxalate, and as for other assays discussed above, data to indicate that such treatment has no effect on detectable PAIg were not reported.

Four dogs with suspected gold-induced IMT were tested 16 times, and each had at least one positive result. In one dog, positive results correlated well with nadirs in platelet concentrations which oscillated directly with increases and decreases in prednisolone therapy; there was a direct correlation ($r = 0.82$) between PAIg and platelet concentration. However, the strongest positive sample had only 1.8 times the mean detectable immunoglobulin of the control platelets. This is a poor differential between positive and negative results in comparison to standard human assays.[475] Despite the relatively weak signal-to-noise ratio, this assay was apparently successful at directly detecting PAIg.

The same assay was used to diagnose IMT in a single dog whose PAIg was over 6 times that of controls.[359] This dog had been part of a control group in a long-term drug study with no clinical or laboratory abnormalities until thrombocytopenia was discovered on day 2,381 (6.5 yr). The platelet concentration was 96,000/μl and it fell to 6,000/μl over the next few weeks. The dog had marked megakaryocytic hyperplasia. This case is interesting because the dog was carefully monitored prior to developing IMT, and there were no known precipitating events.

5. platelet immunofluorescence assays

The Bloom immunoradiometric assay[217] was criticized by Thiem et al.[537] for its low sensitivity, the need for fresh platelets, and the large volume of blood required for harvesting sufficient numbers of platelets. While some of these apparent problems could

be overcome, the authors chose instead to examine indirect assays and compare an indirect solid phase immunoradiometric assay to an indirect flow cytometric immunofluorescence technique. Sera from 4 normal dogs and 5 dogs with clinical diagnoses of IMT were incubated with normal pooled canine platelets. After washing, the platelets were incubated with FITC-SpA, FITC-IgG, or ^{125}I-SpA. The platelets incubated with FITC conjugates were analyzed for fluorescence by flow cytometry while a gamma counter was used to detect radioactivity on the platelets incubated with ^{125}I-SpA. Sera from 4 of 5 dogs were positive (i.e. results were greater than 2 SD above the mean of the controls) with FITC-IgG, while only 2 of 5 were positive with FITC-SpA. There was also reportedly greater separation between negative and positive platelet populations with FITC-IgG, suggesting the superiority of this detector under the assay conditions used. None of the patient sera were positive by the immunoradiometric technique, suggesting it was inferior to the flow cytometric techniques which the authors concluded were promising. Because of the numerous variables in platelet antibody assays, extrapolation of these conclusions to other assay systems is unwarranted.

Moreover, studies such as this are difficult to interpret meaningfully for two major reasons: 1) few samples were analyzed, so the power of the findings is low, and 2) it is not known if the serum samples truly contained PBIg, or if the negatives with the immunoradiometric assay were real and the positives with the fluorescence assays were false due to nonspecific interactions. Each type of assay appeared to have potential since they each linearly detected increasing amounts of PBIg in serial dilutions of rabbit serum containing anti-[canine platelet] antibodies. Pooled normal rabbit serum was used as a negative control, but data were not shown to allow comparisons with positive results.

An indirect microscopic platelet immunofluorescence assay (M-PIFA)[241] and an indirect flow cytometric platelet immunofluorescence assay (FC-PIFA)[240] were recently developed for clinical use. The microscopic PIFA was reportedly chosen for development because platelets are difficult to harvest in adequate numbers for direct assays, because samples for direct assays should be assessed within 24 hours[21] and "because studies in humans have shown that the amount of surface-associated immunoglobulin correlates well with the presence of immune-mediated platelet destruction".[241] However, the cited human studies[516] have assessed PAIg in direct assays and do not apply to PBIg detected by indirect assays.

The general protocol for the M-PIFA is as follows.[241] Serum samples (2μl) from test and control dogs were incubated for 30 minutes at 37°C with 8 million washed normal canine platelets fixed in 1% paraformaldehyde. After washing, the platelets were incubated with a FITC-goat anti-[canine IgG] for 30 minutes at room temperature, washed, and resuspended in a glycerol-PBS solution. Small aliquots of these suspensions were placed on glass slides, each of which included negative control samples of donor platelets incubated with buffer, donor platelets incubated with normal dog serum, and donor platelets incubated with donor serum. Each slide also had a positive control sample of donor platelets incubated with serum from a dog with clinical IMT and a high titer of PBIg. Fluorescence was interpreted by a single technician and graded qualitatively from 0 to 4+.

Potential problems with the assay protocol include the following: 1) test samples were serum instead of plasma, 2) samples were stored at -70°C until analysis, 3) residual thrombin was apparently not inactivated, 4) it was not clear if the 10 normal dog sera

were also frozen before testing or if they were run fresh because they were readily available, 5) the normal donor platelets were collected in EDTA from one healthy dog, 6) donor platelets were fixed in 1% paraformaldehyde, and 7) no comment was made as to whether or not all the secondary antibody used was from 1 lot or if some kind of adjustments had to be made to compare results between lots. If a single lot was used, there was still no mention of the consistency of the positive control's graded result, so it is not clear if the qualitative results could be compared from one assay to the next.

The M-PIFA was evaluated in 10 normal dogs, in 76 thrombocytopenic dogs of which 20 were suspected of having primary IMT, and in 18 nonthrombocytopenic dogs with various diseases. Dogs were considered to have primary IMT when: platelet concentrations were less than 75,000/μl, megakaryocyte density appeared normal to increased on marrow aspirates or core biopsies, and other known causes of thrombocytopenia including rickettsial diseases, DIC, neoplasia, SLE, and drug-induced thrombocytopenia were excluded. Since primary IMT may occur with platelet concentrations greater than 75,000/μl and with a decreased density of megakaryocytes in the bone marrow, the reported sensitivity of the assay related to only a subset of potential IMT presentations.

M-PIFA results were negative in all 10 normal dogs, positive in 14 of the 20 dogs with suspected primary IMT (70%), positive in 15 of the other 56 thrombocytopenic dogs (37%), and positive in 5 of the 18 nonthrombocytopenic diseased dogs (28%). This indicates a moderate sensitivity and a poor specificity for the diagnosis of clinical primary IMT. However, the 15 thrombocytopenic dogs without suspected IMT but with positive results were diagnosed with: lymphoproliferative disease (6), hemangiosarcoma (3), Evans' syndrome (3), steroid responsive idiopathic neutropenia and thrombocytopenia

(1), pure red cell aplasia (1), and SLE (1). Increased PBIg is a potential finding in each of these conditions, so the results may be reasonably specific for primary and secondary IMT but poor for primary IMT as defined.

Of the 5 positive nonthrombocytopenic dogs, 2 had neoplasia, 1 had IMHA, 1 had trauma from being hit by a car, and 1 had recovered from surgery for a portosystemic shunt. The potential for positive results relating to neoplasia and IMHA are clear, though positive results could also have been due to alloantibodies. The dog with the shunt was transfused at some unspecified time before sample procurement and the possibility of transfusion-induced alloimmunization was discussed. However, the potential for pregnancy-related alloimmunization was not mentioned for any of the dogs.

For the 34 positive dogs combined, there was an inverse relationship between intensity of immunofluorescence and the mean platelet concentration of dogs at each fluorescence intensity level ($r = -0.9$ as calculated from the published graph). This suggests that higher PBIg titers occur in those IMT dogs with more severe thrombocytopenias. This is also consistent with more frequent PF3 positivity in samples from dogs with more severe thrombocytopenia.[253,525] It further suggests that the results are, at least on the average, not related to alloantibodies, since no such correlation would be expected between platelet concentration and alloantibody concentration. However, it is still possible that the PBIg is not all antiplatelet antibody; some may be immune complexes or nonspecific aggregates.

The indirect M-PIFA results were compared to D-MIFA results in 12 dogs. The overall sensitivity of the M-PIFA for substantiating a diagnosis of primary IMT (about 70%) was better than that of the D-MIFA (41%), but two dogs had positive MIFA and

negative M-PIFA results. It was suggested that titers may have been low in these 2 dogs, making the M-PIFA results negative, or the MIFA results may have been false positives. Several possibilities to explain the fact that 5 M-PIFA-positive dogs were D-MIFA-negative were suggested: 1) high background fluorescence (low signal-to-noise ratio) impaired sensitivity of the D-MIFA, 2) too few megakaryocytes were present in some D-MIFA samples, and 3) platelets and megakaryocytes may not have identical surface determinants, so some antibodies were reactive with only platelets. Not discussed was the possibility that some M-PIFA positives were the result of nonspecific binding or alloantibodies.

The M-PIFA was compared to an indirect FC-PIFA developed by the same investigators.[240] The FC-PIFA was developed and evaluated because of several potential advantages including: 1) quantification of antibody bound to individual cells; 2) detection of low amounts of fluorescence; and 3) small sample size requirements. As for the M-PIFA, test samples were sera stored at -70°C until testing for reactivity with normal assay platelets harvested from a single dog in the presence of EDTA. Assay platelets were frozen with 5% DMSO until use, at which time the platelets were rapidly thawed at 37°C, washed once, and brought to the appropriate concentration. No data were shown, but the use of frozen versus fresh platelets was evaluated in the FC-PIFA using serum from 3 healthy dogs and 3 dogs with suspected IMT. There was reportedly no statistically significant difference between the fresh and frozen platelets. The potential differences in reactivity between the fixed and frozen platelets were not assessed.

Similar to the M-PIFA, 2μl of test serum were incubated in a buffer containing 3 Mm EDTA with 8 million platelets for 60 minutes at 37°C. After incubation, the

platelets were washed 3 times and then incubated with FITC-goat anti-[canine IgG] for 30 minutes at room temperature. This incubation was also followed by 3 washes and the samples were analyzed with a flow cytometer. Serum samples from 10 normal dogs were used as negative controls to establish a reference range with each assay, and samples were considered positive if the mean fluorescence range was greater than 2 SD above the mean for the clinically normal dogs. It is not clear why a new reference range had to be determined with each assay, since the interassay CV was reported as only 6.14 for the healthy controls. Serum from a dog with clinical IMT and positive M-PIFA and FC-PIFA results was used as a positive control.

Potential problems with the FC-PIFA are similar to those mentioned above for the M-PIFA. Potentially significant differences between the 2 assays, complicating comparisons, include: 1) use of fixed platelets in the M-PIFA but frozen platelets exposed to DMSO in the FC-PIFA; 2) a 30 minute primary incubation for the M-PIFA and a 60 minute primary incubation for the FC-PIFA; and 3) 1% bovine serum albumin (BSA) FC-PIFA buffer but 0.2% BSA in the M-PIFA buffer.

Sera from 10 healthy dogs and 27 dogs with clinical diagnoses of primary or secondary IMT were tested in each assay, and all healthy control dogs were negative in each assay. Of the 27 test dogs, the FC-PIFA was positive in 18 (67%) and the M-PIFA was positive in 15 (55.5%). While the FC-PIFA appeared to be more sensitive than the M-PIFA in this report, it was not statistically significantly so. Eight dogs were negative in both assays, 1 dog was negative in the FC-PIFA but positive in the M-PIFA, and 4 dogs were positive with the FC-PIFA but negative microscopically. All 4 of these had SLE, but there was 1 SLE dog positive with each assay and 1 negative with each assay.

There was a strong direct linear correlation between the M-PIFA scores and the mean FC-PIFA fluorescence scores ($r^2=0.873$). However, there was a great deal of variation in FC-PIFA scores with each microscopic score.

In conclusion, the FC-PIFA was felt to be "a practical, objective, and quantitative method for detecting circulating platelet antibody of the IgG class in dogs suspected of having IMT".[240] While it can certainly detect PBIg in many dogs with suspected IMT, it must be remembered that it cannot differentiate specific autoantibodies from alloantibodies, and it should not be considered a quantitative assay.

6. platelet fragmentation index

Evaluation of blood for circulating platelet microparticles has been suggested as a potentially useful diagnostic approach to supporting a diagnosis of IMT in dogs.[348] This can reportedly be done by determining the fragmentation index of a sample, where fragmentation index is defined as the percentage of platelets less than about 2 Fl in volume.[220,348] There is a single case report (discussed above) where such an assessment was used to support a diagnosis of drug-induced IMT.[220] A circulating platelet lytic factor was detected and considered indicative of immune-mediated platelet destruction[220] The authors concluded that their thrombolytic assay might be useful when PAIg cannot be directly detected due to the inability to collect a sufficient number of platelets to assay.

While an increased fragmentation index may occur in dogs with IMT, data are lacking to indicate what percentage of these dogs may be so affected and what the specificity of this finding is for IMT. Platelet microparticles may be generated in other

platelet consumptive or destructive states unrelated to immunological mechanisms. Further studies are required before the clinical utility of this indirect diagnostic approach is clear.

Similarly, MPV has been suggested as a useful diagnostic parameter in thrombocytopenic dogs.[347] Microthrombocytosis, defined as a low MPV, was retrospectively found to be a specific indicator of clinically diagnosed IMT. It was present in 17 of 31 (55%) dogs with IMT but in only 1 of 37 (3%) thrombocytopenic dogs with either a different diagnosis or no diagnosis.[347] Samples for this study were routine EDTA-anticoagulated whole blood submissions for CBCs, and there were no controls on the time passing between sample collection and sample evaluation. There was also no control group of dogs with known nonimmune thrombocytopenia of the same severity as the thrombocytopenias in the IMT group. Such a control is necessary to determine if low MPVs relate to underlying pathogenesis or simply to marked thrombocytopenia of any cause. It is conceivable that the instrumentation has a bias toward low MPVs when few platelets and more background particles are assessed. While the evaluation of MPV may be a useful diagnostic consideration, more carefully controlled studies of this indirect approach are needed before firm interpretations of the results can be made.

7. other

In evaluating the potential immunological contribution to thrombocytopenia in dogs treated with gold compounds, an electron microscopic method using a SpA-colloidal gold conjugate was developed.[217] Washed or unwashed platelet-rich buffy coat samples

were incubated with a SpA-colloidal gold conjugate for 15 min at 37°C. After washing, the cells were suspended in 1% glutaraldehyde and processed for electron microscopy. Both of the two dogs tested in this way were positive for membrane-bound immunoglobulin when the cells were unwashed, but both were negative with washing. As the authors suggested, further studies were needed to determine if immunoglobulin was removed during washing steps, perhaps due to low affinity binding of primary antibodies, or if the detected immunoglobulin in unwashed samples was just nonspecifically adsorbed to the platelet surface. Until such work, the results of this assay are questionable.

It has been suggested that the quantitative NBT reduction test may be a convenient indirect assay for detecting "antiplatelet antibody" in dog sera.[461] In such an assay, sera would be incubated with platelets and then the platelets would be incubated with neutrophils to test for the presence of platelet opsonization and consequent phagocytosis with NBT reduction. This would be a very indirect method and should not be considered a useful clinical assay for the direct assessment of PAIg.

Immunoblotting and immunoprecipitation of canine samples have been done to identify the specificity of PAIg or PBIg.[237,339] Lewis[339] eluted PAIg from platelets of dogs with IMT and used radioimmunoprecipitation to show that GPIIb and/or IIIa were the targets in 4 of 17 dogs tested. While it is likely that the PAIg bound specifically to the glycoproteins in a Fab-mediated manner, it has not been proven. Therefore it is likely, but not proven, that GPIIb/IIIa has target epitopes for antiplatelet autoantibodies in some dogs with IMT.

D. Summary

There have been many recent advances in the development of assays for detecting antibodies responsible for platelet destruction in dogs with IMT. Most of these assays detect PBIg and therefore their results need to be interpreted with caution. The most promising of the recent assays is the direct ELISA of Lewis which detects PAIg.[21,160] Like any direct assay, its major limitation in the clinical arena is the paucity of cells in many dogs with IMT. However, this assay has the potential to provide a great deal of useful information about the pathogenesis of thrombocytopenia in many canine conditions. Ideally, several well characterized assays for canine PAIg should undergo widespread clinical use such that each may support or refute the findings of the others.

As the development and use of veterinary assays for PAIg and PBIg continue, several major goals deserve attention:

1) to differentiate among autoantibodies, alloantibodies, Fc-binding, and immune adherence,
2) to differentiate different subtypes of canine IMT, if they exist,
3) to be able to identify drug-induced IMT,
4) to determine the clinical utility of assays, thereby supporting or refuting the belief that, compared to the PF3 assay, better direct assays "would be impractical for routine use because the assumption of an immune basis can safely be made for the majority of clinical cases of recurrent, severe thrombocytopenia",[22]
5) to determine the role of antibodies in various thrombocytopenic states including those of neoplasia and infectious diseases,

6) to determine if complement-mediated immune adherence is operative in various canine thrombocytopenic states,

7) to apply assays to veterinary transfusion medicine if platelet refractoriness becomes a problem, and

8) to use as research tools to investigate aspects of pathogenesis and therapy for canine IMT, the results of which may also be applicable to the human disease.

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IMMUNE-MEDIATED THROMBOCYTOPENIA IN DOGS: ASSAY DEVELOPMENT, A ROLE FOR COMPLEMENT, AND ASSESSMENT IN A TOXICOLOGIC STUDY

Volume II

By

Michael A. Scott

A DISSERTATION

Submitted to
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DOCTOR OF PHILOSOPHY

Department of Pathology

1995

STAPHYLOCOC

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CHAPTER 1

STAPHYLOCOCCAL PROTEIN A BINDING TO CANINE IgG AND IgM

Abstract

Staphylococcal protein A (SpA) binds with high affinity to immunoglobulins from many species, making it a useful reagent for immunoassays and immunoglobulin purification procedures. However, its use is limited by poor reactivity with some immunoglobulin subclasses, including human IgG_3 and murine IgG_1. To clarify conflicting reports of SpA's reactivity with canine immunoglobulins, we evaluated the binding of canine IgG and IgM to Cowan I strain SpA. IgG and IgM were purified from pooled normal canine plasma by affinity chromatography with polyclonal anti-IgG and anti-IgM antibodies. The purified IgG and IgM were assessed for SpA reactivity by affinity chromatography using a SpA-agarose column. The relative proportions of total chromatographed IgG or IgM in the flow-through (SpA-nonbindable) and eluate (SpA-bindable) fractions were determined by absorbance at 280 nm or by densitometry of protein bands on SDS-PAGE gels. The IgG and IgM in each immunoglobulin fraction were also nonspecifically adsorbed to microtitration plates and tested for reactivity with ^{125}I-SpA using a solid phase immunoradiometric assay (IRMA). Approximately 20% of canine IgG and 33% of canine IgM did not bind to the SpA affinity column and were

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also unreactive with ^{125}I-SpA using the IRMA. Incomplete reactivity of SpA with canine IgG and IgM limits the usefulness of SpA in canine immunologic procedures.

Introduction

Staphylococcal protein A (SpA) is a 42 kD bacterial type I Fc receptor secreted from, or found on the surface of, most strains of *Staphylococcus aureus*.[1-3] Its high affinity binding[4] to CH2 and CH3 determinants[5] on the Fc portion of IgG from many species[1,6] has made it a useful immunoglobulin detector in a wide variety of immunoassays. Likewise, it has been useful in immunoglobulin purification procedures. The major limitations of SpA relate to either too broad or too narrow a spectrum of reactivity for the particular application of interest. Because SpA reacts with some non-IgG immunoglobulins from several species,[6] its breadth of reactivity is too great when immunoglobulin class-specific interactions are desired. Its reactivity is too narrow, however, to detect or purify all IgG subclasses in some species; for example, SpA does not bind well to human IgG_3 and murine IgG_1.[2]

The binding spectrum of SpA to canine immunoglobulins has not been clearly established. SpA has been reported to bind from 67[7] to essentially 100%[8,9] of canine IgG, and from 36[7] to 90%[8,9] of canine IgM. Reports[10] that SpA has no reactivity with canine IgM, and that SpA-Sepharose can be used to obtain pure canine IgG, conflict with most findings.

While using SpA-Sepharose to purify canine IgG, it appeared that a significant amount of the IgG would not bind to SpA. These results were contrary to the most recent reports of the SpA-bindability of canine IgG.[8,9] Consequently, to clarify these

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conflicting observations, we investigated the binding of canine IgG and IgM to commercially available SpA (Cowan I strain) using affinity column and solid state systems.

Methods

Canine IgG and IgM were purified from pooled citrated plasma by affinity chromatography with polyclonal antibodies. The SpA-reactivity of each purified immunoglobulin was assessed by SpA affinity chromatography. The relative amounts of protein in the column flow throughs (non SpA-binding) and eluates (SpA-binding) were determined by absorbance at 280 nm or by densitometry of protein bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. SDS-PAGE was also used to evaluate immunoglobulin purity. Additionally, the binding of flow through and eluate fractions to SpA was assessed with a solid phase assay similar to immuno-radiometric assays for cell-associated immunoglobulin.

Affinity columns

Three immunoglobulin affinity columns were prepared with the Affi-Gel^{R} Hz Hydrazide Gel system (Bio-Rad Laboratories, Richmond, CA), one each with: 1) a polyclonal rabbit anti-[canine IgG(whole molecule)] antibody (rabbit anti-G) (Sigma Chemical Company, St. Louis, MO); 2) an affinity purified polyclonal goat anti-[canine IgG(γ)] antibody (goat anti-γ) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD); and 3) an affinity purified polyclonal goat anti-[canine IgM(μ)] antibody (goat anti-μ) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Specifically, 2 or 3

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mg of affinity isolated, antigen-specific immunoglobulin were resuspended in 1 or 2 ml coupling buffer (50 mM acetate, 150 mM NaCl, pH 5.5) and dialyzed against 6 liters of coupling buffer overnight at 4°C. The immunoglobulin was then oxidized by incubation for 1 hr in the dark at room temperature with a 1/10 volume of fresh stock sodium periodate solution (20.8 mg/ml). This reaction was stopped with the addition of glycerol to a final concentration of 20 mM, and the periodate was removed by overnight dialysis in coupling buffer at 4°C. The oxidized immunoglobulin was incubated overnight at room temperature with 1 ml Affi-Gel^R that had been thoroughly washed in coupling buffer. The slurry was then transferred to a column at 4°C and equilibrated with application buffer (phosphate buffered saline with 0.025% NaN_3 (PBS), pH 7.2). Coupling efficiency ranged from 71 to 77% for the three columns, with 0.9 to 1.8 mg immunoglobulin bound per column.

The SpA affinity column was a 1.5 ml column of Cowan I strain protein A covalently linked to cross-linked 4% beaded agarose (Sigma). Its binding capacity for human IgG was 20-25 mg/ml. It was equilibrated in PBS, pH 7.2 for the anti-G column experiment and pH 8.0 for the anti-γ and anti-μ column experiments.

Immunoglobulin purification

Plasma was pooled from citrated blood samples of either 4 or 10 (anti-G column) clinically healthy dogs, half of each sex, and each of a different breed. Aliquots of the pooled plasma were incubated in the columns for 15 min before allowing all nonbound material to flow through. Bound immunoglobulin was eluted with PBS, pH 3.0, and the pH of each eluate was immediately adjusted to 7.2 with 0.1 M NaOH. Multiple eluates

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from multiple cycles on each column were pooled and the immunoglobulins were concentrated by centrifugation in Centriprep-10 concentrators (Amicon Inc., Beverly, MA). For the anti-G eluate, the concentrated IgG was dialyzed in 6 liters of water overnight at 10°C and then lyophilyzed. The lyophilyzed protein was resuspended in 300 μl of PBS, pH 7.2, to a concentration of 9.32 mg/ml. The anti-γ IgG and the IgM were concentrated in Centriprep-10 and Centricon-100 concentrators (Amicon Inc., Beverly, MA) to 5.63 and 1.31 mg/ml, respectively.

SpA affinity chromatography

In separate experiments, carefully measured volumes of each immunoglobulin preparation were loaded onto the SpA-agarose affinity column at less than 1/3 the bed volume and less than 1/10 the column's binding capacity for human IgG. After incubation for 1 hr at 4°C, application buffer was allowed to flow and the entire flow through was collected. The bound immunoglobulin was then eluted with PBS, pH 3.0, and the entire eluate peak was collected in a single fraction. The pH of the eluate was immediately adjusted to 7.2 with NaOH. The absorbance (280 nm) of each unconcentrated flow through, eluate, and pre-SpA fraction was then measured in duplicate with a spectrophotometer (Perkin Elmer) to estimate the protein recovery. The eluates from the anti-γ and anti-μ columns were nearly pure, so the absorbances at 280 nm provided a reliable measure of the relative amounts of protein in the 3 samples from these 2 columns.

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SDS-PAGE and densitometry

Pre-SpA and post-SpA immunoglobulins fractions were assessed for purity by SDS-PAGE under reducing and nonreducing conditions using the discontinuous buffer system of Laemmli.[11] For the experiment using the anti-G eluate, there was significant contamination of the IgG by IgM. Consequently, absorbance readings could not be used to assess the relative amounts of SpA-bindable and SpA-nonbindable IgG. Instead, protein bands on the SDS-PAGE gels were assessed densitometrically to determine the relative amounts of IgG in each fraction. To this end, flow through and eluate fractions were electrophoresed in triplicate lanes such that the Coomassie blue-stained bands had densities in the linear range of the densitometer at 595 nm. By cutting the gels, the bands were rearranged so that they could be scanned in tandem and the densitometer's computer could calculate the relative densities of each band. By knowing the total volumes of flow-through and eluate, and the relative amounts of IgG in each as assessed by densitometry, the percentage of IgG in each fraction could be calculated. The linear range of the densitometry readings was determined by densitometrically evaluating gels containing bands of human IgG in increasing amounts from 2 to 30 μg.

Solid phase assays

A solid phase immunoradiometric assay (IRMA) was used to assess the binding of the flow through and eluate fractions of the SpA affinity column to the following ^{125}I-labeled antiglobulin reagents: 1) SpA (Sigma); 2) a polyclonal goat anti-[canine IgG(γ)] (^{125}I-anti-γ) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD); 3) a polyclonal goat anti-[canine IgG_1] (^{125}I-anti-IgG_1) (Bethyl Laboratories, Inc., Montgomery,

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Texas); 4) a polyclonal goat anti-[canine IgG_2] (^{125}I-anti-IgG_2) (Bethyl); and 5) a polyclonal goat anti-[canine IgM(μ)] (^{125}I-anti-μ) (Kirkegaard and Perry). Proteins were radiolabeled by modifications of the method of Fraker and Speck.[12] Briefly, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma) was dissolved in dimethyl chloride and 0.6 μg were deposited in the base of a glass test tube by evaporation. To this were added 25 μg of protein (1 mg/ml) and 0.3 μCi iodine-125 (NaI). After 10-15 minutes of incubation with mixing, the radiolabeled protein was separated from the free iodide by filtration through a 10 ml Sephadex G25 filtration column (Pharmacia Biotech, Piscataway, NJ).

Flow through and eluate proteins were diluted in PBS, pH 7.2, to 10 μg/ml, and then in serial quarterly dilutions to 2.5 and 0.625 μg/ml. The diluted proteins were then nonspecifically adsorbed onto wells of a microtitration plate (Immulon-2 Removawell plates, Dynatech Laboratories, Inc., Chantilly, Virginia) in triplicate 50 μl volumes overnight at 4°C. After adsorption, wells were washed with blocking buffer (PBS containing 3% bovine serum albumin and 0.5% Tween, pH 7.2) and then incubated with 300 μl blocking buffer for 1 hr at room temperature. The blocking buffer was removed and the wells were washed 3 times with 300 μl rinses of washing buffer (Tween-free blocking buffer). After washing, 50 μl PBS containing 10 μg of each antiglobulin reagent were added to each set of eluate and flow through wells, including blanks. The antiglobulin reagents were incubated in the wells for 1 hr after which the fluid was removed and the wells were washed five times with washing buffer. Each well was removed and transferred to a gamma counter (Model 1190, TmAnalytic, Bensenville, IL)

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Results

SDS-PAGE

By SDS-PAGE, the product of the anti-G column had predominant IgG bands with significant IgM bands as well. The product of the anti-γ column was almost entirely IgG, with very faint IgM bands. Similarly, the product of the anti-μ column was predominantly IgM with very faint IgG bands.

The SpA column flow through (FT) and eluate (E) of the anti-G IgG were assessed by SDS-PAGE using a 5% gel to see if there was any detectable difference in apparent molecular masses. There was not.

Quantitative SpA chromatography

As analyzed by SDS-PAGE, the products of the anti-γ and anti-μ columns were nearly pure IgG and IgM, respectively. For the following calculations of binding percentages, the immunoglobulins were considered pure. The SpA-bindable immunoglobulin in each case was therefore defined by:

$$\%SpA\text{-}bindable = \frac{C_E V_E}{C_T V_T} \times 100 = \frac{A_E V_E}{A_T V_T} \times 100$$

where C_E and C_T are the concentrations of the eluates and pre-SpA total immunoglobulin, respectively, A_E and A_T are their respective absorbances, and V_E and V_T are their

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respective volumes. Since for each protein, concentration is equal to the absorbance divided by the protein's extinction coefficient, the extinction coefficients cancel out of the equation. Implicit in the equation is the assumption that SpA-bindable and SpA-nonbindable immunoglobulins have the same absorbances at 280nm.

From this equation, 82% of the IgG purified by anti-γ column chromatography bound to SpA-agarose (Table 1.1). Conversely, 18% of the same IgG did not bind to SpA-agarose. Of the IgM purified by anti-μ column chromatography, about 2/3 bound to the SpA-agarose (Table 1.1). IgM recovery was 93.6% rather than 100% as for IgG, probably due to the smaller amounts of IgM used. Because of this, the SpA-bindable IgM can be reported as 69% of the recovered IgM or as 65.1% of the total IgM added to the column (Table 1.1). About 30% of the IgM did not bind to SpA-agarose under the conditions used (Table 1.1).

Recovery for the anti-G product containing IgG and IgM was roughly 95% of the protein added to the SpA column (Table 1.1). Since the extinction coefficients of IgG and IgM are similar, and most of this mix was IgG, use of the same extinction coefficient provided a rough measure of the relative amounts in each fraction. More accurate estimation of SpA bindability, however, required densitometric assessment of the SDS-PAGE bands. The anti-G column was initially equilibrated to pH 7.2. To confirm that a higher pH would not allow more immunoglobulin to bind the SpA-agarose, the FT from this experiment was rechromatographed through a SpA column equilibrated at pH 8.0. No protein was detected in the eluate, so SpA bindability was not improved at pH 8.0 as compared to pH 7.2. Also, failure of any of the FT immunoglobulin to bind to

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Table 1.1. Immunoglobulin recovery in flow through (FT) and eluate (E) fractions from SpA affinity chromatography.

	anti-G	anti-γ	anti-μ
pooled plasma from n dogs	10	4	4
immunoglobulin	IgG + IgM	IgG	IgM
concentration (mg/ml)	(9.21)[1]	5.63	1.31
volume loaded onto SpA column (μl)	250	444	483
protein loaded (mg)	(2.30)	2.50	0.63
FT volume (ml)	9.83	13.80	11.40
FT A_{280}	(0.102)	0.046	0.019
FT protein (mg)	(0.72)	0.45	0.18
E volume (ml)	8.70	16.00	14.00
E A_{280}	(0.238)	0.179	0.035
E protein (mg)	(1.48)	2.05	0.41
% protein recovery	(95.6)	100.0	93.6
% SpA-bound	NA[2]	82.0	69[3]/65.1
% not SpA-bound	NA	18.0	31[3]/28.6

[1]values in parentheses are approximations based on using an extinction coefficient of 1.4 for the mixture of IgG and IgM

[2]not applicable because IgG and IgM are mixed but do not have the same percentage SpA bindability

[3]based on recovered protein/based on total protein

Densitometry

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Densitometry

Because the anti-G antibody was not heavy chain specific, the E contained both IgG and IgM. The contribution of any IgA that might have been present was considered minor because the normal circulating concentration of canine IgA is only about 5% of the combined IgG and IgM concentrations.[13] Preliminary SDS-PAGE analysis of the FT and E fractions was used to determine how much of each to electrophorese such that the bands would have densities within the linear range of the densitometer. The FT was electrophoresed at twice the volume of the E. Densitometrically, 31.8% of the recovered IgG was in the FT band (68.2% was in the E band). This is based on the mean value of three determinations, (31.6, 31.5, and 32.3%), on each of 3 distinct sets of SDS-PAGE lanes.

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Since the SDS-PAGE band for FTIgG was generated with twice the volume of FT as compared to E, the ratio of the IgG concentration of the FT to that of the E was:

$$\frac{concentration\ of\ FT\ IgG}{concentration\ of\ E\ IgG} = \frac{(0.5)(31.8\%)}{68.2\%} = 0.233.$$

The ratio of the total IgG in the FT to that in the E could therefore be calculated from the total volumes of FT and E collected:

$$\frac{IgG_{FT}}{IgG_E} = 0.233\ x\ (9.83ml/8.70ml) = 0.263,$$

so

$$IgG_{FT} = 0.263\ x\ IgG_E.$$

Since

$$IgG_{FT} + IgG_E = IgG_T\ (total\ IgG),$$

$$(0.263\ x\ IgG_E) + IgG_E = IgG_T.$$

Therefore,

$$IgG_E\ x\ 1.263 = IgG_T,$$

$$\frac{IgG_E}{IgG_T} = \frac{1}{1.263} = 0.792$$

and the E IgG was 79.2% of the total IgG. Therefore 20.8% of the IgG purified on the anti-G affinity column and recovered from the SpA column was SpA-nonbindable. This agrees with the 18% figure determined spectrophotometrically for the anti-γ IgG.

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Table 1.2 Solid phase
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Fraction	FT
0.0	0.2 ± 0.0
1.25	0.2 ± 0.0
2.5	0.8 ± 0.0
5.0	2.8 ± 0.2

IRMA

An IRMA was used to confirm that the activity of SpA in the chromatography studies was the same as the activity of radiolabeled SpA in a solid phase system. The SpA-nonbindable IgG and IgM from the chromatographic studies, i.e. the FT fractions of each column, were not detected by ^{125}I-SpA in the IRMA (Tables 1.2–1.4). Each column E, however, was detected equally well by ^{125}I-SpA and either ^{125}I-anti-γ for IgG or ^{125}I-anti-μ for IgM. Note the presence of some IgM in the anti-G column product (Table 1.2).

The IRMA was also used to determine if polyclonal anti-[canine IgG_1] and polyclonal anti-[canine IgG_2] antibodies would have different reactivities with the SpA-bindable and SpA-nonbindable IgG. These antibodies react to two electrophoretically distinct canine IgG subpopulations, but the relationship of these subpopulations to other published classifications is not clear. In any case, each antibody detected SpA-bindable and SpA-nonbindable IgG (Table 1.3).

Table 1.2. Solid phase binding of iodinated antiglobulin reagents to SpA column FT and E fractions of immunoglobulin purified by anti-[IgG(whole molecule)] affinity chromatography.

concentration for adsorption (μg/ml)	% bound (mean ± S.D.)					
	^{125}I-SpA		^{125}I-anti-γ		^{125}I-anti-μ	
	FT	E	FT	E	FT	E
0.0	0.2 ± 0.0	0.2 ± 0.0	1.0 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
0.625	0.2 ± 0.0	6.8 ± 0.2	20.6 ± 0.3	26.3 ± 0.8	2.4 ± 0.0	4.2 ± 0.2
2.5	0.8 ± 0.0	45.0 ± 3.2	33.8 ± 1.0	36.7 ± 0.2	6.7 ± 0.5	10.5 ± 0.2
10.0	2.8 ± 0.2	68.7 ± 0.3	38.2 ± 0.3	39.6 ± 1.2	10.2 ± 0.3	13.7 ± 0.4

Table 1.3. Solid phase
and E fracti

[illegible]	^{25}I-Sp
[illegible]	FT
0.0	0.1
	± 0.0
0.625	0.1
	± 0.1
2.5	0.2
	± 0.2
10.0	0.4
	± 0.4

Table 1.4. Solid phase
and E fracti

[illegible]	
0.0	0.
0.625	0.
2.5	0.
10.0	0.

Table 1.3. Solid phase binding of iodinated antiglobulin reagents to SpA column FT and E fractions of IgG purified by anti-[IgG(γ)] affinity chromatography.

concentration for adsorption (μg/ml)	% bound (mean ± S.D.)							
	^{125}I-SpA		^{125}I-anti-γ		^{125}I-anti-G_1		^{125}I-anti-G_2	
	FT	E	FT	E	FT	E	FT	E
0.0	0.1 ± 0.0	0.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
0.625	0.1 ± 0.1	3.1 ± 0.2	11.7 ± 0.1	10.3 ± 0.8	3.4 ± 0.1	2.0 ± 0.0	12.7 ± 0.4	14.2 ± 0.1
2.5	0.2 ± 0.2	10.9 ± 0.1	24.3 ± 0.5	22.7 ± 1.2	9.9 ± 0.2	5.9 ± 0.1	26.3 ± 1.2	34.0 ± 0.7
10.0	0.4 ± 0.4	47.4 ± 2.4	37.4 ± 1.0	34.8 ± 1.6	25.1 ± 0.4	18.5 ± 0.3	43.4 ± 1.6	54.7 ± 1.7

Table 1.4. Solid phase binding of iodinated antiglobulin reagents to SpA column FT and E fractions of IgM purified by anti-[IgM(μ)] affinity chromatography.

concentration for absorption (μg/ml)	% bound (mean ± S.D.)			
	^{125}I-SpA		^{125}I-anti-μ	
	FT	E	FT	E
0.0	0.1 ± 0.1	0.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
0.625	0.1 ± 0.0	1.2 ± 0.1	9.3 ± 0.3	9.0 ± 0.2
2.5	0.2 ± 0.0	5.7 ± 0.1	18.1 ± 0.2	17.4 ± 0.3
10.0	0.9 ± 0.1	41.2 ± 0.7	24.0 ± 0.5	23.8 ± 0.9

Discussion

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Discussion

SpA may not be as useful for the detection and purification of canine immunoglobulins as has been suggested.[8,9] Protein A from the Cowan I strain of *Staphylococcus aureus* bound only about 80% of the canine IgG and 67% of the canine IgM purified by heavy chain specific (γ and μ) polyclonal antibodies. This was true under conditions of column chromatography as well as in a solid phase immunoradiometric assay.

Similar results for IgG were generated by two approaches. In the first approach, a significant amount of IgM was co-purified with IgG on an anti-IgG (whole molecule) affinity column. Consequently, the relative amounts of SpA-bindable and SpA-nonbindable IgG were determined by densitometric evaluation of Coomassie blue-stained bands on SDS-PAGE gels. In the second IgG experiments, IgG was purified with a γ-chain specific antibody. IgM co-purification was negligible. In this study, relative amounts of SpA-bindable and SpA-nonbindable IgG could be measured spectrophotometrically. The results of these approaches were remarkably similar, finding that 20.8 and 18% of canine IgG was SpA-nonbindable in the first and second methods, respectively. Use of plasma pooled from 10 (first approach) and 4 (second approach) dogs of different breeds and genders suggests that these figures are generally applicable, though variation among individual dogs was not assessed.

These findings contrast with reports of less SpA reactivity,[7] as well as with two reports that SpA binds to at least 98 and 90% of normal canine IgG and IgM, respectively.[8,9] It has been suggested that the greater binding seen by Warr and Hart[9] may have been due to application of serum to SpA-Sepharose at a higher pH (pH 8.0) than was used previously.[14] However, we saw no difference in recovery when IgG was

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applied at pH 7.2 as compared to pH 8.0. Different results may also have been due to use of SpA from different strains of *S. aureus,* though the strains of origin were not specifically stated in any one of these reports. We evaluated SpA from the Cowan I strain because it is the most frequent natural source of SpA for immunologic procedures.[1,2]

Other explanations for disparate results may relate to the investigators' techniques. In the early studies by Goudswaard,[7] incomplete protein recoveries (38.5 to 102%) after SpA chromatography, relatively crude starting immunoglobulin material (ammonium sulfate precipitates), and limited sensitivity of immunoglobulin quantition (radial immunodiffusion) may have contributed significant error to the assessment of SpA reactivity. Warr and Hart[9] also measured immunoglobulin concentrations in samples before and after SpA affinity chromatography, but they chromatographed whole serum, and used a competitive radioimmunoprecipitation assay to measure immunoglobulin concentrations. Again, protein recovery was a problem. Less than 1% of the initial detectable IgG was present in the column FT after 3 passages, but only 60% of the starting amount was detected in the eluted fraction. Almost 40% of the IgG was therefore unaccounted for. Similarly, only 33% of the initial IgM was detectable in the FT and E fractions together. The apparent loss of immunoglobulin may relate to insensitivity of the immunoglobulin assay. Alternatively, eluted protein may actually have been lost during dialysis and concentration steps.

Most recently, an indirect ELISA was used to measure immunoglobulin concentrations before and after serum was exposed to SpA.[8] The serum was from dogs immunized with ragweed extract, and the ELISA detected only those immunoglobulins

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that reacted with ragweed extract coated onto microtitration plates. It is therefore likely that only a fraction of each immunoglobulin class was detectable. The recovery of immunoglobulins ranged from a low of 38% for IgE to 100% for IgG and IgA; 69% of the IgM was reportedly recovered. The major concerns about this study are that protein recovery was inconsistent and/or incomplete, and only some immunoglobulins were detectable.

In contrast, protein recovery was consistently good in our studies. The evaluation of essentially pure proteins or protein bands, as opposed to serum or serum fractions, allowed more precise and accurate quantitation, and the relative amounts of immunoglobulins in the FT and E fractions could be calculated simply with proportions based on volumes and absorbances. Moreover, the immunoglobulins evaluated were not restricted to any particular subpopulations; rather, they were all those immunoglobulins reactive with heavy chain specific polyclonal antibodies. Such antibodies are generally presumed to have class-wide reactivity.

It is not known if SpA-nonbindable IgG in dogs represents a distinct immunologic subclass of IgG as is true of human and murine IgG, or if each subclass of canine IgG contains SpA-bindable and SpA-nonbindable immunoglobulins. We tested the FT and E IgG to see if these fractions correlated with IgG_1 and IgG_2 subclasses as defined immunoelectrophoretically by the antibody supplier (Bethyl Laboratories, Montgomery, TX). They did not. Gourdswaard also could not show IgG subclass specificity of SpA using 3 different antibodies to canine IgG subclasses immunoelectrophoretically classified as IgG_{a+b}, IgG_c, and IgG_d.[7] This contrasts with human and murine IgG, in which cases SpA binds poorly to human IgG_3 and to murine IgG_1.[2] However, the canine studies do

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not exclude the possibility that SpA-nonbindable IgG is a particular IgG subclass, be it IgG_1, IgG_{2a}, IgG_{2b}, or IgG_{2c} as currently classified,[15] or some other uncharacterized subpopulation.

The current results impact several common uses of SpA: 1) canine immunoglobulin purification methods employing SpA column chromatography may omit significant and uncharacterized subpopulations of IgG and IgM, 2) the complete removal of IgG from IgG-containing samples may not be possible with SpA chromatography, 3) SpA-nonbindable IgG will not immunoprecipitate with SpA despite its reactivity with the desired antigen, and 4) the use of SpA as an antiglobulin reagent for canine IgG may result in false negative results with a variety of immunoassays. We have experience with an IRMA for the detection of canine platelet-associated immunoglobulin (PAIg).[16] Only once has there been positivity with ^{125}I-anti-IgG(γ) but not ^{125}I-SpA.

Although SpA-based assays for human PAIg will not detect $PAIgG_3$, human patients with IMT usually have multiple classes of antiplatelet antibodies with reactivity to multiple platelet epitopes.[17] IgG_3 is frequently contributory to the detectable PAIgG, but it rarely occurs without other IgG subclasses.[18,19] Therefore, SpA can usually reliably detect human PAIgG, but it cannot accurately quantify it. In dogs, if SpA-bindable IgG is also usually produced concurrently with SpA-nonbindable IgG, the major limitation of SpA would again relate to quantitation.

The benefits of SpA may outweigh its limitation for use in immunoassays. SpA is relatively inexpensive, stable, and easily radiolabeled. It has a high binding affinity with a constant binding ratio that allows quantitation of SpA-bindable immunoglobulins.[1,20] Moreover, it will bind other immunoglobulin classes in dogs such that it may

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detect PAIg in cases where a specific anti-IgG detecting antibody would not. These characteristics probably make SpA a suitable reagent in immunoassays when qualitative results are acceptable. However, quantitation of canine immunoglobulins in clinical assays using SpA (Cowan I) will be limited by the SpA-nonreactivity of 20% of canine IgG and 33% of canine IgM.

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CHAPTER 2

AN IMMUNORADIOMETRIC ASSAY FOR THE DETECTION OF CANINE PLATELET-ASSOCIATED IMMUNOGLOBULINS

Abstract

As an aide to improving our understanding of immune-mediated thrombocytopenia (IMT) in dogs, we developed and characterized a sensitive immunoradiometric assay (IRMA) for canine platelet surface-associated immunoglobulin (PSAIg). This assay also detects platelet-bindable immunoglobulin (PBIg) in the form of autoantibodies, alloantibodies, and xenogeneic antibodies. PBIg was detected by incubating plasma with normal canine platelets fixed in 0.01% glutaraldehyde. While the reactivity of unfixed refrigerated platelets decreased dramatically over days, these fixed platelets maintained reactivity to 3 different plasma antibodies for months to years. The platelet reactive immunoglobulins were reproducibly detected in a dose-response fashion with ^{125}I-labeled staphylococcal protein A (SpA). Antibodies to canine IgG(γ) and IgM(μ) were used to define the class of platelet-reactive antibodies and to detect SpA-nonbindable immunoglobulins. Plasma from a dog with clinically diagnosed IMT and a high titer of PBIg that immunoprecipitated GPIb was used as a positive control for SpA and the anti-IgG(γ) detector. The exposure of blood samples to 4°C or room temperature (RT) led to a progressive increase in PSAIg which could convert negative samples to apparent positives

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within hours. The storage of isolated plasma at 4°C or RT also led to progressive and dramatic increases in PBIg, mostly IgM. False positive results due to this nonspecific PSAIg and PBIg, mostly IgM, were avoided by using fresh 37°C blood samples and 37°C plasma incubation temperatures. Clinically, the assay detected moderate to marked increases in PSAIg, usually IgG, for 96% of 24 dogs given a diagnosis of IMT. Of the other 19 thrombocytopenic dogs tested, 5 had increases in PSAIg that were considered clinically significant, and it is likely that immune-mediated platelet destruction played a principal role in the pathogenesis of thrombocytopenia for 4 of these dogs. This assay appeared to be useful for differentiating dogs with IMT from dogs with nonimmune thrombocytopenia.

Introduction

Immune-mediated thrombocytopenia (IMT) appears to result primarily from accelerated platelet destruction due to increased platelet surface-associated immunoglobulin (PSAIg[a]). Confirming the presence of increased PSAIg with reliable direct assays may be useful for identifying dogs with IMT.

Thrombocytopenia occurred in about 5% of 18,910 dogs retrospectively evaluated at one veterinary teaching hospital, with about half the thrombocytopenic dogs having fewer than 150,000 platelets/μl.[1] The cause of thrombocytopenia was undetermined in about a third of the cases, but thrombocytopenia was frequently associated with neoplasia, inflammation, and infection. Thrombocytopenia was considered immune-mediated in about 5% of the cases, though specific testing for platelet-reactive

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immunoglobulin was not done. Direct assays for PSAIg may improve the largely clinical differentiation of immune-mediated from nonimmune thrombocytopenias. They may also identify roles for PSAIg in the pathogeneses of such canine thrombocytopenias as those commonly associated with neoplastic and infectious diseases.

Indirect assays have been developed for detecting canine platelet-bindable immunoglobulin (PBIg[b]) in patient plasma or serum samples.[2-6] These samples are convenient and unaffected by the paucity of cells in many thrombocytopenic dogs. However, these indirect assays have not had great sensitivity for clinically diagnosed canine IMT,[2,4-6] and high nonspecific background binding has been a problem.[2] Furthermore, these assays for PBIg do not differentiate among antiplatelet autoantibodies, antiplatelet alloantibodies, immune complexes, and aggregates of normal IgG present in stored samples.

High background signals and mediocre sensitivity have also limited the utility of direct megakaryocyte immunofluorescence testing (DMIFT).[5,7-9] Other problems with DMIFT include difficulties in collecting sufficient numbers of megakaryocytes for testing,[5,7,8,10] the exposure of nonpathologic cytosolic immunoglobulin due to smearing trauma with nonsuspension techniques,[11] and incompletely shared antigenicity between platelets and megakaryocytes.[11] The recent use of platelet volume and fragmentation for diagnosing canine IMT is an indirect method with unproven specificity for IMT.[12-14]

Relatively little direct testing for canine PSAIg has been attempted clinically, and when used in toxicologic settings for detecting drug-induced antibodies, signal-to-noise

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ratios have been relatively low.[15,16] Recently, however, a direct ELISA for PSAIg was used clinically and found to detect PSAIg in most dogs suspected of having IMT.[4] Moreover, PSAIg eluted from the platelets of some dogs with IMT radioimmunoprecipitated GPIIb and/or IIIa, suggesting that as for human IMT, GPIIb/IIIa has target epitopes for antiplatelet autoantibodies in some dogs with IMT.[17]

We have developed and characterized a sensitive immunoradiometric assay (IRMA) for detecting PSAIg on canine platelets and PBIg in canine plasma. The principal immunoglobulin detector was radiolabeled staphylococcal protein A (SpA). Radiolabeled antibodies to canine IgG and IgM were used in conjunction with SpA to determine the class of immunoglobulin and to detect SpA-nonbindable immunoglobulins. Sample storage and assay temperatures profoundly influenced the results, with increased PSAIg and PBIg occurring under some sample handling conditions. We suggest that for any assay of canine PSAIg or PBIg, strict sample management may be required to prevent the apparent positivity of normal samples.

Methods

Dogs

Blood donor dogs were maintained by the University Laboratory Animal Resources facility which follows USDA and NIH guidelines for animal use and care. Approval for use was obtained from the All University Committee on Animal Use and Care. The dogs were thoroughly preconditioned before use.

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Blood collection and platelet isolation

For normal dogs, blood was drawn by jugular venipuncture through 18 gauge needles into plastic syringes containing 1/10 volume citrate to give a final citrate concentration of 10.5 mM (0.32%). Platelets were harvested by differential centrifugation for 2-4 min at 1300 x g. Centrifugation was often repeated once or twice to maximize platelet yields.

Radiolabeling

The following antiglobulin reagents were radiolabeled by modifications of the method of Fraker and Speck:[18] staphylococcal protein A (SpA) (Sigma, St. Louis, MO), a polyclonal rabbit anti-[canine IgG(whole molecule)] (anti-G) (Sigma), a polyclonal goat anti-[canine IgG(γ)] (anti-γ), and a polyclonal goat anti-[canine IgM(μ)] (anti-μ) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Briefly, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma) deposited on the base of a glass test tube was incubated with 25 μg of protein (1 mg/ml) and 0.3-0.5 mCi ^{125}iodine (NaI, NEN Dupont). After 10-15 minutes of incubation with mixing, radiolabeled protein was separated from free iodide by filtration through a 10 ml Sephadex G25 filtration column (Pharmacia Biotech, Inc., Piscataway, NJ). The labeled protein was pooled, diluted with PBS/BSA (phosphate buffered saline (PBS) with 3% bovine serum albumin (BSA) and 0.025% sodium azide, pH 7.1) to stock concentrations of 1.2 μg/ml, and frozen in aliquots until use. Heavy chain specificity of the anti-γ and anti-μ antibodies was confirmed by immunoblotting, and the same lot of each antibody was used in all studies.

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Positive control

The assay positive control (PC) was citrated plasma obtained by plasmapheresis from a dog with clinically diagnosed IMT. The specificity of the PC antibody was assessed by radioimmunoprecipitation using normal canine platelets surface-labeled by a modification of the lactoperoxidase technique of Philips.[19] To 1 X 10^9 washed platelets in 800 μl PBS were added 200 μl (1 mg/ml) lactoperoxidase (Sigma) in PBS and 0.75 mCi ^{125}iodine. After the slow addition of 50 μl of 3 mM H_2O_2, the radiolabeled platelets were washed in PBS and resuspended in PBS/BSA. Platelet aliquots were incubated for 1 hr with PC plasma, normal autologous plasma, or PBS, and then washed and resuspended in lysis buffer (150 mM NaCl, 10 mM Tris, with 1% Triton X-100, 1 mM PMSF, and 24 μM leupeptin). After lysis, a 50% slurry of 4% beaded agarose/SpA in PBS/BSA was incubated with each sample for 1 hr. The SpA-agarose was then washed, and nonreducing sample buffer (370 Mm Tris (pH 6.8) and 0.1% SDS) was added to each sample before heating them for 10 min in a boiling water bath. The agarose was pelleted and the supernatants were assessed under nonreducing and reducing (with 4% ß-mercaptoethanol) conditions by SDS-PAGE on a 5-12% linear gradient gel with the discontinuous buffer system of Laemmli.[20] Molecular weight standards were electrophoresed with each gel and visualized with Coomassie Blue. After staining, the gels were dried for autoradiography.

Fixation

For fixation of platelets in glutaraldehyde (grade I, Sigma), fresh platelets were washed 3 times in 37°C PBS, pH 6.5, with 1 μM prostaglandin E_1 (PgE_1). Washed

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platelets were then incubated at a concentration of 100,000 platelets/μl for 30 min in PBS with 0.01% glutaraldehyde. Glutaraldehyde was removed by washing with PBS before resuspending the platelets in PBS/BSA.

The reactivity of refrigerated fixed platelets was evaluated over time for 3 positive antibodies: 1) the assay PC antibody, 2) a rabbit anti-[canine whole platelet] plasma, and 4) a human plasma with specific reactivity to the Pl^{A1} epitope of GPIIIa (anti-Pl^{A1}).[21] This antibody also reacted with canine GPIIIa as identified by immunoblotting (data not shown). Dose-response curves were generated with fixed platelets for each antibody and compared to those generated with 1 week-old, refrigerated, unfixed platelets.

Assay conditions

Sample Storage and Assay Temperature

After a supply of platelets with reproducible reactivity was established, several studies were conducted to investigate the high nonspecific background binding seen with refrigerated samples and room temperature assay conditions. For these studies, platelets and plasma were harvested from citrated blood collected from normal blood donor dogs. The samples were stored either before or after cell separation at room temperature (RT, 20-22°C), 4°C, or at 37°C. To achieve a 37°C assay temperature, incubations were done in a 37°C incubator, wash buffer was maintained at 37°C, and the plate was warmed on a heating plate during manipulations. Centrifugation was done at room temperature.

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SpA Binding

The length of incubation required to saturate the binding of SpA to PSAIg was determined in polypropylene microfuge tubes which allowed rapid washing and removal of unbound SpA at frequent intervals. The number of platelets and volumes of reagents were each 3 times those of the standard plate assay method, and washes were 5 times the volumes. Normal fixed platelets were first incubated with PC plasma to provide a relatively large but not saturating amount of PSAIg. One set of 3 tubes contained platelets that had been incubated for 120 min with twice the PC plasma to verify that the binding capacity of SpA was not saturated. At each time point, unbound SpA was removed by washing and the tips of triplicate microfuge tubes were removed for assessing platelet-associated radioactivity in a gamma counter (TmAnalytic, Bensenville, IL).

Test Platelet Number

This experiment was done to optimize the number of platelets to assay for PSAIg, and to determine the effect of increasing numbers of platelets on the detection of PSAIg by SpA. Platelets from a dog with clinical IMT and a positive test for PSAIg were added to wells at increasing numbers from 2.5 X 10^6 to 12.5 X 10^6 platelets per well. The standard assay methods were then used.

Standard Assay Method

Platelets were harvested from fresh citrated blood at 37°C by serial centrifugation 2 to 3 times at 1300 x g. Platelet-rich plasma (PRP) was removed and pooled after each

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centrifugation. In some cases, the remaining blood was resuspended in PBS and the centrifugation procedure was repeated to obtain a better yield. PgE_1 was added to the PRP (1 μM final) before washing the platelets 3 times with 37°C assay buffer (PBS/BSA, pH 7.1). Contaminating erythrocytes, if present, were largely removed by rapid differential centrifugation in a microfuge. Washed platelets were resuspended in assay buffer, and the platelet concentrations were determined with a Neubauer hemocytometer.

Five million test platelets were added to each test well of a U-bottom microtitration plate and pelleted at 850 x g for 8 minutes in a Beckman J6B floor centrifuge. The buffer was removed and the platelets were resuspended and incubated for 1 hr at RT in assay buffer containing 10 ng of ^{125}I-labeled antiglobulin reagents. After 3 resuspending washes in assay buffer, aliquots of the test platelets were removed from each well and assessed for platelet-associated radioactivity. Results were reported as percentages of the added radioactivity that remained with the platelets (% bound). When the number of harvested platelets allowed it, each of 3 antiglobulin detectors was used, and each sample was assessed in triplicate.

For the indirect assay, 5 μl of test plasma was incubated for 1 hr at 37°C with normal canine platelets fixed in 0.01% glutaraldehyde. The plasma was prewarmed to 37°C for 30 min before use. After 3 resuspending washes, the detecting reagents were added and the assay proceeded as for the direct assay. PC plasma was tested concurrently as a positive control for SpA and anti-γ. The anti-μ antibody was periodically tested for its ability to detect canine heat-aggregated IgM bound to human C1q (Sigma) nonspecifically coated onto wells of microtitration plates. It was also tested in an assay for erythrocyte surface-associated immunoglobulin, where the positive control

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Evaluation of antiglobulin reagents

Concentration Dependency

The effect of increasing concentrations of antiglobulin reagents on detection of constant amounts of PSAIg was evaluated. PC immunoglobulin was bound to normal fixed platelets and detected by the antiglobulin reagents over a concentration range of 0.1-1.0 μg/ml using standard assay conditions.

Similar studies were done with IgG nonspecifically adsorbed to Immulon II Removawell microtitration plate wells (Dynatech Laboratories, Inc., Chantilly, VA). Nonspecific adsorption was done overnight at 4°C with 50 μl of 10 μg/ml IgG in 50 mM carbonate buffer, pH 9.6. After adsorption, the wells were washed 3 times with blocking buffer (PBS/BSA with 0.5% Tween) and then incubated with 200 μl blocking buffer for 1 hr. Blocking buffer was removed and 50 μl of each antiglobulin reagent were added at increasing concentrations from 0.1 to 1.2 μg/ml. After a 4 hr incubation, the wells were washed 5 times with PBS/BSA before removal and transfer to a gamma counter.

Dose-response Curves

The detection of increasing amounts of PSAIg by the antiglobulin reagents was assessed under standard assay conditions. SpA and the polyclonal anti-IgG antibodies were directly compared using normal fixed platelets incubated with increasing amounts

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Coefficient of variation

The interassay and intra-assay coefficients of variation (CVs) for the assay were based on repeated use of the PC plasma. No CV could be calculated for the use of anti-μ, because there was no positive control for the anti-μ antibody in this assay.

Reference range

A reference range was established in 20 clinically healthy dogs of a variety of breeds, half of each gender. The standard assay method was used with blood maintained at 37°C until use within 3 hr of collection. The reference range was established concurrently with PC standard curves so that the cut-off value using SpA could be adjusted for any decreased reactivity of the detecting antibodies that might occur with storage.

Clinical evaluation

PSAIg was assayed in 43 dogs evaluated for thrombocytopenia. SpA alone was used in early cases, SpA and anti-γ later, and finally anti-μ was added to the assay procedure. Diagnoses of IMT were based on exclusion of other known causes of thrombocytopenia and on response to therapy. In addition to routine serum chemistries

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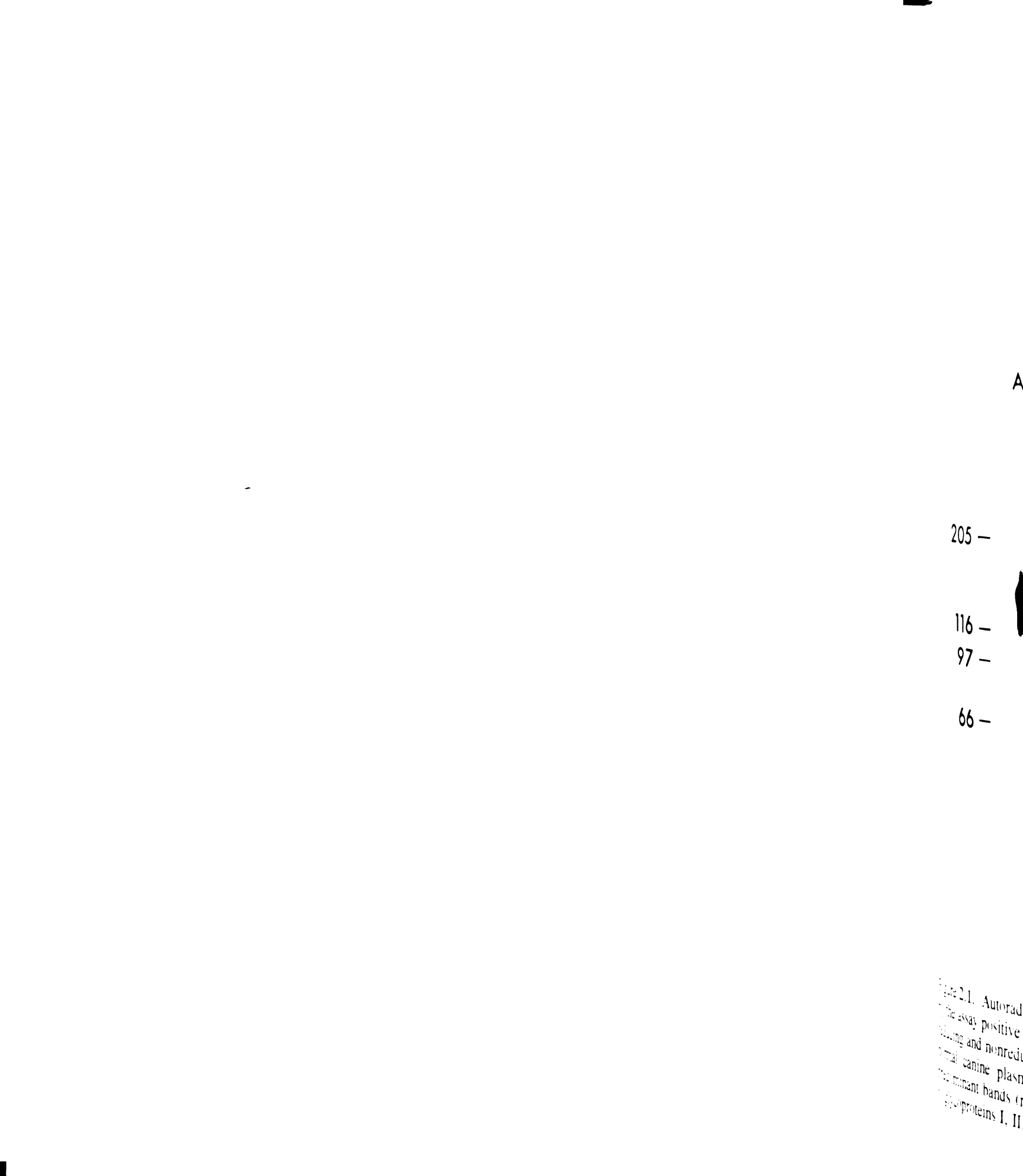
Results

Positive Control

The positive control plasma was from a cocker spaniel with clinical IMT and a high titer of PBIgG. The binding of this antibody to platelets reached saturation within 30 min (data not shown), and it was detectable with SpA and anti-γ but not anti-μ. The antibody did not react with erythrocytes in direct binding or adsorption studies. When platelets were surface-labeled with ^{125}iodine, the antibody immunoprecipitated GPIb (Figure 2.1). It therefore reacted with GPIb or with part of the GPIb complex that does not radiolabel with ^{125}iodine and that does not dissociate from it with Triton X-100 treatment.[22,23]



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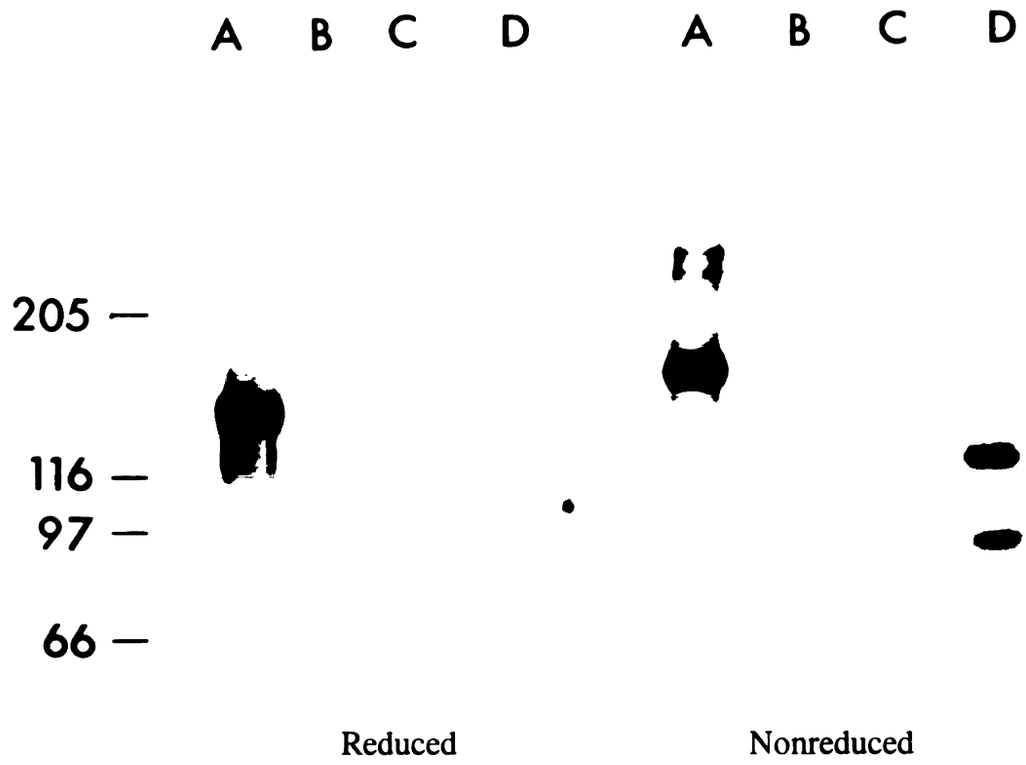

Figure 2.1. Autoradiograph of ^{125}I-labeled platelet surface proteins immunoprecipitated by the assay positive control plasma (A). Proteins were separated by SDS-PAGE under reducing and nonreducing conditions on a 5-12% gradient gel. Negative controls were normal canine plasma (B) and PBS (C). Labeled platelet lysates (D) had three predominant bands (nonreduced) corresponding in descending order of molecular mass to glycoproteins I, II, and III.

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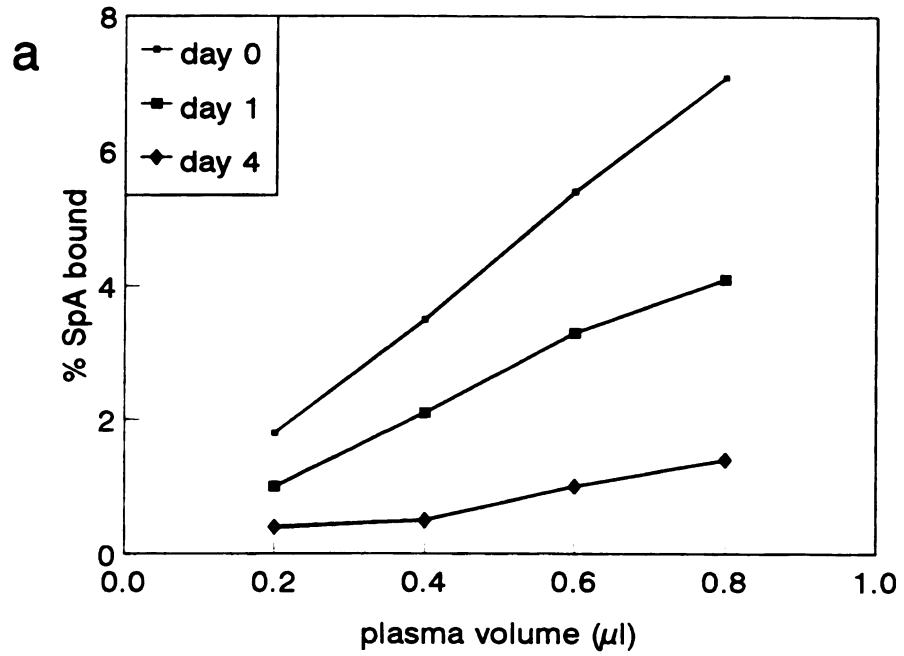
Fixation

When normal platelets were washed and stored at 4°C in assay buffer, the detectable PSAIg decreased daily. Microscopically, the platelets appeared to fragment during storage. When used as normal test platelets for detecting PBIg in plasma, stored platelets also yielded daily decreases in detectable PBIg with positive plasma samples (Figure 2.2a,b). This lack of reproducibility with stored platelets initially hampered efforts to optimize the assay conditions and standardize the results.

Figure 2.2. Decreasing reactivity of washed canine platelets with refrigerator storage for 4 days. Reactivity was tested with a human anti-Pl^{A1} antibody (a) and the positive control canine autoantibody (b). Data were generated in duplicate.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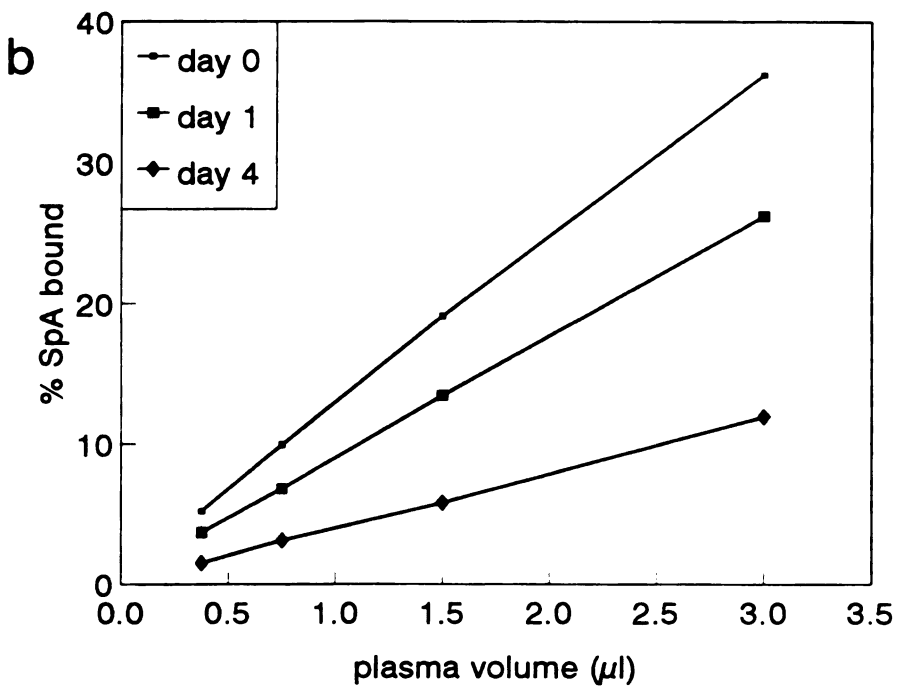
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Figure 2.2

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In an effort to overcome this problem, platelet fixation was evaluated as a means of maintaining consistent platelet reactivity. Pilot studies with several fixation times and concentrations of glutaraldehyde and paraformaldehyde suggested that 30 min fixation in 0.01% glutaraldehyde might successfully maintain platelet reactivity. Use of 1 and 2% paraformaldehyde caused platelet clumping which was noted microscopically.

In the 84 day temporal study with fixed (0.01% glutaraldehyde) and unfixed platelets, the reactivity of unfixed platelets decreased rapidly over the first week of storage. For the entire 28 or 84 days tested, fixed platelets maintained reactivity to PBIg in plasma from a dog with IMT, from a rabbit immunized with canine platelets, and from a human patient with posttransfusion purpura and anti-Pl^{A1} antibodies (Figure 2.3). Single batches of fixed platelets gave reproducible results with the PC antibody for at least 2 years (data not shown).

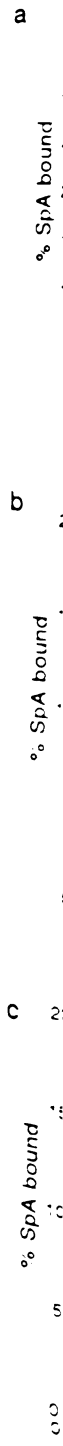
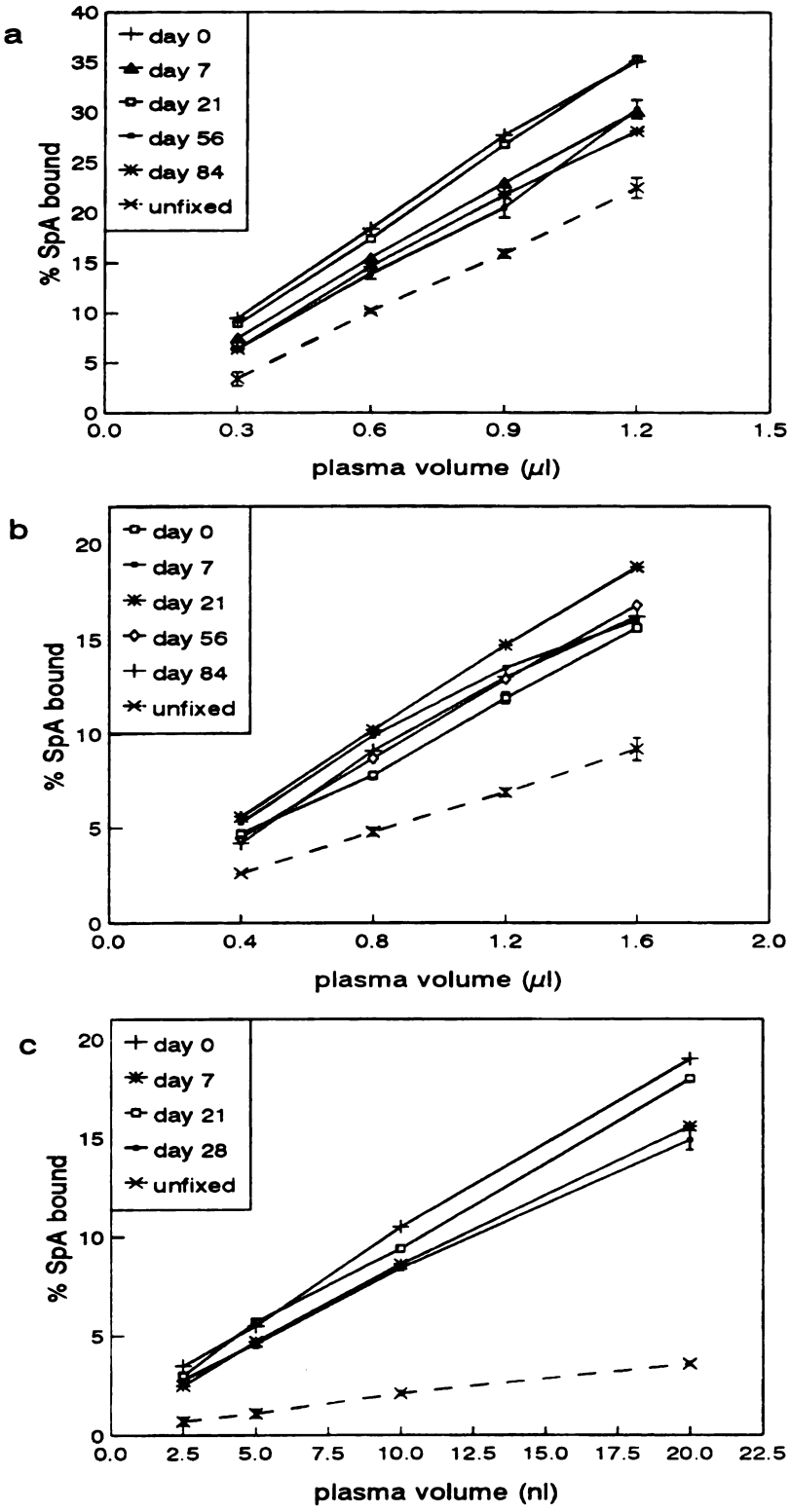


Figure 2.3. Reactivity of fixed platelets to a) the positive control canine plasma, b) human anti-Pl^{A1} plasma, and c) rabbit anti-[canine platelet] serum was compared to week-old unfixed platelets (dashed lines). Reactivity was assessed over 84 (a,b) or 28 (c) days. Errors ($\pm$ S.D.) are shown for the unfixed platelets and for the lowest curve in the fixed platelet groups.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plasma volume (μl)
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day 0
day 7
day 21
day 56
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plasma volume (μl)
c
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Figure 2.3

Assay conditions

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Indirect Assay

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Assay conditions

Sample Storage and Assay Temperatures

The duration and temperature of sample storage, and the assay temperature greatly affected the results for the direct and indirect assays. With each assay, increased nonspecific binding of immunoglobulin occurred whenever platelets had prolonged contact with plasma at 4°C or RT.

Indirect Assay

Normal plasma stored in the refrigerator or at room temperature for 2 days had considerably more PBIg than paired fresh-frozen samples when assayed at room temperature (Table 2.1). This nonspecific binding was markedly reduced by warming the plasma to 37°C and maintaining a 37°C assay temperature until after the unbound plasma was removed from the assay platelets by washing (Table 2.1). The nonspecific binding in fresh-frozen plasma samples was also reduced by this procedure.

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Table 2.1. Effects of plasma storage temperature and plasma incubation temperature on SpA-detectable platelet-bindable immunoglobulin for three dogs.

plasma storage temperature	% SpA bound ± S.D.*					
	Dog 1		Dog 2		Dog 3	
	RT[1]	37°C[2]	RT	37°C	RT	37°C
4°C	16.5 ± 2.0	2.9 ± 0.6	16.2 ± 0.2	2.2 ± 0.1	18.1 ± 0.6	3.9 ± 0.7
RT	14.4 ± 1.2	3.4 ± 0.1	9.2 ± 0.3	2.8 ± 0.1	19.4 ± 1.7	6.1 ± 0.4
-65°C	5.1 ± 0.1	1.5 ± 0.1	1.8 ± 0.2	1.1 ± 0.3	7.6 ± 0.4	2.2 ± 0.5

* means of triplicates
[1] RT = assay done completely at RT
[2] 37°C = assay done at 37°C until after plasma was removed from assay platelets by washing

To further investigate the effects of sample storage temperature on PBIg results generated with a 37°C primary incubation temperature, the PBIg in whole blood and plasma samples stored at RT, 4°C, and -65°C were compared over time for 3 dogs (Figure 2.4). Normal plasma samples stored either at 4°C or at RT usually had progressive increases in PBIg sometimes beginning within 24 hr. These increases were less marked than at lower assay temperatures. The increased PBIg could be removed by plasma filtration through a 0.22 μm filter. When samples were stored at 4°C or RT as whole blood instead of plasma, PBIg results were relatively low and stable, similar to plasma stored at -65°C.

Figure 2.4. Comparison of sample storage conditions on SpA-detectable PSAIg for three normal dogs (a,b,c). Samples were tested fresh (F) and then daily with storage as refrigerated whole blood (RWB), room temperature whole blood (RTWB), refrigerated plasma (RP), room temperature plasma (RTP), and plasma frozen at -65°C. In each group, consecutive bars represent consecutive days. The day 5 bars for RP and RTP are divided, the lower portion representing PSAIg after sample filtration (0.22 μm filter).

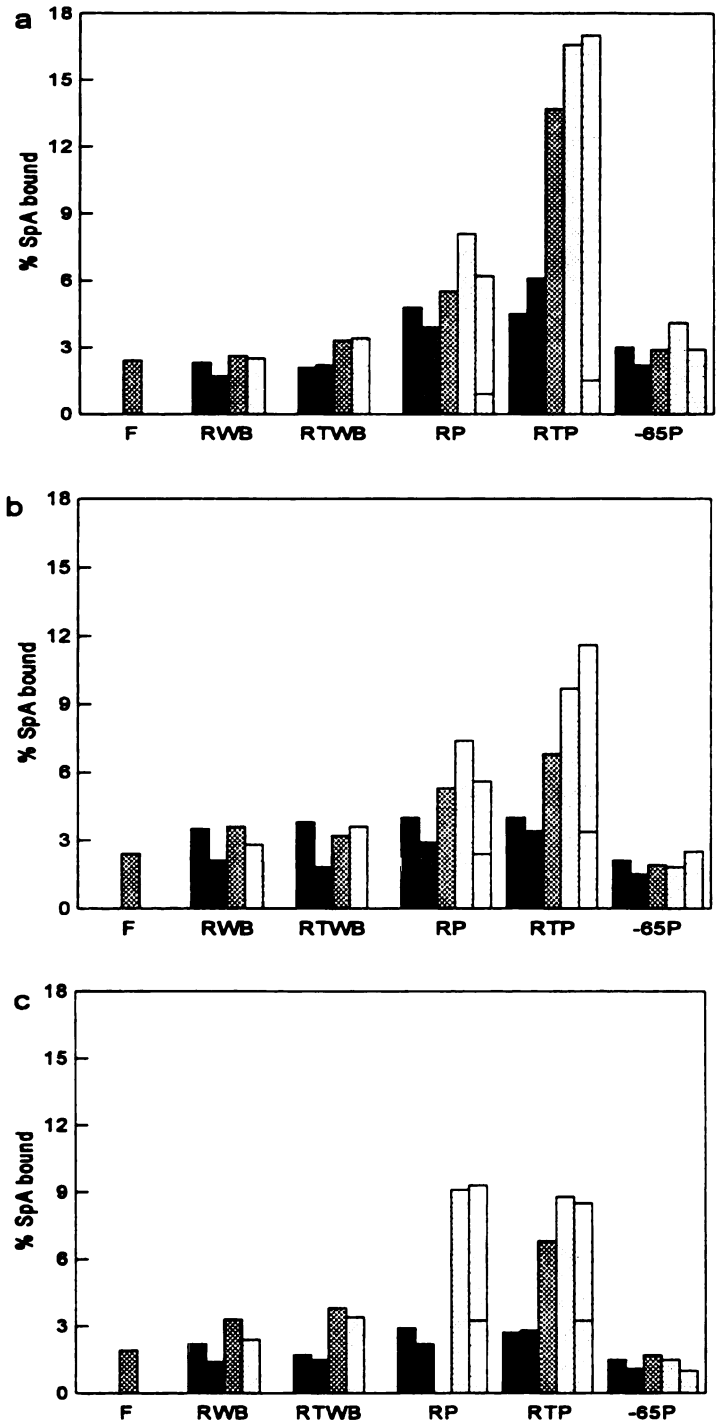

Figure 2.4

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To determine the class of immunoglobulin deposited on fixed platelets by plasma stored at 4°C, the PBIg in the plasma of three dogs was assessed with SpA, anti-γ, and anti-μ detectors (Table 2.2). The SpA-bindable PBIg, averaging 11.4 times the background for fixed platelets alone, was due principally to IgM which was detected at an average of 5.6 times the background amount. Minimal PBIgG was present in these normal samples, being detected at an average of only 1.4 times background.

Table 2.2. Determination of the class of platelet-bindable immunoglobulin responsible for nonspecific binding.

	% bound ± S.D.*		
	SpA	anti-γ	anti-μ
Buffer	0.5 ± 0.0	0.8 ± 0.0	1.2 ± 0.2
Plasma 1**	5.0 ± 0.3	1.5 ± 0.2	7.2 ± 0.4
Plasma 2	6.3 ± 0.3	1.0 ± 0.2	5.7 ± 0.2
Plasma 3	5.8 ± 0.1	0.8 ± 0.3	7.2± 0.3

* means of triplicates

** plasma samples from 3 normal dogs had been stored 24 hr at 4°C and 5 μl each were assayed with a 37°C primary incubation temperature

Direct Assay

With platelets from normal dogs, PSAIg consistently increased with blood storage time, but much more with blood stored at 4°C or RT than at 37°C (Table 2.3). The increases in PSAIg began within hours of blood collection, though to variable degrees among dogs, and they were progressive. Blood refrigeration generally caused the greatest increases. After 6.5 hr of refrigeration, the SpA-bindable PSAIg for dog 2 had increased to 15 times that of fresh platelets. By 27 hr, the SpA-bindable PSAIg of

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refrigerated platelets from dog 1 was 60 times the fresh value. These increases were accompanied by increases in IgG and IgM as detected by the anti-γ and anti-μ antibodies, but the nature of these reagents did not allow quantitation (see below). For both dogs, PSAIg increased little in 13 hr samples stored at 37°C. However, storage of blood at 37°C for more than a few hours markedly decreased platelet yields and altered cell centrifugation properties. Similarly, platelet yields were decreased for refrigerated samples because platelets separated more with the buffy coats.

Table 2-4. Effects of storage time and temperature on detectable platelet surface associated immunoglobulin for normal dogs.

	% bound ± S.D.*								
	SpA			anti γ			anti μ		
Storage time (hr)	*RT*†	4°C	37°C	RT	4°C	37°C	RT		

Table 2.3. Effects of storage time and temperature on detectable platelet surface-associated immunoglobulin for normal dogs.

		% bound ± S.D.*								
		SpA			anti-γ			anti-μ		
	Storage time (hr)	RT†	4°C	37°C	RT	4°C	37°C	RT	4°C	37°C
Dog 1	0.0	0.3 ± 0.0 (fresh)			1.0 ± 0.0 (fresh)			1.5 ± 0.0 (fresh)		
	6.5	0.6±0.1	0.2±0.1	N.A.	0.6±0.0	0.5±0.0	N.A.	2.1±0.1	1.1±0.1	N.A.
	13.0	1.9±0.1	1.4±0.2	0.5±0.1	1.7±0.1	0.8±0.1	1.1±0.1	3.8±0.1	2.6±0.2	1.7±0.1
	27.0	4.0±0.1	18.1±1.4	—	1.6±0.1	5.4±0.3	—	5.8±0.4	14.3±0.3	
Dog 2	0.0	0.7±0.3 (fresh)			0.6±0.0 (fresh)			3.1±0.0 (fresh)		
	6.5	2.3±0.6	10.9±0.9	N.A.	0.7±0.0	1.1±0.1	N.A.	4.3±0.2	7.6±0.2	N.A.
	13.0	6.3±0.2	—	1.1±0.2	1.0±0.1	—	1.1±0.0	7.4±0.3	—	4.2±0.2
	27.0	13.0±0.4	—	—	1.9±0.1	—	—	10.3±0.2	—	—

* means of triplicates

† sample storage temperature

RT= room temperature, N.A. = not attempted, — = insufficient platelets

The negative control blanks gave similar results for each assay, ranging from 0.3 to 0.4% for SpA, 0.9 to 1.1% for anti-γ, and from 1.1 to 1.4% for anti-μ.

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Because some clinical samples were difficult to obtain fresh, we determined the PSAIg results for normal dogs using samples that were maintained at room temperature for 24 hr. For 10 dogs, these values were compared to PSAIg results for fresh samples and for 24 hr samples maintained at RT but heated to 37°C for 30 min prior to platelet harvesting. Refrigeration and 37°C storage were not evaluated in this study because of marked decreases in platelet yields occurring with these storage conditions. For samples from these 10 normal dogs, the mean SpA-bindable PSAIg for unheated 24 hr samples (7.0%) was 17.5 times the mean for fresh samples (0.4%), and the values ranged from 1.4 to 19.2% (Figure 2.5). IgM appeared to contribute more (4.2 x fresh) to the increase than did IgG (2.8 x fresh), though again, these results were not quantitative. By heating the blood for 30 min before platelets were collected, the mean SpA-bindable PSAIg was decreased by more than half, but it was still 7.5 times that of fresh platelets.

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Figure 2.5. Increas
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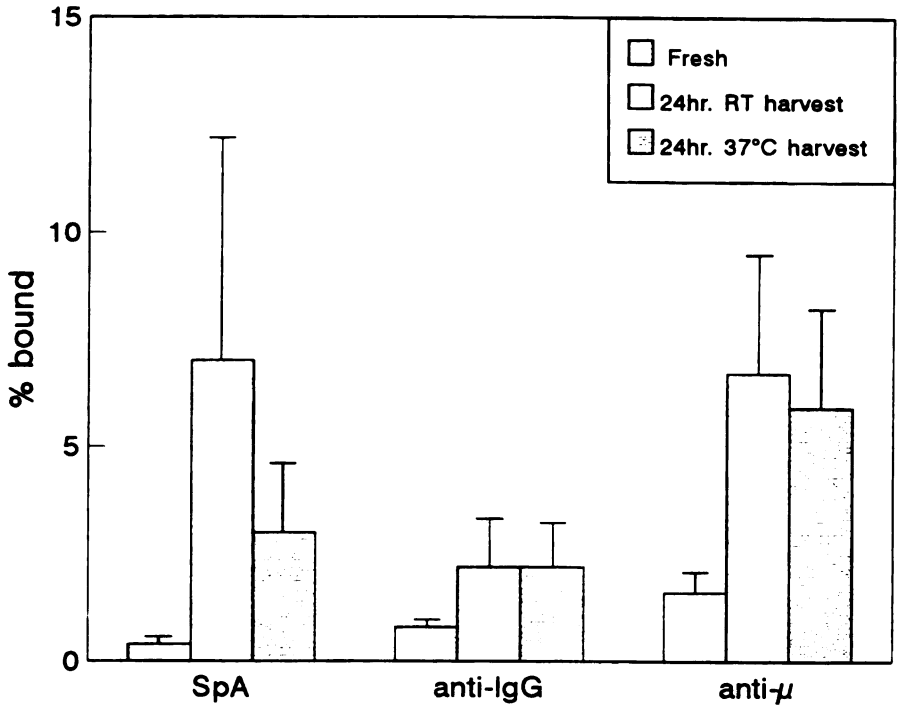

Figure 2.5. Increased PSAIg (% bound ± S.D.) with room temperature (RT) storage of whole blood from ten normal dogs. Platelets were harvested and assayed fresh, at 24 hr without warming to 37°C, and at 24 hr after warming the blood to 37°C for 30 minutes.

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SpA Binding

The binding of SpA to a relatively large but not saturating amount of PSAIg under the standard assay conditions appeared to be complete by 30 min (Figure 2.6). However, a 60 min incubation period was chosen to help assure complete detection of PSAIg. Similar studies were not done with the polyclonal antiglobulin reagents because they were intended only for qualitative use. One hour incubations were used for all antiglobulin reagents. Plasma incubations were also done for one hour because positive antibodies were maximally bound by 30 min (data not shown).

% SpA bound

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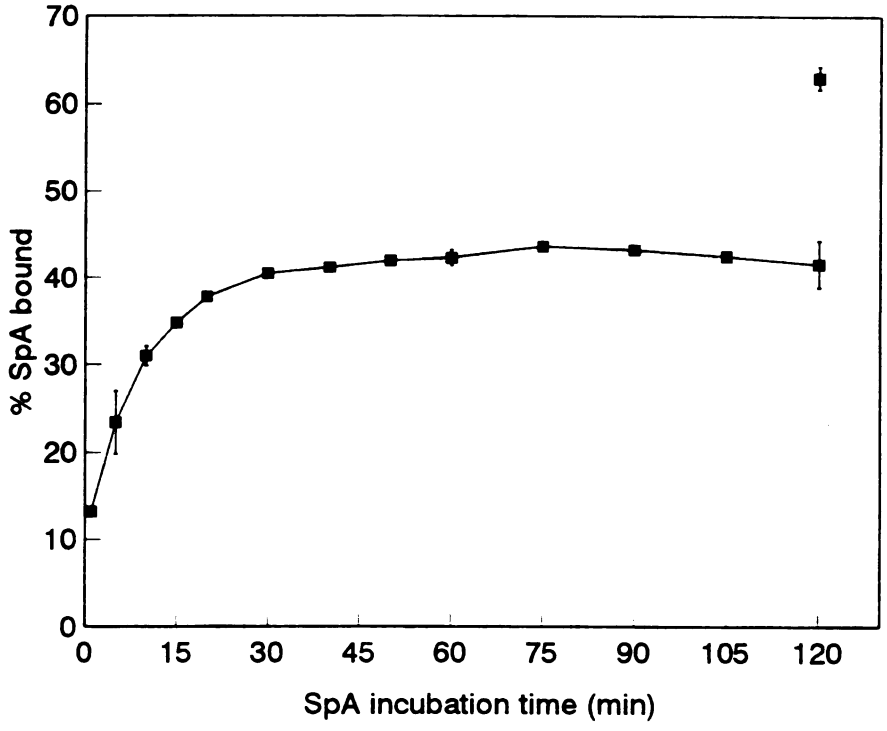

Figure 2.6. Time to saturation for the binding of SpA to PSAIg. Error bars (± S.D.) are smaller than data symbols where not shown.

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Test Platelet Number

There was a direct linear relationship between the number of PSAIg-positive platelets assayed and the PSAIg detected by SpA (Figure 2.7). Because of the difficulty in harvesting many platelets from severely thrombocytopenic dogs, 5 X 10^6 was chosen as the routine number of platelets to test.

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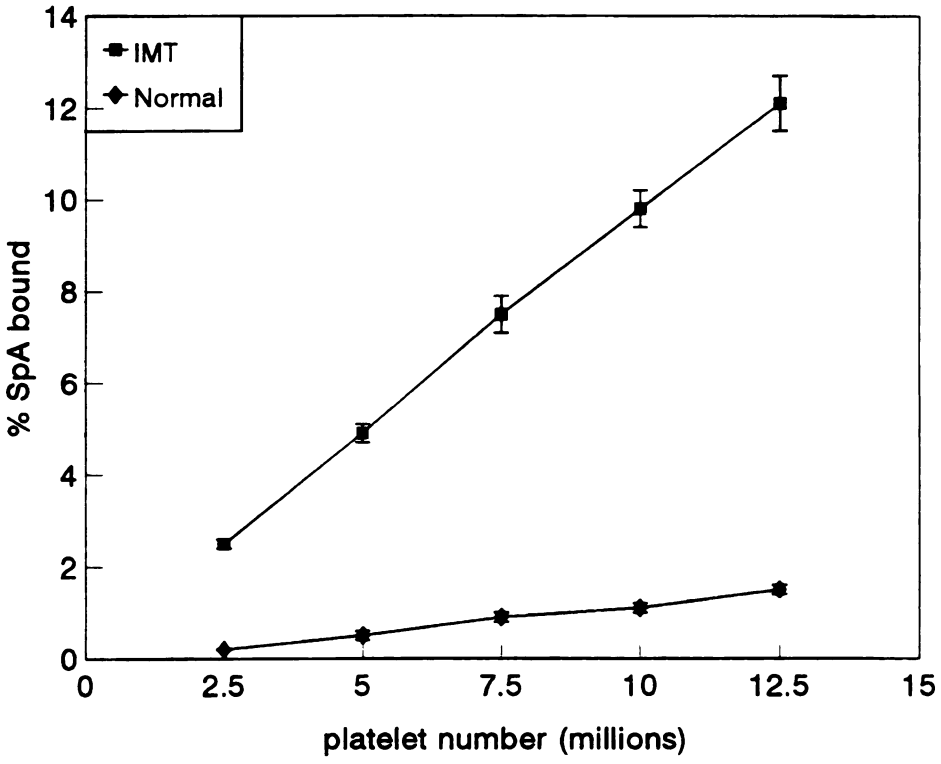

Figure 2.7. Direct relationship between platelet number and SpA-detectable PSAIg. Platelets were from a dog with clinical IMT and a normal dog. Error bars (± S.D.) are smaller than data symbols where not shown.

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Nonspecific background binding of the antiglobulin reagents was very low in the direct assay. It was higher in the indirect assay, partly due to plasma and detector interactions with the well walls. By resuspending platelets and removing them from the wells prior to assessing them for platelet-associated radioactivity, nonspecific interactions with the plastic were not measured.

When erythrocytes visibly contaminated platelet samples, they could usually be removed by short, rapid differential centrifugation in a microcentrifuge. When they were not completely removed, they were washed and tested concurrently with platelets to exclude a contribution of the erythrocytes to the final PSAIg results.

To assess the effect of lipemia on results for PBIg and PSAIg, blood was collected from a normal dog immediately before and 90 min after the dog was fed cream. Results for PSAIg and PBIg were compared between pre- and post-cream samples. For PBIg, PC-spiked and unspiked lipemic plasma were tested using amounts of PC plasma at the threshold of the assay's sensitivity. Lipemia did not affect the results in this dog (data not shown).

Evaluation of antiglobulin reagents

Concentration Dependency

The effect of increasing concentrations of antiglobulin reagents on detection of a constant amount of PSAIg was evaluated for SpA and the anti-γ antibody (Figure 2.8). Over the concentration range tested, 0.1-1.0 μg/ml, the amount of SpA that bound to antibody-coated platelets was constant. Therefore, the platelet IgG-to-SpA binding ratio

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was not influenced by the SpA concentration. The anti-γ detector, however, bound increasingly to the PSAIg in proportion to its concentration through most of the concentration range tested. The binding of a different polyclonal antibody, anti-G, to IgG-coated wells also increased with increasing concentrations (0.1-1.2 μg/ml) of anti-G (data not shown).

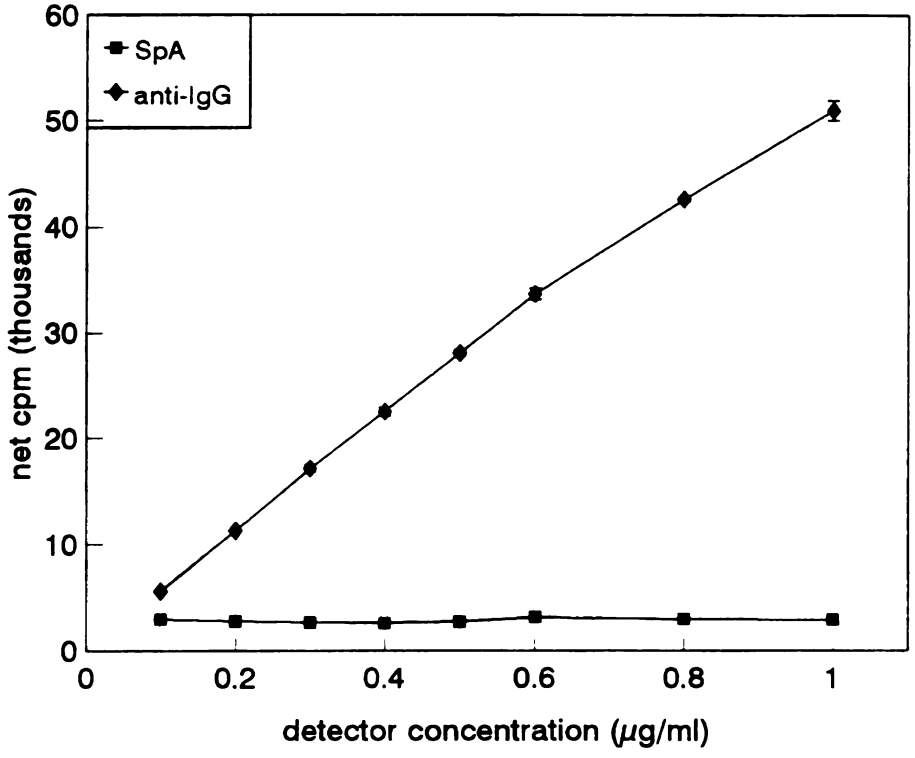

Figure 2.8. Concentration dependency of the binding of polyclonal anti-γ (anti-IgG) to PSAIg as compared to the constant binding of SpA. Error bars (± S.D.) are smaller than data symbols where not shown.

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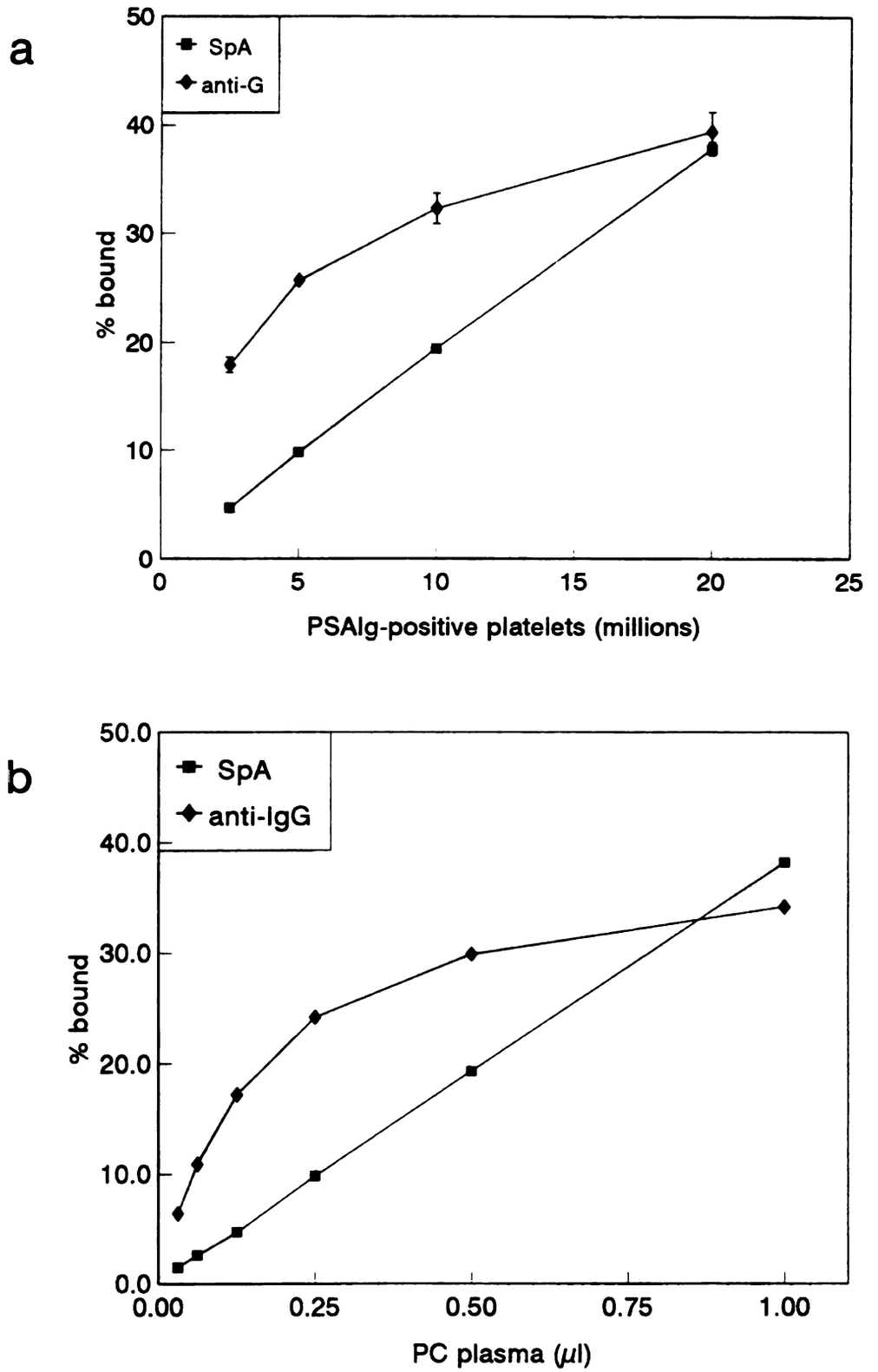
Because the concentration of SpA did not affect its binding, small changes in the incubation volume of SpA with platelets did not affect the results. Even when SpA was added to PSAIg-positive platelets in volumes ranging from the usual 50 μl up to 150 μl, there was little to no difference in the results for PSAIg (data not shown).

Dose-response Curves

Because SpA had a constant binding ratio to IgG, there was a direct relationship between PSAIg and "% SpA bound" (Figures 2.3 and 2.7). This 1:1 relationship contrasted with that of anti-G (Figure 2.9a) and anti-γ (Figure 2.9b) which did not linearly detect linear increases in PSAIg. They only began to approach linear detection at very high ratios of detector to IgG, when their binding to IgG approached saturation. In our assays with IgG nonspecifically adsorbed to microtiter plate wells where a much wider range of detector:IgG ratios was possible, and ratios of at least 500:1 (detector:IgG) appeared to be necessary for linear detection by the anti-G antibody (data not shown). However, these polyclonal antibody detectors produced a greater signal with small amounts of IgG than did SpA.

Figure 2.9. Linear binding of SpA to increasing PSAIg contrasted with nonlinear binding of anti-G (a) and anti-γ (anti-IgG) (b). In (a), error bars ($\pm$ S.D.) are smaller than data symbols where not shown. Data in (b) were based on duplicate determinations.


a
SpA
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b
SpA
anti-IgG
% bound
PC plasma (μl)

Figure 2.9

Coefficients

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Coefficients of variation

The assay CVs increased with radioactive decay of antiglobulin reagents because of counting error at low cpm. Therefore, the counting time was extended when decay was considerable. The CVs for SpA and anti-γ are shown in Table 2.4. The interassay CVs were calculated for 8 independent assays over a 2-month period using PC plasma. For SpA, CVs were calculated at each point of a standard curve. For anti-γ, only the highest concentration of PC plasma was used. The intra-assay CVs were determined for 8 concurrent repetitions of the PC standard curve (SpA) or highest concentration of PC (anti-γ). While the CV for anti-μ could not be determined for the PSAIg assay because there was no positive IgM control, triplicate determinations with plasma samples containing PBIgM were of similar reproducibility to the results with the anti-γ antibody.

Table 2.4. Interassay and intra-assay coefficients of variation (%) for SpA and anti-γ detectors.

	SpA			anti-γ
	high	low	mean	
interassay	8.6	4.2	6.6	5.0
intra-assay	4.1	1.6	2.4	4.1

Reference range

PSAIg reference ranges for 20 normal dogs were generated using platelets harvested from blood less than 3 hr old and maintained at 37°C. These results were (mean $\pm$ S.D.): for SpA, 0.5 $\pm$ 0.2; for anti-γ, 0.7 $\pm$ 0.2; and for anti-μ, 1.7 $\pm$ 0.6%. The cutoff for a positive result was selected as the mean plus 3 standard

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deviations. For fresh samples, these cutoffs were 1.1, 1.3, and 3.5 for SpA, anti-γ, and anti-μ, respectively. In contrast to these normal samples, there was sometimes a delay of up to about 30 min before clinical blood samples were placed at 37°C.

Clinical evaluation

The detectable PSAIg of positive samples from dogs with clinical IMT decreased dramatically within a day when washed test platelets were stored either at RT or at 4°C in assay buffer. Samples for PSAIg were therefore assayed within 3 hr of sample preparation.

The clinical results are tabulated in Tables 2.5 and 2.6. Many dogs were not tested for PSAIg until therapy had begun, so the platelet concentrations at presentation (initial) and on the assay date (assay) often differed. Except where noted, dogs with a diagnosis of IMT were treated with prednisone, usually at 1 mg/lb BID, and many dogs received concurrent antibiotics. In sporadic cases, vincristine, cyclosporine, cyclophosphamide, danazol, and azathioprine were additionally used.

Of the 24 dogs with clinical diagnoses of IMT, 23 (96%) had positive results for increased PSAIg (Table 2.5). The single dog that was negative (#24) was discharged within a day and lost to follow-up before response to therapy could be evaluated; its diagnosis was therefore uncertain. Of the 19 dogs given a diagnosis other than IMT (Table 2.6), 9 (47%) had positive results based strictly on the normal reference range. However, 4 of the 9 positive values were considered clinically insignificant (1.4, 1.5, 1.6, and 1.9% for dogs #9, 13, 12, and 14, respectively) and likely the result of slight delays in sample handling. Two of the remaining 5 dogs had diagnoses of idiopathic

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thrombocytopenia and neutropenia which may have been immune-mediated. In one of these dogs (#18), the platelet and neutrophil concentrations increased dramatically within 5 days after prednisone therapy was eventually instituted. The other dog (#17) was quickly discharged and lost to follow-up. The seventh dog (#19) was diagnosed with chronic renal failure. Its platelet concentrations persisted in the 45,000-70,000/μl range until prednisone therapy was instituted, after which they were 119,000/μl on day 6 and 318,000/μl on day 33. This dog also may have had IMT. The eighth of these 9 dogs (#16) had zinc-induced hemolytic anemia and developed thrombocytopenia transiently for 1 day following a transfusion of whole blood. The dog was positive for PSAIg while thrombocytopenic (27,000/μl), but was negative the next day when the platelet concentration had increased to 101,000/μl. This reaction is similar to the transient transfusion-induced thrombocytopenia occurring in dogs with antibodies to incompatible erythrocytes,[24,25] and it may involve immune adherence and transient increases in PSAIg. The last of these 9 dogs had pyometra and possible septicemia (#15), and appeared to have mild but real increases in PSAIgG and PSAIgM.

Table 2.5

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Table 2.5. Platelet surface-associated immunoglobulin (PSAIg) results for dogs diagnosed with immune-mediated thrombocytopenia.

		Platelet Concentration (/μl)		PSAIg Results (% bound)			
	Breed	Initial	Assay	SpA	anti-γ	anti-μ	Platelet Responses†
1.*	Belgian Sheepdog	7,000	7,000	58.2	24.6	10.5	47,800 on day 3
			47,800	33.0	11.2	6.6	104,000 on wk 3
			220,000	2.0	—	—	220,000 on day 47
2.	Brittany Spaniel	5,000	5,000	41.4	13.8	5.1	264,000 on day 9
3.*	Cocker Spaniel	9,000	9,000	47.0	—	—	109,000 on day 3
			109,000	6.1	11.6	2.7	226,000 on day 5
4.	Labrador Retriever	4,000	4,000	25.0	—	—	198,000 on day 8
	(relapse)	14,000	79,000	4.7	8.3	2.4	210,000 on day 4
5.*	Cocker Spaniel	37,000	65,000	34.7	22.0	5.5	126,000 on day 5
6.	Dalmatian	3,000	57,000	20.0	10.8	3.3	131,000 on day 5
7.*	Poodle-mix	23,000	263,000	22.1	22.6	1.9	263,000 on day 7
8.	Australian Shepherd	18,000	next day	23.2	26.6	2.0	162,000 on day 5
9.	Chesapeake Bay Retriever	5,000	66,000	8.0	19.8	6.0	127,000 on day 4
10.	Cocker-mix	8,000	8,000	27.9	19.4	4.1	5,000 on day 6
11.	Cocker Spaniel	7,000	7,000	1.9	—	—	39,000 on day 4
			39,000	—	8.6	2.5	sent home
12.	Cocker Spaniel	10,000	10,000	9.3	9.7	—	40,000 on day 9
13.	Cocker Spaniel	7,000	107,000	11.0	18.4	—	107,000 on day 4
14.**	Borzoi	5,000	5,000	21.8	2.2	—	160,000 on day 4
15.	Labrador Retriever	8,000	8,000	10.8	—	—	231,000 on day 4
16.	Cocker Spaniel	2,000	6,000	60.0	—	—	24,000 on day 3 sent home
17.*	Mix	22,000	22,000	10.6	—	—	76,000 on day 3
18.	Cocker Spaniel	15,000	9,000	25.0	—	—	206,000 on day 5
19.**	Dandie Dinmont	3,000	3,000	8.9	—	—	4,000 on day 4 elected euthanasia
20.	Cocker Spaniel	5,000	next day	14.0	—	—	48,000 on day 3
			285,000	0.4	—	—	285,000 on day 10
21.	Mix	33,000	87,000	29.6	—	—	87,000 on day 4
22.	German Shepherd	39,000	next day	21.2	—	—	109,000 on day 4
23.	Mix	53,000	next day	24.0	—	—	131,000 on day 3
24.*	Cocker Spaniel	7,000	7,000	0.2	—	—	sent home

* thrombocytopenia was an incidental finding with no clinical signs; ** possible systemic lupus erythematosus; † follow-up platelet concentrations as available; days from initial evaluation; positive values are those greater than 1.1% for SpA, 1.3% for anti-γ, and 3.5% for anti-μ

Table 2.6. Platelet surface-associated immunoglobulin (PSAIg) results for thrombocytopenic dogs not given a diagnosis of immune-mediated thrombocytopenia.

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Table 2.6. Platelet surface-associated immunoglobulin (PSAIg) results for thrombocytopenic dogs not given a diagnosis of immune-mediated thrombocytopenia.

	Breed	Diagnosis	Platelet Concentration (/μl) Initial	Assay	SpA	anti-γ	anti-μ	Outcome
1.	Mix	ehrlichiosis	80,000	38,500	0.8	0.4	0.3	unknown
2.	Pit Bull	DIC, nasal bleeding; suspect neoplasia	48,000	36,000	0.7	—	—	euthanasia
3.	Dalmatian	seizures, thrombocytopenia	123,000	109,000	0.8	0.9	1.6	no increase
4.	Miniature Schnauzer	pancytopenia with chemotherapy	35,000	35,000	0.6	—	—	41,000 on day 5 786,000 on day 36
5.	Golden Retriever	lameness, pyrexia, possible cyclic thrombocytopenia	187,000	187,000	0.8	2.2	1.2	250,000 on day 3, no therapy
6.	Miniature Schnauzer	CNS disease with seizures, splenomegaly	139,000	139,000	0.9	—	—	unknown
7.	Mix	bacterial endocarditis	34,000	next day	0.5	—	—	responded to antibiotics
8.	German Shepherd	splenic hemangiosarcoma	68,500	68,500	1.1	0.9	—	euthanasia at surgery
9.	Scottish Deerhound	pneumothorax from pulmonary bullae	120,000	120,000	1.4	—	—	130,000 at 1 wk; no response to prednisone

Table 2.6 (cont.)

	Platelet Concentration (/μl)

Table 2.6 (cont.)

	Breed	Diagnosis	Platelet Concentration (/μl) Initial	Assay	SpA	anti-γ	anti-μ	Outcome
10.	Mix	multiple myeloma 2 yr after IMT	83,000	next day	2.8*	—	—	pancytopenia persisted
11.	Welsh Corgi	pancytopenia with marrow neoplasia	11,000	11,000	3.4*	0.7	—	died on day 7
12.	Old English Sheepdog	unknown, possibly granulocytic leukemia	58,000	58,000	1.6	3.0	3.0	42,000–116,000 for 1 mo despite doxycycline and prednisone
13.	West Highland Terrier	idiopathic pancytopenia	98,000	112,000	1.5	2.8	3.5	271,000 on day 9
14.	Soft-Coated Wheaton Terrier	idiopathic pancytopenia	12,000	2 days later	1.9	—	—	119,000 on day 6 with antibiotics
15.	Boxer	pyometra, septicemia	102,000	103,000	3.2	5.5	6.9	surgery and no follow-up
16.	Dachshund	zinc-induced hemolytic anemia	178,000	27,000	5.7	18.3	6.1	transient thrombocytopenia for 1 day after transfusion
				101,000	0.2	0.6	—	

Table 2.6 (cont.)

Platelet Concentration (/μl)

Table 2.6 (cont.)

	Breed	Diagnosis	Platelet Concentration (/μl) Initial	Assay	SpA	anti-γ	anti-μ	Outcome
17.	Collie	idiopathic thrombocytopenia, neutropenia	127,000	201,000	21.1	24.3	1.2	unknown
18.	Australian Terrier	idiopathic thrombocytopenia, neutropenia	29,000	29,000	4.6	3.0	14.7	141,000 on day 5 after prednisone (neutrophils also responded)
19.	Mix	chronic renal failure	47,000	64,000	24.4	—	—	60,000 on day 12 of antibiotics; 119,000 on day 6 and 318,000 on day 33 after prednisone

* 24-hr-old samples; SpA mean ± S.D. for 10 normal dogs was 3.0 ± 1.6%; for other dogs, positive values are those greater than 1.1% for SpA, 1.3% for anti-γ, and 3.5% for anti-μ

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Of the 23 positive dogs with diagnoses of IMT, 9 were tested with SpA alone and 3 were tested with SpA and anti-γ. Of the 11 dogs tested with SpA, anti-γ, and anti-μ, 6 had increases in IgG and not IgM, while 5 had increased IgG and IgM, with IgG predominating. One dog with possible systemic lupus erythematosus (#14) had a strong positive with SpA and a weak positive with anti-γ, suggesting that the PSAIg was IgM or perhaps IgA. One dog (#11) with a weak positive result using SpA had a moderately strong positive result the next day with anti-γ, suggesting that the PSAIg may have been predominantly SpA-nonbindable IgG. For the few dogs tested serially (Table 2.5, dogs 1, 3, and 20), initial high values for PSAIg decreased as platelet concentrations increased in response to immunosuppressive therapy.

Of the 23 dogs with diagnoses of IMT and positive results, 9 (39%) were Cocker Spaniels. The female:male ratio of these 23 dogs was 13:10, and 11 of the females and 9 of the males were neutered. Ages at presentation ranged from 1.5 to 11.25 yr with a mean of 6.75 yr. For 5 of the 23 dogs (22%), thrombocytopenia was detected unexpectedly on routine blood exams for procedures including dentistry, cervical radiographs, and aural surgery. These dogs had no hemorrhage or other clinical signs of disease. Most dogs with IMT, however, had the typical range of hemorrhagic signs including mucosal and cutaneous petechiae and ecchymoses, gastrointestinal hemorrhage, ocular hemorrhage, epistaxis, and prolonged bleeding from venipuncture sites. IMT was diagnosed throughout the year with no obvious seasonality, though testing was not done such that a seasonal pattern could be definitively evaluated.

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Discussion

As stated by Christopoulos,[26] "It is logical to argue that the sensitivity and specificity of any new method for PSIgG [platelet surface IgG] detection should refer to its ability to reproducibility detect known amounts of IgG on the platelet surface rather than the compatibility of its results with the subjective clinical diagnosis of ITP [IMT] which, after all, is a heterogeneous entity and a diagnosis of exclusion." This is perhaps especially applicable to canine studies, because the contribution of PSAIg to the thrombocytopenia of many canine diseases is unknown. In these studies, we have developed and characterized a sensitive IRMA which can detect defined antibodies in a reproducible and linear fashion.

Linear dose-response curves for PSAIg and PBIg were possible with SpA because of its high binding affinity and constant binding ratio.[27,28] In contrast, two polyclonal antibodies to canine IgG bound increasingly to IgG as their concentrations increased. Increasing concentrations presumably shifted the binding equilibrium such that lower affinity binding sites on IgG progressively became occupied. The high concentrations of detectors required for constant detector-to-IgG binding ratios and linear binding curves were impractical for routine use in the IRMA. As used, these detectors could not produce a signal proportional to the amount of PSAIg present, and they were not useful for relative quantitation of PSAIg among samples.

The polyclonal detectors were useful, however, for defining the class of immunoglobulin present and for detecting any PSAIg or PBIg that was SpA-nonreactive. Under the conditions of this IRMA, SpA did not bind to about 20% of canine IgG or 33% of canine IgM.[29] The concurrent use of SpA, anti-γ, and anti-μ therefore allowed

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detection of all PSAIgG and PSAIgM, and provided immunoglobulin classification in addition to relative quantitation. SpA was very sensitive, detecting PBIg linearly with nanoliter to microliter volumes of plasma from immunized rabbits and from dogs or people (anti-PI^{A1}) with clinical IMT. When small volumes of PC plasma were assayed, the polyclonal detectors had greater sensitivity than SpA because of the amplified signals generated by their multivalent binding. However, nonspecific PSAIg would be amplified to the same degree as specific PSAIg, so the polyclonal detectors may be no better than SpA at detecting pathologic PSAIg and differentiating it from nonspecific immunoglobulin in clinical samples.

This IRMA avoided many of the problems associated with some previous human and canine assays for platelet-reactive antibodies. Importantly, the direct assay detected only surface immunoglobulin, not internalized immunoglobulin.[30-32] Similarly, because the indirect assay used whole platelets, nonpathologic plasma antibodies reactive with cytosolic epitopes were not detectable.[33-35] Nonspecific binding was minimized by sample handling procedures, assay temperature, and the measurement of only platelet-associated radioactivity without the radioactivity nonspecifically associated with the plastic. This low background binding combined with the chosen amounts of platelets and SpA allowed for the generation of strong signal-to-noise ratios. In contrast to some assays, the positive control for this IRMA was a perfect match for the target analyte of the assay: canine antiplatelet antibody in a citrated sample. EDTA was not used because of its association with greater nonspecific PSAIg,[36,37] and its potential to generate reactive neoepitopes.[38-40] Furthermore, EDTA may dissociate canine $\alpha_{IIb}\beta_3$ as it does the human complex,[41] thus removing important complex-dependent antigens.[42,43]

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A standard curve was incorporated into the assay so that the results could be corrected for any change in SpA reactivity, thus allowing for meaningful comparisons over time. This was accomplished by using platelets fixed in 0.01% glutaraldehyde. Fixed platelets had consistent and long-term reactivity, and they did not require thawing or counting with each use; they simply required resuspension. However, fixed platelets may not maintain reactivity to all antibodies, especially those such as some drug-induced antibodies which may require membrane perturbation for binding.[44] Fixed platelets may also cause false positive results for PBIg due to aldehyde-induced neoantigens.[39,45] Therefore, fresh platelets or isolated exosolic membrane proteins may be preferable for PBIg detection.

The principal problem we encountered with this IRMA related to increases in detectable PSAIg and PBIg, mostly IgM, in samples stored at room temperature or 4°C. PSAIg sometimes increased markedly within hours of whole blood storage at these temperatures, while PBIg values increased dramatically only when plasma was isolated before storage. Presumably, PBIg formed in whole blood but was removed from the plasma by associating with platelets and contributing to PSAIg. When clinical samples had significant increases in PSAIgM in addition to PSAIgG, it signaled the possibility of sample handling problems. Indeed, this occurred with old samples or samples that had been mistakenly refrigerated. Fresh samples optimized platelet yields and prevented these false positive results.

These studies extend the findings of Lewis[36] who used a SpA-based ELISA and showed that canine PSAIg increased with sample storage time. While the anticoagulant contributed to this effect,[36] the storage of samples on ice may have been of primary

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importance. In our studies, time-related increases in PSAIg were minimal for citrated blood stored at 37°C for 24 hr.

These temperature and storage studies emphasize the need for uniform[36] and timely handling of control and test samples when assaying for canine PSAIg or PBIg. Even with careful management, however, false positive results for PBIg may occur with fresh-frozen samples. Unpredictably and uncommonly, fresh-frozen plasma samples stored in multiple aliquots at -65°C from certain normal dogs converted to strong positives, reacting with all test platelets including autologous ones. This reactivity could be removed by filtration through 0.22 μm filters, but it was not further characterized.

Clinically, PSAIg was increased in 96% of 24 dogs with diagnoses of IMT, the single negative dog not having a well-supported diagnosis. Of 19 dogs with other diagnoses, clearly positive results for PSAIg occurred in 4 dogs that may reasonably have had an immune-mediated component to their thrombocytopenias, and in one dog with a pyometra and probable septicemia. PSAIg may be increased in human septicemic patients.[46-48] Given the uncertainties of the pathogenesis of thrombocytopenia in these 5 dogs, it is possible that significant increases in PSAIg occurred only in dogs with an immune-mediated component to their thrombocytopenia.

Increases in PSAIg are not specific for any one disease. PSAIg may be antiplatelet antibody, antibody bound specifically to adsorbed antigens, or immunoglobulin bound directly or indirectly to the platelet via immunologically nonspecific mechanisms. However, increased PSAIg suggests an immune-mediated acceleration of platelet destruction that may be associated with thrombocytopenia when platelet production has not increased enough to compensate for destruction. The detection of

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increased PSAIg may therefore be helpful in directing immunosuppressive therapy.

Continued use and critical evaluation of this and other assays will define their clinical utility for a variety of uses. The assessment of PSAIg may be helpful for: 1) supporting clinical diagnoses which have relied largely on apparent positive responses to immunosuppressive therapy and on the presumed elimination of other causes of thrombocytopenia; 2) confirming diagnoses of drug-induced IMT; 3) determining the contribution of PSAIg to the thrombocytopenia of other canine diseases; and 4) diagnosing and managing platelet incompatibility problems that may arise with the continued evolution of canine transfusion medicine. As this and other assays for canine PSAIg evolve, so too will our understanding of the interaction of immunoglobulins with canine platelets and the contributions of immune-mediated platelet destruction to thrombocytopenia in dogs. The studies reported here emphasize the importance of stringent sample management during the course of future investigations.

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CHAPTER 3

CANINE AGGREGATED IgG INDUCES COMPLEMENT-MEDIATED, NOT Fc-MEDIATED, CLUMPING OF CANINE PLATELETS

Abstract

The binding of immune complexes or IgG aggregates to human platelet FcγRII (CD32) can stimulate signal transduction and platelet activation. This appears to be important in human patients with heparin-induced thrombocytopenia. Canine platelets have not been characterized with respect to their responses to immunologic stimuli, and it is not known if canine platelets express Fcγ receptors. We used platelet aggregometry to compare the responses of human and canine platelets to heat-aggregated IgG (HAIgG). Canine and human HAIgG caused Fc-mediated aggregation of human gel-filtered platelets (GFP) that was blocked by the anti-FcγRII monoclonal antibody IV.3. In contrast, canine GFP were unresponsive to HAIgG. In platelet-rich plasma, however, HAIgG induced delayed and submaximal clumping of canine platelets that was not blocked by Fc fragments of canine IgG. This platelet clumping also occurred in citrated whole blood and GFP suspended in plasma or serum plus hirudin. Heating plasma or serum at 56°C for 5 min prevented clumping, as did 5 mM EDTA or 5 mM EGTA. Studies with complement-depleted plasma and canine plasma naturally deficient in C3 showed that the HAIgG-induced clumping of canine platelets required complement, and specifically C3.

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Similar clumping occurred with zymosan and bacterial lipopolysaccharide, other known activators of complement. This complement-mediated clumping of canine platelets may be an important contributor to thrombocytopenia in canine diseases associated with circulating immune complexes or other complement activators.

Introduction

Immune-mediated platelet destruction with subsequent thrombocytopenia is believed to be mediated by increased phagocytosis of antibody-coated platelets. In human patients, immunoglobulins can be associated with the platelet surface through immunologically specific, $F(ab')_2$, or immunologically nonspecific interactions. In dogs, only immunologically specific binding has been documented.[1,2] The potential for nonspecific interactions of immunoglobulins with canine platelets, and the mechanisms involved, are unknown.

Immunologically nonspecific binding of immunoglobulins to platelets includes Fc-mediated binding in species with platelet Fc receptors. Human platelets and megakaryocytes express Fc receptors for IgG, FcγRII (CD32), which are glycoproteins belonging to the immunoglobulin gene superfamily.[3-6] Platelets of pigs, sheep, goats, cattle, and nonhuman primates also reportedly express Fc receptors, while platelets of rabbits, dogs, mice, and horses reportedly do not.[3,7] However, data to support these reports are generally unavailable except in the case of rabbits which have been clearly shown to lack FcγRII analogues.[8] Data to confirm the presence or absence of Fcγ receptors on canine platelets are lacking.[9]

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The Fc receptors on human platelets bind the Fc portion of monomeric IgG with low affinity and that of complexed IgG with higher affinity.[3,10] The affinity for naturally occurring immune complexes (ICs) is unknown, but heat or chemically aggregated IgG bind well and have been used widely in vitro as substitutes for true ICs to investigate Fc-mediated platelet interactions. Despite some functional polymorphism of human platelet FcγRII, the Fc-mediated binding of aggregated IgG, ICs, or aggregated Fc fragments to FcγRII consistently causes platelet aggregation and granule release which are blockable with monomeric IgG, Fc fragments, and monoclonal antibodies to FcγRII.[3,11] This Fc-mediated platelet activation appears to result from signal transduction via G protein-mediated activation of phospholipase C.[12]

The in vivo consequences of Fc-mediated IC binding are perhaps best described for human patients with heparin-induced thrombocytopenia (HIT). In HIT, immunoglobulins interact with heparin and platelet factor 4 to form ICs which bind to platelet Fc receptors.[13] The consequences of Fc binding include accelerated platelet phagocytosis and platelet activation leading to accelerated platelet consumption, the generation of platelet microparticles,[14] and the development in some patients of platelet-rich thrombi. Fc-bound circulating ICs from infectious, neoplastic, autoimmune, and other drug-induced diseases may similarly contribute to thrombocytopenia. However, the potential for Fc-mediated thrombocytopenias in dogs is dependent upon whether or not canine platelets express Fc receptors.

We have used HAIgG and platelet aggregometry to assess canine platelets for a functional response to HAIgG analogous to the FcγRII-mediated response of human platelets. Our investigations show that canine platelets are responsive to nonspecific

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immunologic stimulation in the form of HAIgG. However, the mechanism of interaction between HAIgG and canine platelets is markedly different from the Fc-mediated stimulation of human platelets by HAIgG.

Methods

Materials

Collagen, adenosine diphosphate (ADP), phorbol 12-myristate 13 acetate (PMA), calcium ionophore A23187, platelet activating factor (PAF), lipopolysaccharide (from *Escherichia coli*, serotype 0128:B12), prostaglandin E1 (PgE_1), and papain were purchased from Sigma Chemical Company (St. Louis, MO). The monoclonal antibody IV.3 was purchased from Medarex, Inc. (West Lebanon, NH).

Recombinant hirudin (Behring; Marburg, Germany) was a generous gift of Dr. Robert Roth. Stock hirudin at 40 U/μl was added to serum to give a final concentration of 300 U/ml after GFP were added. Whenever serum was used with less than 60 min heating at 56°C, hirudin was added to inhibit the effects of thrombin.

C3-deficient and control canine plasma were generously provided by Dr. Jerry A. Winklestein at The Johns Hopkins University School of Medicine.[15-17] Functional C3-like activity exists in affected dogs at less than 3-10% that of control dogs,[15,17] and antigenically detectable C3 concentrations are even more deficient.[15,16]

Zymosan was prepared by boiling 1 gram zymosan A (Sigma) per 200 ml water for 30 min, decanting, and bringing to 6 mg/ml in phosphate buffered saline (PBS). Cobra venom factor (CVF) from Naja naja (Calbiochem-Novabiochem Corporation, La Jolla, CA) contained 1.25 U/μl. For complement depletion, CVF was incubated in

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plasma (7.5 U/ml) for 1 hr at 37°C and then at room temperature for another hour. When zymosan was used for complement depletion, plasma was incubated with 6 mg/ml zymosan, twice, for 30 minutes each time at 37°C. After pelleting the zymosan, the plasma was kept at room temperature for at least an hour until being used. Results were compared directly to plasma processed concurrently and identically except without activators. All other chemicals were reagent grade.

Dogs

The blood donor dogs were maintained by the University Laboratory Animal Resources facility which follows USDA and NIH guidelines for animal use and care. Approval for use was obtained from the All University Committee on Animal Use and Care. Preconditioning after purchase included physical examination, deworming, heartworm testing and subsequent prophylaxis, routine vaccinations, dipping for ectoparasites, and observation for a minimum of 30 days before use.

Blood collection and platelet isolation

Blood was drawn by jugular venipuncture through 18 gauge needles into plastic syringes containing 1/10 volume citrate to give a final citrate concentration of 10.5 mM (0.32%). Traumatic attempts were generally aborted and discarded. Platelet-rich plasma (PRP) was harvested from whole citrated blood by differential centrifugation within 15 min of blood collection. PRP was pooled from each of 2 or 3 collections after serial centrifugations for 2-4 min at 1300 x g. Gel-filtered platelets (GFP) were obtained by filtering 5 ml of PRP through a 50 ml bed volume of Sepharose CL-2B (Pharmacia

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Biotech, Inc., Piscataway, NJ) equilibrated with calcium-free Tyrode's buffer (140 mM NaCl, 5.6 mM glucose, 6 mM $NaHCO_3$, 2.7 mM KCl, 3.6 mM NaH_2PO_4, and 0.35% (w/v) bovine serum albumin (BSA), pH 7.2). The first 5 ml of platelet-rich effluent were collected and recalcified to 1 mM calcium with 5 μl of 1 M $CaCl_2$.

IgG

Human IgG (Cohn fraction II) was a gift from the Michigan Department of Public Health. Canine IgG was purified from normal citrated plasma by staphylococcal protein A-agarose (Sigma) affinity chromatography followed by exclusion chromatography with Sephacryl-300 HR (Pharmacia) to remove contaminating immunoglobulins. Only fractions in the peak corresponding to monomeric IgG were pooled. The pooled fractions were dialyzed against water, lyophylized, and resuspended in PBS, pH 7.2. The final product was assessed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions using Coomassie Blue to visualize the bands. Only bands for IgG were present. IgG was alternatively purified by ammonium sulfate precipitation followed by diethylaminoethyl Sephadex ion exchange chromatography (Pharmacia) and finally Sepharose G-200 (Pharmacia) exlusion chromatography to remove contaminating IgM and albumin. The purification method did not alter the outcome of the studies.

Fc and Fab purification

Fc and Fab fragments were prepared by papain digestion of canine IgG in digestion buffer (0.1 M phosphate, 10 mM cysteine, and 2 mM EDTA, pH 7.0). The

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IgG was purified by the staphylococcal protein A (SpA) method and was therefore all SpA-bindable. Papain was added at a ratio of 1 mg papain to 100 mg IgG. After a 4 hr incubation at 37°C, the incubation mixture was placed on ice. The digestion buffer was exchanged for PBS, pH 7.2, by repeated dilutional centrifugations in Centriprep-10 concentrators (Amicon, Beverly, MA), and then by dialysis in PBS overnight at 4°C. Undigested IgG was removed by Sephacryl 300 exclusion chromatography, and SpA affinity chromatography was used to separate the Fcs (SpA-bindable) from the Fabs (SpA-nonbindable). SDS-PAGE confirmed removal of whole IgG from the Fcs and Fabs which each had apparent molecular masses of about 50 kD.

Heat aggregation

IgG was aggregated by heating 50 to 100 μl aliquots of 5 to 84 mg/ml solutions for 30 to 60 min at 56°C in a water bath. For some studies, samples were heated at 65°C for 5-10 min to produce larger, visible, and easily pelleted aggregates.

Aggregometry

Optical aggregometry was done in a Chrono-log lumiaggregometer at 37°C with stirring at 900 rpm. Because the effect of HAIgG was similar at all platelet concentrations tested, platelet concentrations of PRP and GFP were not adjusted to a constant. Consequently, comparisons of tracings were made only within single aggregometry sessions.

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Lactate dehydrogenase

ADP, calcium ionophore, and HAIgG were added to PRP to assess lactate dehydrogenase (LDH) release from stimulated platelets. After 15 or 60 min, platelets were pelleted by centrifugation and 100 μl of supernatant were tested by kinetic assay (Sigma) in a Perkin Elmer spectrophotometer at 340 nm. LDH release was reported as a percentage of the LDH released from platelets lysed by Triton X-100. Test results were also compared to controls using PRP treated similarly but without the addition of agonists; this assessed background plasma LDH and any LDH released from platelets with stirring over time. Data are means of duplicate determinations.

Particle counting

For analysis of platelet clumping in 37°C whole blood, HAIgG-treated and PBS-treated control samples from each of 2 dogs were analysed in pairs on a Technicon H-1 hematology analyzer (Miles, Inc., Tarrytown, PA). Data were collected before addition of HAIgG or PBS, and at 15 and 60 min after their addition. Blood smears were made at each time and evaluated to confirm platelet clumping.

Results

Human HAIgG caused full-scale aggregation of human GFP (Figure 3.1a). Aggregation was consistently blocked with plasma or by preincubating the platelets with IV.3, a monoclonal antibody to the human platelet FcγRII (Figure 3.1a). Similarly, canine HAIgG (K9HAIgG) was capable of fully aggregating human GFP (Figure 3.1a),

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Addition of human HAIgG to human PRP caused no detectable aggregation. In contrast, the addition of human or canine HAIgG to canine citrated PRP caused a delayed and submaximal clumping of platelets without an initial decrease in light transmittance that is associated with shape change (Figure 3.1c). Unheated monomeric canine IgG did not induce platelet clumping (Figure 3.1c). K9HAIgG produced at temperatures greater than 56°C, such that IgG aggregates were precipitable and visible, induced clumping in PRP more rapidly and to a greater (but still submaximal) extent than did the same amount of HAIgG produced at 56°C. Increasing concentrations of HAIgG shortened the lag time, increased the rate of aggregation (slope), and increased the amplitude of maximal light transmittance (Figure 3.1c). However, aggregations remained submaximal, corresponding approximately to the amplitude of primary reversible aggregation induced by threshold concentrations of ADP (Figure 3.1d). Macroscopically, the clumping also appeared incomplete in that there were many fine platelet aggregates as opposed to the fewer, larger aggregates seen after maximal aggregation by such platelet agonists as ADP, collagen, and PAF (Figure 3.2).

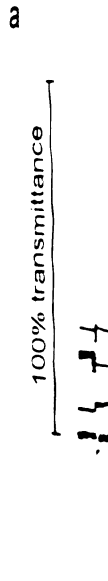
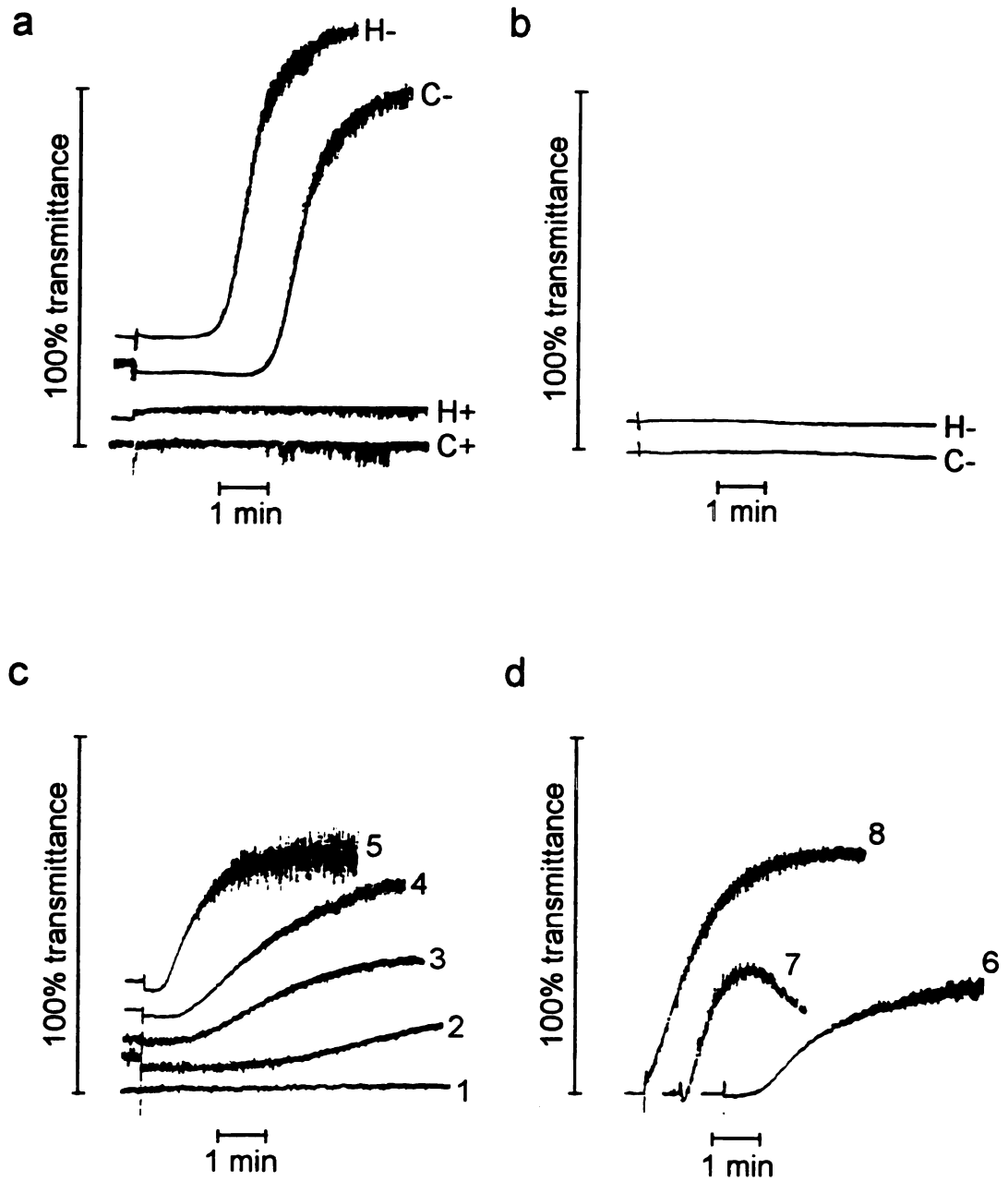


Figure 3.1. Aggregometry curves using: a) human GFP with human (H) and canine (C) HAIgG plus (+) and minus (-) monoclonal antibody IV.3, b) canine GFP with human and canine HAIgG, and c) canine PRP with canine HAIgG. Unheated monomeric IgG (1) is compared to increasing concentrations of HAIgG (32 μg/ml (2), 64 μg/ml (3), 128 μg/ml (4)) and to 128 μg/ml HAIgG formed at 59°C (5). In (d), the maximum light transmittance with HAIgG (6) in PRP is compared to that of reversible (7) and irreversible (8) ADP aggregations.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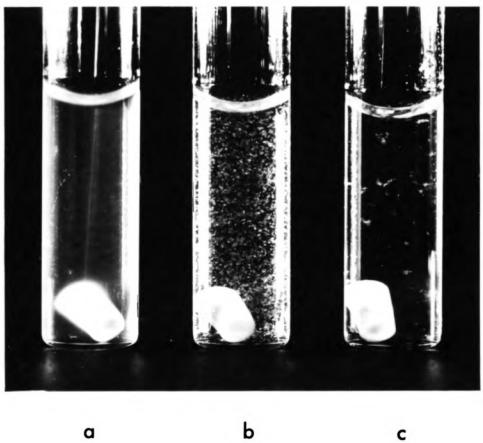

Figure 3.2. Photographs of aggregometry cuvettes containing a) unstimulated platelets, b) numerous small clumps of platelets corresponding to the maximal response to 0.4 mg/ml HAIgG, and c) fewer large clumps of platelets corresponding to full-scale aggregation after stimulation with 2.5 μg/ml collagen.

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Since the monoclonal antibody IV.3 did not bind to canine platelets (data not shown), blocking experiments with it were not indicated. Instead, potential Fc-mediation was evaluated by preincubation of PRP with concentrations of K9Fcs and K9HAFcs equimolar to those of the added K9HAIgG. Canine Fcs and HAFcs did not inhibit HAIgG-induced platelet clumping.

The findings described for PRP also occurred when K9HAIgG was added to canine 300 μl GFP in the presence but not in the absence of 100 μl canine citrated plasma, heparinized plasma, or hirudin-serum (Figure 3.3). Hirudin was added to serum to inhibit the aggregatory effects of residual thrombin; we found that even when serum was heated for 45 min at 56°C, 100 μl of serum added to 300 μl GFP caused delayed hirudin-inhibitable, and therefore thrombin-mediated, aggregation. The addition of 5 mM EDTA or 5 mM EGTA prevented HAIgG-induced platelet clumping in this system, while calcium replenishment (5 mM) again supported it. When HAIgG and plasma or serum were preincubated before addition to GFP, the lag time to platelet clumping was eliminated (Figure 3.3). This indicated that the lag time was related to an interaction of HAIgG with plasma rather than with platelets, and suggested the possibility of an enzymatic process involving plasma proteins.

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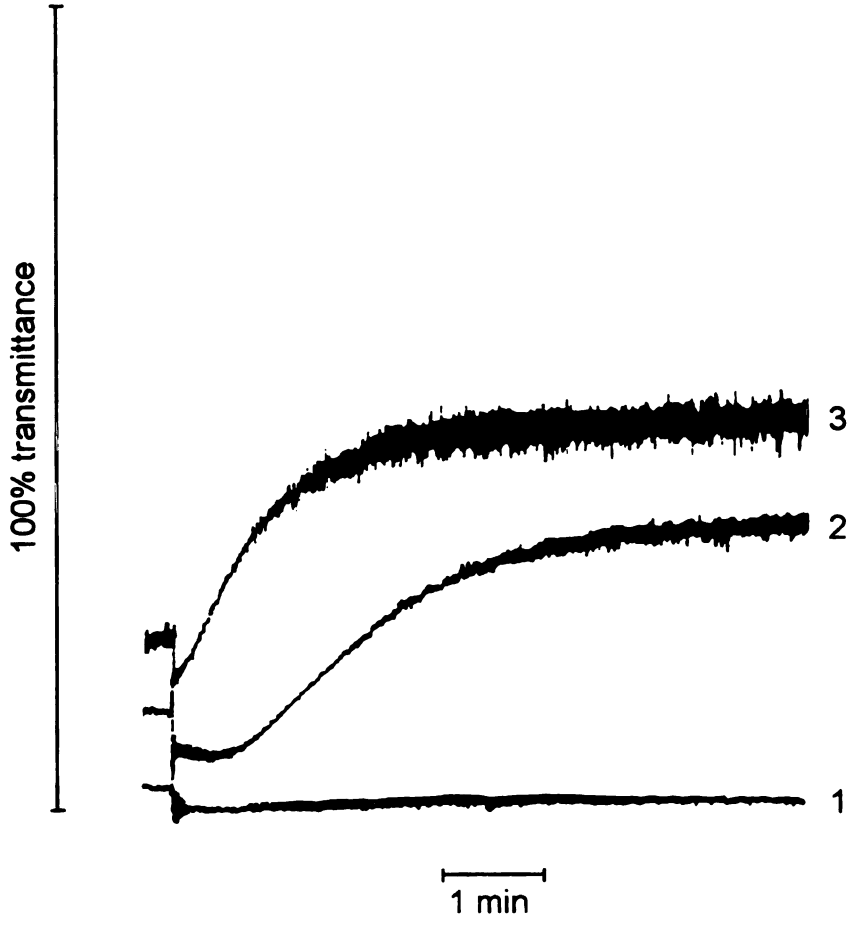

Figure 3.3. Aggregometry curves for canine GFP (300 μl) incubated with HAIgG in the presence of 100 μl citrated plasma (2,3) or Tyrode's buffer (1). Tracings indistinguishable from curve 2 were generated with heparinized plasma and serum plus hirudin. For curve 3, HAIgG and plasma were incubated for 10 min before addition to GFP.

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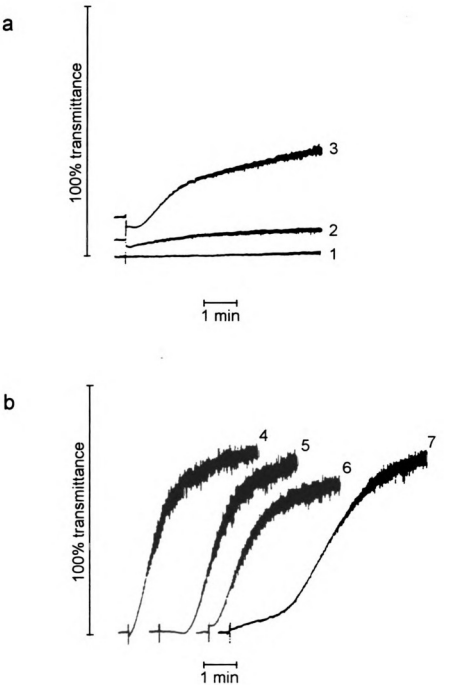
Heat lability of the cofactor(s) was shown by the prevention of clumping when the added serum or plasma was first heated for 5 or 30 min at 56°C (Figure 3.4a). Heated serum treated with hirudin did not inhibit aggregations induced by ADP, collagen, PAF, or PMA (Figure 3.4b). Clumping was also markedly but not completely inhibited by heating plasma for 20 min at 50°C (Figure 3.4a), a process known to inactivate the alternative complement pathway.[18]

Figure 3.4. Effects of heating serum (56°C, 5 min) or plasma (50°C, 20 min) on their support of HAIgG-induced clumping of GFP. HAIgG (a) or platelet agonists (b) were added to 300 μl GFP and 100 μl heated serum plus hirudin (1,4-7), heated plasma (2), or unheated serum (3). Platelet agonists were PAF (4), collagen (5), ADP (6), and PMA (7).



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Evidence that HAIgG-induced clumping was complement-mediated and not Fc-mediated was that zymosan and bacterial lipopolysaccharide (LPS), other known complement activators,[19,20] produced plasma/serum-dependent platelet clumping with the same characteristic delay and submaximal aggregation (Figure 3.5). The effects of LPS and zymosan were also prevented by use of plasma or serum (with hirudin) heated for 5 min at 56°C (Figure 3.5).

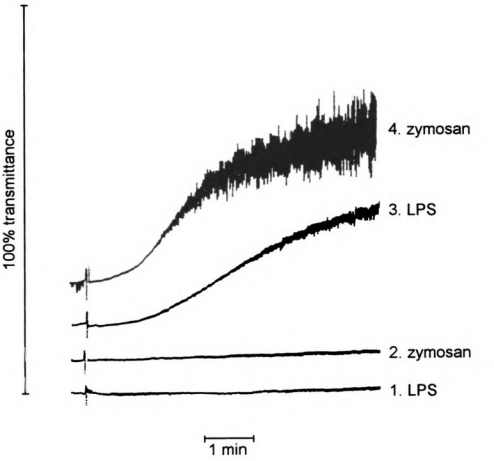

Figure 3.5. Aggregometry curves generated with 0.7 mg/ml zymosan (2,4) or 25 μg/ml LPS (1,3) in 300 μl GFP with 100 μl serum plus hirudin. Serum was either unheated (3,4) or heated (1,2) at 56°C for 5 min.

To specifically investigate the participation of complement in the support of HAIgG-induced platelet clumping, plasma was pretreated with zymosan to activate complement and effectively deplete it from the plasma. The zymosan was then pelleted by centrifugation before the treated plasma was added to GFP. As compared to control plasma, the capacity of zymosan-treated plasma to support platelet clumping by HAIgG was markedly reduced or absent (Figure 3.6a). Collagen and ADP aggregations were unaffected.

CVF was also used to deplete plasma of complement, and the effects were similar; plasma depleted of complement by CVF did not support HAIgG-induced clumping (Figure 3.6b). In contrast, CVF caused mild or no inhibition of aggregations induced by calcium ionophore and PAF (Figure 3.6b). However, CVF's effects were not specific as it also inhibited aggregation induced independently by collagen and ADP (Figure 3.7).

Figure 3.6. Effects of complement depletion by zymosan (a) and cobra venom factor (b) on aggregometry with HAIgG and control agonists added to 300 μl GFP plus 100 μl complement-depleted (1,3,4,5,7,8) or control plasma with complement activity (2,6). Effects of HAIgG in complement-depleted plasma (1,5) were compared to its effects on control plasma (2,6). Control agonists were collagen (3), ADP (4), PAF (7), and calcium ionophore (8).

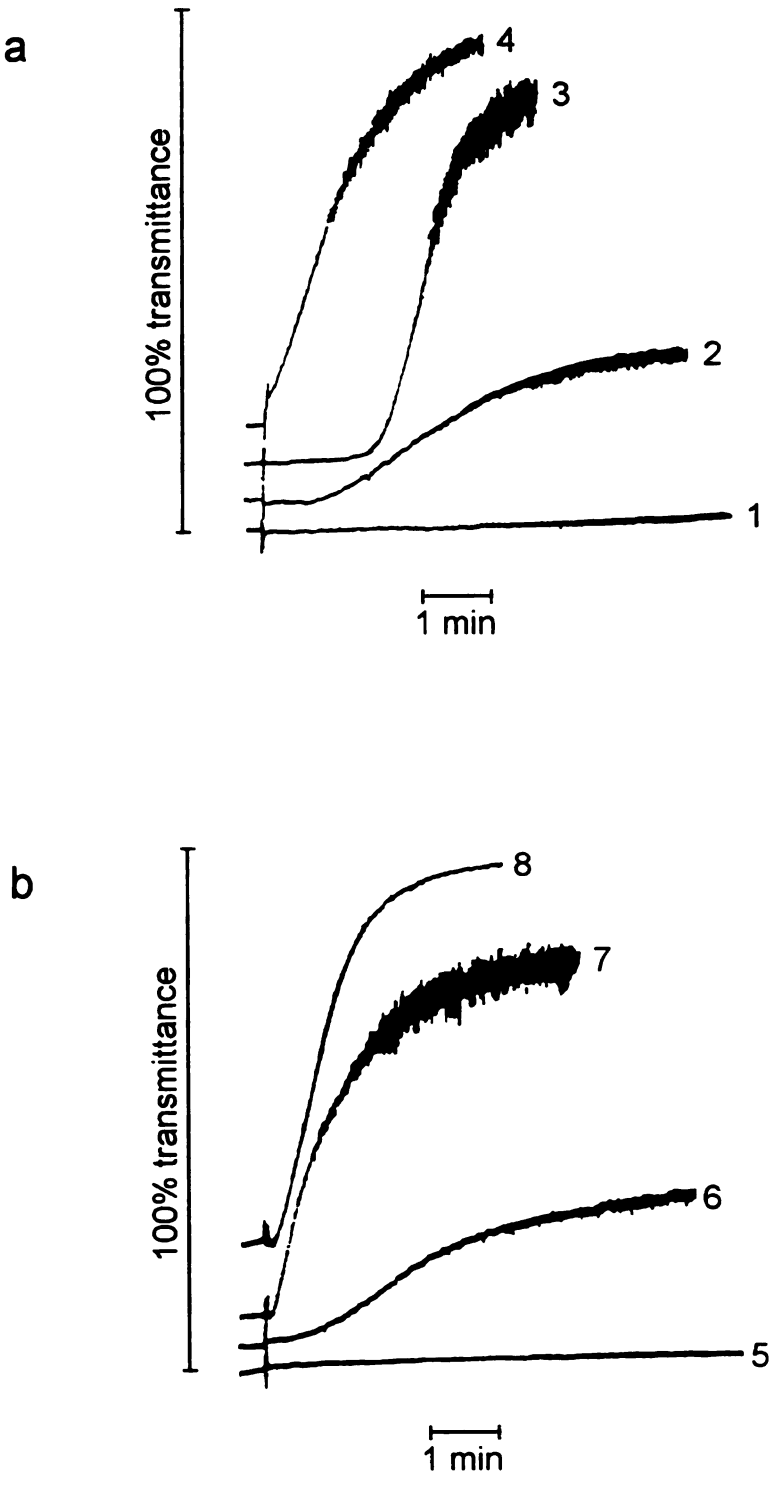
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Figure 3.6

The effects of CVF on ADP aggregation were evaluated by altering the order of addition of CVF and ADP to platelets. When platelets were exposed to CVF-treated plasma before the addition of ADP, inhibition was greater than when platelets were exposed to CVF-treated plasma and ADP simultaneously by adding GFP to a mixture of CVF-treated plasma and ADP (Figure 3.7a). This suggested an effect of CVF or CVF-treated plasma on the platelets. A plasma requirement for the inhibition of collagen-induced aggregation was shown by substituting Tyrode's buffer for plasma; CVF did not inhibit collagen-induced aggregation in the absence of plasma, but it delayed aggregation in the presence of plasma, even without a preincubation period (Figure 3.7b, curve 6). Inhibition was complete when plasma was incubated with CVF for 75 min at 37°C (Figure 3.7b, curves 4 and 5).

Figure 3.7. Effects of cobra venom factor (CVF) on aggregations induced by ADP (a) and collagen (b) using 300 μl GFP and 100 μl CVF-treated plasma, control plasma, or Tyrode's buffer. In (a), ADP (20 μM final) was added to GFP plus CVF-treated plasma (1), to GFP plus control plasma (3), or to GFP simultaneously with CVF-treated plasma (2). In (b), collagen (5 μg/ml final) was added to GFP plus plasma incubated with CVF for 75 min at 37°C (4), to GFP plus CVF-treated Tyrode's buffer (7), to GFP plus Tyrode's buffer (8), to GFP plus untreated plasma (9), to GFP simultaneously with CVF-treated plasma (5), and to GFP with unheated plasma immediately after the addition of CVF, thus avoiding preincubation of CVF with plasma (6).

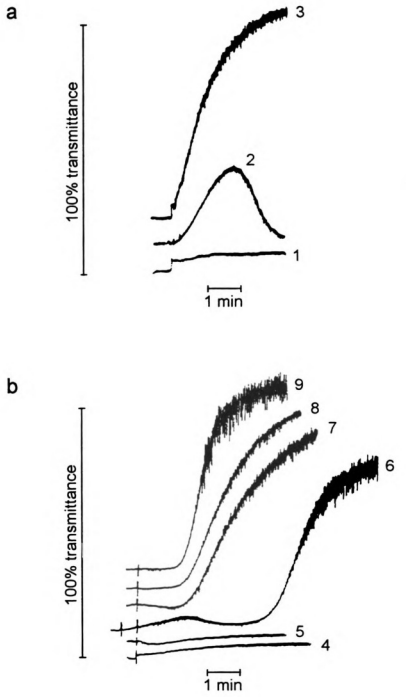
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Figure 3.7

Further evidence of a complement requirement came from use of canine C3-deficient plasma. Canine plasma deficient in C3 did not support clumping by K9HAIgG, but control plasma handled under identical conditions did (Figure 3.8).

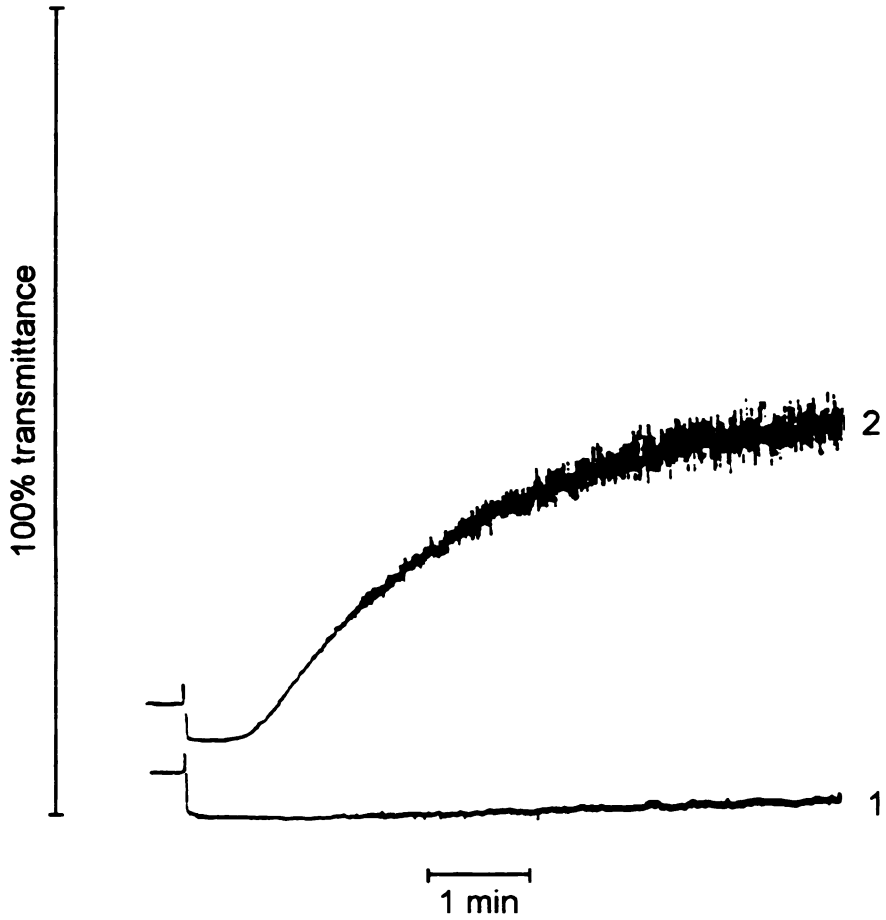


Figure 3.8. Aggregation curves for HAIgG added to 300 μl GFP plus 100 μl C3-deficient (1) or normal control (2) plasma. Results with a second pair of C3-deficient and normal dogs were the same.

The addition of 100 μg of K9HAFabs to PRP induced delayed and relatively low amplitude platelet clumping, perhaps limited by the size and number of aggregates that could form by heating a relatively dilute solution (4 mg/ml). HAFabs also accelerated and mildly amplified the platelet response to HAIgG. K9HAFcs, however, produced very delayed and very mild platelet clumping.

Because complement played a pivotal role in HAIgG-induced platelet clumping, platelet lysis was assessed by measuring LDH concentrations in platelet suspensions. By 15 min, calcium ionophore induced the release of detectable LDH (Table 3.1), while ADP and HAIgG did not. LDH was not elevated above negative controls in the supernatants of clumped platelets even 60 min after the addition of HAIgG.

Table 3.1. Lactate dehydrogenase released from platelets during aggregometry.

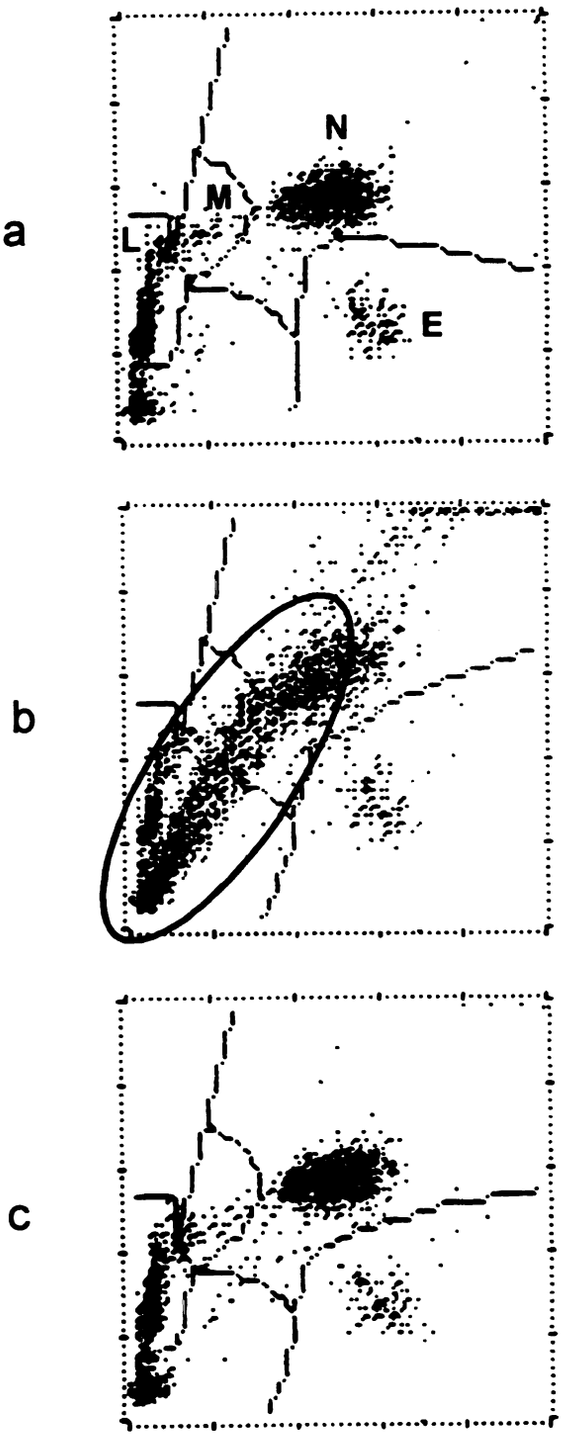
Treatment	Release[1]
no agonist	0.3
ADP (20 mM)	0.4
calcium ionophore (40 mM)	2.6
HAIgG (0.22 mg/ml)	0.3
no agonist (60 min)	0.7
HAIgG (60 min)	0.7

[1]Values are the % of LDH release compared to the supernatant of platelets lysed with Triton X-100.

The effects of HAIgG were also evaluated in citrated whole canine blood, a somewhat more physiologic system than the PRP and GFP systems. Fifteen minutes

after HAIgG or PBS were added to citrated blood, platelet clumps were detected by an automated blood analyzer (Technicon H-1), and apparent platelet concentrations were 173,000/μl and 239,000/μl in the control samples from two dogs, but only 15,000/μl and 17,000/μl in the HAIgG-treated samples (Figure 3.9). By 1 hr, the apparent platelet concentration had increased to 102,000/μl and 59,000/μl. Platelet clumping was marked on blood smears from HAIgG-treated samples but not on control smears.

Figure 3.9. Technicon H-1 analysis of cells in citrated whole blood from 1 of 2 test dogs before (a) and 15 min after the addition of 0.25 mg/ml HAIgG (b) or matching volumes of PBS (c). The apparent platelet concentrations of each sample for dog 1 (D1) and dog 2 (D2) are recorded next to the corresponding representative cytogram. An ellipse surrounds the region where platelet clumps are recorded. N=neutrophils, E=eosinophils, M=monocytes, L=lymphocytes.



Apparent platelet concentrations (/μl)

D1: 264,000
D2: 196,000

D1: 17,000
D2: 15,000

D1: 239,000
D2: 173,000

Figure 3.9

Discussion

These studies show that canine platelets lack functional Fcγ receptors analogous to the human FcγRII which mediate aggregation induced by HAIgG. This species difference was established by the failure of canine GFP to aggregate in response to canine or human HAIgG in the absence of plasma. In contrast, human GFP did aggregate in response to canine and human HAIgG, and this aggregation was prevented by plasma and by the Fc-reactive monoclonal antibody IV.3. This species difference is important for understanding the in vitro and in vivo responses of canine platelets to immunologic stimuli.

Although HAIgG did not induce Fc-mediated aggregation of canine GFP in Ca-Tyrode's buffer, canine GFP did clump when exposed to HAIgG in the presence of plasma or serum. HAIgG also induced marked clumping of platelets in citrated whole blood, suggesting that the results may be applicable to whole blood and in vivo conditions. Because this plasma/serum-dependent clumping was not inhibitable by preincubation with aggregated or unaggregated Fc fragments of canine IgG, it did not appear to be Fc-mediated. Instead, this HAIgG-induced clumping was mediated by the canine complement system.

A complement requirement for HAIgG-induced clumping of canine platelets was evidenced by: 1) canine plasma naturally deficient in C3 did not support HAIgG-induced platelet clumping, 2) plasma depleted of complement by preincubation with CVF or zymosan did not support HAIgG-induced platelet clumping, 3) known complement activators, LPS and zymosan, induced similar clumping, 4) clumping was prevented by heating plasma or serum at 56°C for 5 min, and 5) clumping required divalent cations.

This complement dependency explains the plasma or serum requirement and the delay that always occurred before clumping. As expected, the delay was eliminated by preincubating HAIgG with plasma to activate complement before the plasma was added to GFP.

HAIgG may activate either the classical or the alternative complement pathways, but the data suggest that the alternative complement pathway was of primary importance in HAIgG-induced platelet clumping. HAIgG-induced platelet clumping was markedly inhibited by heating plasma at 50°C for 20 min, a treatment that removes alternative complement pathway activity from canine serum while preserving classical pathway activity.[18] We confirmed that this heat treatment inactivated the canine alternative pathway by showing that it prevented the lysis of rabbit erythrocytes by canine serum (data not shown). We also confirmed that the lysis of rabbit erythrocytes by canine serum was mediated by the alternative pathway by showing that it was prevented by 5 mM EDTA but not by 5 mM EGTA (data not shown).[21] Therefore, the marked but incomplete inhibition of platelet clumping after plasma was heated at 50°C for 20 min suggests that the alternative complement pathway was of principal importance but that the classical pathway also contributed to platelet clumping.

Consistent with a primary role for the alternative pathway were the findings that K9HAFabs were good inducers of platelet clumping while K9HAFcs were at best only weakly active. Fc-mediated complement activation occurs by the classical pathway in human systems, while activation of the alternative pathway by human IgG is Fab-mediated.[22,23] If the same is true for canine IgG, the poor response to Fc fragments suggests that activation of the classical pathway may be inefficient at inducing platelet

clumping without the amplification provided by an intact alternative pathway. However, it is also possible that Fab fragments have a required role in platelet clumping in addition to activating complement.

Because C3 is pivotal for both the alternative and classical complement pathways, lack of platelet clumping in the presence of C3-deficient plasma did not differentiate the activity of one pathway from that of the other. However, it clearly established that C3 was required for the effect. The loss of activity after plasma was depleted of alternative and common pathway factors by treatment with the alternative pathway activator CVF suggests that the classical pathway components C1, C4, and C2 were not sufficient to induce platelet clumping.[24,25] It is possible, however, that CVF treatment inhibited HAIgG-induced platelet clumping because of effects other than complement depletion.

Nonspecific inhibitory activity of CVF on canine platelet aggregometry has been noted previously.[26] The inhibition of collagen-induced aggregation in dogs was thought to relate to a complement requirement. However, collagen aggregated canine GFP in the absence of plasma. Instead, the data suggest that CVF convertases formed in the presence of plasma may have promiscuous enzymatic activity that affects ADP and collagen receptors or activation pathways. Any inhibitory effects of CVF on canine platelet aggregation should be interpreted cautiously.

Because complement activation was responsible for HAIgG-induced platelet clumping, we assessed whether or not LDH leakage occurred as a result of platelet lysis from surface assembly of complement proteins. The lack of detectable LDH leakage for up to an hour after HAIgG was added to PRP indicates that significant lysis did not occur in this system. This differs from the response of rabbit platelets to ICs which includes

aggregation, granule release, and lysis.[27] The submaximal aggregometry tracings and lack of detectable platelet lysis with canine platelets indicate species differences with respect to complement-mediated platelet effects.

The full in vivo and in vitro significance of complement-mediated platelet clumping in dogs remains to be elucidated. In vitro, the findings may help explain the mild to moderate rises in aggregation tracing baselines sometimes occurring during aggregometry with canine PRP. We found that the addition of citrated plasma to GFP sometimes induced platelet clumping with the same features as that induced by HAIgG. This was usually mild and most notable with traumatic venipunctures. Heating the plasma to inactivate complement prevented the effect. The addition of serum (with hirudin), especially hemolyzed serum, caused a more pronounced platelet clumping. This effect was also prevented by heating the serum for 5 min at 56°C, as well as by complement depletion with zymosan. The greater and more consistent effect seen with serum is expected in light of the fact that complement can be activated by plasmin, thrombin, kallikrein, and Hageman factor.[28] We suggest that complement activation in citrated samples is responsible for the mild platelet clumping sometimes occurring in untreated canine PRP samples. The degree of this effect may vary with the dog, the citrate concentration used and therefore also the dog's hematocrit, general sample handling, and perhaps most importantly, the quality of the venipuncture.

The in vivo significance of complement-mediated, HAIgG-induced clumping of canine platelets is currently unknown. However, the fact that platelet clumping occurred in whole blood suggests that HAIgG, ICs, and other complement activators may affect platelet function or kinetics in vivo. Further studies are required to determine if

complement-induced platelet clumping contributes significantly to shortened platelet survival and thrombocytopenia in neoplastic, infectious, autoimmune, or drug-induced canine diseases. Whereas functional Fcγ receptors are important in the pathogenesis of such human diseases as heparin-induced thrombocytopenia, complement activation may be more important in the pathophysiology of canine diseases associated with thrombocytopenia.

In summary, we have shown that: 1) canine platelets lack functional Fc receptors analogous to those on human platelets, and 2) HAIgG-induced submaximal clumping of canine platelets requires complement, and specifically C3. However, these studies did not dissect the mechanism by which HAIgG and complement clump canine platelets. The mechanism of this effect is addressed in an accompanying manuscript.

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CHAPTER 4

AGGREGATED IgG INDUCES AGGLUTINATION OF CANINE PLATELETS BY IMMUNE ADHERENCE

Abstract

In previous studies, we showed that heat-aggregated IgG (HAIgG) caused canine platelets to clump in the presence of plasma containing active complement and C3. In the studies reported here, we characterize the mechanism of HAIgG-induced clumping of canine platelets. Platelet metabolism was not required, as evidenced by: 1) the HAIgG-induced agglutination of glutaraldehyde-fixed platelets suspended in plasma, and 2) the failure of prostaglandin E_1, sphingosine, or a combination of antimycin A and 2-deoxy-D-glucose to prevent clumping. RGDS peptides were also not inhibitory, indicating that fibrinogen binding to the platelet integrin $\alpha_{IIb}\beta_3$ was not required. By transmission electron microscopy, clumped platelets appeared unactivated, and they had surface-associated HAIgG that sometimes appeared to form interplatelet bridges. HAIgG was not platelet-associated when complement was heat-inactivated. When insoluble HAIgG was incubated with plasma and then washed, an increased amount of HAIgG-associated C3 was detectable using a ^{125}I-anti-C3 antibody. These washed C3-HAIgG aggregates caused platelet clumping in the absence of plasma. C3-HAIgG did not induce clumping after incubation with an anti-C3 antibody, indicating that C3 mediated the

platelet-HAIgG interaction necessary for agglutination. Therefore, HAIgG caused passive agglutination of canine platelets by immune adherence (IA). Under certain conditions, agglutination progressed to aggregation with ATP secretion. This suggests that IA can stimulate signal transduction in canine platelets. The agglutination and aggregation of canine platelets resulting from immune adherence may be important in the pathogenesis of canine thrombocytopenic conditions associated with circulating immune complexes or other complement activators.

Introduction

Heat-aggregated IgG (HAIgG) causes platelet clumping when it is added to canine platelets in the presence of active canine complement.[1] The phenomenon specifically requires C3, suggesting the hypothesis that HAIgG-induced platelet clumping may be a consequence of immune adherence. The studies reported here were undertaken to evaluate this hypothesis by determining the roles of platelet metabolism and complement in the mechanism of HAIgG-induced clumping of canine platelets.

Immune adherence is the complement-mediated attachment of antigen-antibody-complement complexes to erythrocytes or platelets[2] expressing complement receptors. The complement-mediated binding of immunoglobulins to the high affinity C3b receptor (CR1, CD35) on human and monkey erythrocytes is a well described example of immune adherence.[3] The CR1-mediated adherence of immune complexes (ICs) to erythrocytes in these species is an important mechanism of IC processing. CR1 receptors are absent from human platelets.[3,4] Though several other receptors for complement proteins have been identified,[3,4] their significance is largely unknown.

There is a generally held belief that the primary effector cell of immune adherence and immune complex (IC) processing has shifted with evolution from the platelets of subprimates to the erythrocytes of primates.[2,4-6] However, a platelet complement receptor analogous to the CR1 receptor on human erythrocytes has been identified only on rabbit platelets.[5] In vivo and in vitro studies indicate that platelets are the principal effector cells of immune adherence and immune complex processing in rabbits,[7,8] though there is evidence to suggest that rabbit erythrocytes can also participate and that they do have CR1-like cofactor activity.[9]

To date, neither the presence nor the absence of specific complement receptors on canine platelets has been confirmed. However, the immune adherence potential of canine platelets has been suggested by finding that the addition of bacteria to canine blood resulted in aggregates of platelets, leukocytes, and bacteria.[2] Bacteria and other soluble and insoluble antigens also adhered to washed canine platelets in the presence of a heat-labile serum factor.[2]

In this report, we explore the mechanism of HAIgG-induced platelet clumping in dogs and show that it is due to immune adherence. We further show that while platelet clumping and immune adherence are passive processes that do not require platelet metabolism, platelet activation does occur under certain circumstances.

Methods

Materials

Collagen, adenosine diphosphate (ADP), calcium ionophore A23187, thrombin, prostaglandin E1 (PgE_1), 2-deoxy-D-glucose, antimycin A, sphingosine, papain, RGDS

peptide, the control RKDVY peptide, guinea pig complement, and control caprine IgG were purchased from Sigma Chemical Company (St. Louis, MO). Goat anti-[canine C3] was from Bethyl Laboratories, Inc. (Montgomery, TX). HB43, a murine monoclonal antibody to human IgG(Fc), was purchased from the American Type Culture Collection (Rockville, MD). Chrono-lume luciferase luciferin reagent (LLR), the ATP standard, and arachidonic acid were from Chrono-log (Haverton, PA). Rabbit complement serum was from Pel-Freeze Clinical Systems (Brown Deer, WI). All chemicals not specifically noted were reagent grade.

Recombinant hirudin (Behring; Marburg, Germany) was a generous gift of Dr. Robert Roth, Michigan State University. Stock hirudin at 40 U/μl was added to serum to give a final concentration of 300 U/ml after GFP were added. For whole blood anticoagulation, 500 U/ml of hirudin were used. This prevented blood coagulation for at least 24 hr at room temperature.

Dogs

The blood donor dogs were maintained by the University Laboratory Animal Resources facility which follows USDA and NIH guidelines for animal use and care. Approval for use was obtained from the All University Committee on Animal Use and Care. The dogs were preconditioned as previously described.

Blood collection and platelet isolation

Blood was drawn by jugular venipucture as previously described[1] to give final whole blood citrate concentrations of 10.5 mM (0.32%). Platelet rich plasma (PRP) was

harvested by differential centrifugation as previously described.[1] Sepharose CL-2B (Pharmacia Biotech, Inc., Piscataway, NJ) equilibrated with calcium free Tyrode's buffer (140 mM NaCl, 5.6 mM glucose, 6 mM $NaHCO_3$, 2.7 mM KCl, 3.6 mM NaH_2PO_4, and 0.35% (w/v) bovine serum albumin (BSA), pH 7.2) was used to obtain gel-filtered platelets (GFP). GFP were recalcified to 1 mM calcium with 5 μl of 1 M $CaCl_2$. The gel filtration procedure has been reported.[1] Human plasma and cells were obtained from normal laboratory volunteers using the same anticoagulation as for the dogs.

IgG and immunoglobulin fractions

Human IgG (Cohn fraction II) was a gift from the Michigan Department of Public Health. Canine IgG was purified from normal citrated plasma as previously described.[1] IgG was judged pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fc and Fab fragments were prepared by papain digestion of canine IgG as previously described.[1] SDS-PAGE confirmed removal of whole IgG from the Fcs and Fabs which each had apparent molecular masses of about 50 kD.

Heat aggregation of IgG

IgG was aggregated by heating 50 to 100 μl aliquots of 5 to 84 mg/ml solutions for 30 to 60 min at 56°C in a water bath. For some studies, samples were heated at 65°C for 10 min to produce larger, visible, and easily pelleted aggregates.

Aggregometry

Optical aggregometry was done in a Chrono-log lumiaggregometer at 37°C with stirring at 900 rpm. Because the effect of HAIgG was similar at all platelet concentrations tested, platelet concentrations of PRP and GFP were not adjusted to a constant. Consequently, comparisons of tracings were made only within single batches of platelets and not between them.

For blocking experiments with RGDS and the control RKDVY peptides, the peptides (0.2 mM final) were incubated with platelets for 20 min at 37°C before the addition of agonists. Volumes were kept constant.

For some experiments, precipitated, pelletable HAIgG was incubated with plasma for 15 min (0.75 mg/ml) to form complement-fixed aggregates (C3-HAIgG). C3-HAIgG was then pelleted, washed once in calcium-Tyrode's buffer (Tyrode's buffer with 1 mM $CaCl_2$), resuspended in Ca-Tyrode's buffer, and used for aggregometry at a final concentration of 0.75 mg/ml.

For blocking experiments with the anti-C3 antibody and control goat IgG, washed activated aggregates were pelleted, incubated for 30 min at room temperature with 50 μg of control or test antibody in 60 μl volumes, washed once in 1 ml of Ca-Tyrode's, resuspended in 50 μl Ca-Tyrode's buffer, and added to GFP at a final concentration of 0.75 mg/ml.

ATP secretion

To reduce the proaggregatory effect of the LLR which contains a high concentration of magnesium, half the recommended amount was added to each cuvette.

When the recommended 40 μl of LLR were added to 400 μl platelet suspensions before the addition of 10 mM ADP, 1.5 μM ATP was released; use of only 20 μl LLR led to the release of only 0.1 μM ATP. In another experiment, the same ADP stimulation led to 0.5 μM ATP release with 20 μl reagent, 0.8 μM with 40 μl LLR, and 0.8 μM with 20 μl reagent plus magnesium chloride to reach a magnesium concentration equivalent to that in 40 μl LLR. The magnesium in the LLR therefore increased the measured ATP release. When 20 or 40 μl or reagent were added in a volume-controlled manner to plasma without platelets, the addition of ADP resulted in the same amount of ATP detection (0.1 μM). Doubling the ADP doubled the detected ATP. This constant increase in measured ATP was apparently due to a small amount of ATP in the ADP, and it was not included in the measurement of release for ADP aggregations. The plasma studies showed that the increase in ATP release detected with platelets was clearly due to an effect of LLR on the platelets, not merely to the increased LLR concentration.

Platelet fixation

For fixation of platelets in glutaraldehyde (grade I, Sigma), fresh platelets were washed 3 times in 37°C phosphate buffered saline (PBS), pH 6.5, with 1 μM PgE_1. Washed platelets were then incubated at a concentration of 100,000 platelets/μl for 30 min in PBS with either 0.01% or 0.001% glutaraldehyde. Glutaraldehyde was removed by washing with PBS before resuspending the platelets in PBS plus 3% BSA. For fixation in paraformaldehyde, platelets were similarly washed and then fixed in paraformaldehyde (4% final) for 48 hr at 4°C. Fixed platelets were washed twice in

PBS and resuspended in PBS with 3% BSA. For aggregometry, fixed platelets were pelleted and resuspended in fresh plasma.

Transmission electron microscopy (TEM)

Aggregations were done with PRP or GFP in a total volume of 500 μl. Equal volumes of phosphate buffered 0.1% glutaraldehyde were added at the appropriate times for fixation, incubated in the aggregometer for 1 min, and then the contents were transferred so that the platelets could be pelleted in a microcentrifuge at 12,800 x g for 20 sec. The supernatants were removed by aspiration, and 1 ml of phosphate buffered 3% glutaraldehyde was layered gently over each pellet and allowed to sit overnight. The samples were then post-fixed in 1% osmium tetroxide, rinsed, and stained en bloc with 2% uranyl acetate. After dehydration through a series of graded ethanols followed by propylene oxide, the samples were infiltrated and embedded with Polybed-Araldite epoxy resin which was allowed to polymerize at about 74° C for 2 days. Thin sections (70-90 nm) were stained with saturated aqueous uranyl acetate for 1 hr and lead citrate for 2-3 min. Sections were examined at 60 kV with a Philips 301 electron microscope.

C3 assay

Canine IgG (30 mg/ml) was aggregated by heating 15 min at 65°C to form large aggregates. HAIgG was added to fresh or heat-inactivated plasma at 0.75 mg/ml and incubated for 15 min at 37°C. Aggregates were then washed and resuspended in Ca-Tyrode's, and 75 and 225 μg of test and control (incubated with heat-inactivated plasma) HAIgG were pelleted in tubes for triplicate incubations with ^{125}I-labeled goat anti-[canine

C3] or ^{125}I-labeled control antibody. The control antibody was HB43; its purpose was to indicate the degree of nonspecific trapping of radiolabeled immunoglobulin in the IgG aggregates.

Results

By TEM, platelet clumping was evidenced by only a few small, loose platelet clumps. Platelets were unevenly distributed on thick sections, but most of the clumps did not remain cohesive through centrifugation and processing. Apparently, the HAIgG-induced platelet clumps had relatively weak interplatelet bridges. The platelets clumped by HAIgG appeared quiescent without shape change, granule centralization, or noticeable loss of granules (Figure 4.1a). When complement was active and HAIgG induced platelet clumping, platelets were coated with aggregates of HAIgG (Figure 4.1b). These aggregates of IgG sometimes appeared to bridge platelets together, and they remained attached to platelets after several washes (Figure 4.1c). When complement was inactivated by heating to prevent platelet clumping, the HAIgG aggregates were present among platelets instead of on them (Figure 4.1d).

Figure 4.1. Transmission electron micrographs of canine platelets after aggregometry with HAIgG formed at 56°C (a) and 59°C (b,c,d). HAIgG (d insert and arrows) was associated with the surfaces of clumped platelets (a,b,c), but not with unclumped platelets incubated with HAIgG in the presence of heat-inactivated plasma (d). a) X 14,000, b) X 16,850, c) X 22,000, d) X 13,650

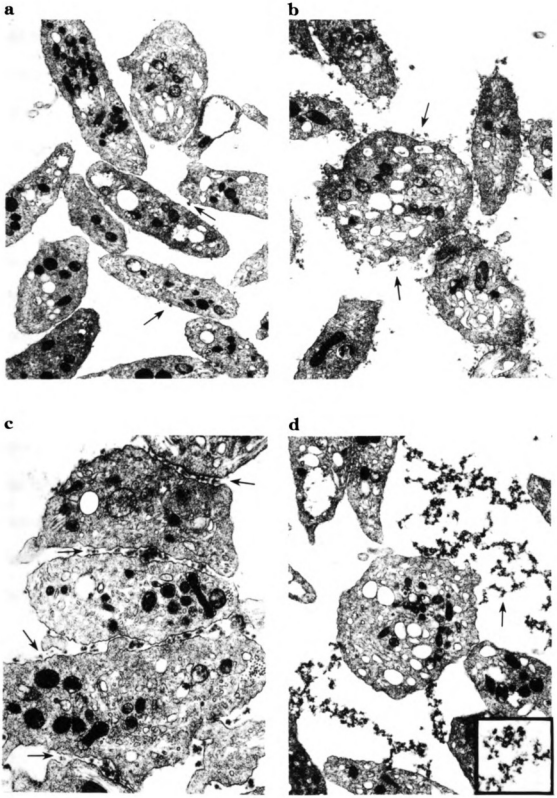

Figure 4.1

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(Fab)

These findings suggested that complement activation altered HAIgG such that it bound to platelets and formed interplatelet bridges which mediated platelet clumping. This hypothesis was investigated by incubating insoluble, pelletable HAIgG with plasma to form "activated" HAIgG that could be added to GFP in the absence of plasma. When the insoluble aggregates of "activated" HAIgG were washed 3 times by pelleting centrifugations and added to GFP, immediate increases in light transmittance were noted and the platelets again clumped (Figure 4.2). The delay before clumping that always occurred when HAIgG was added to plasma in the presence of platelets was eliminated. Clumping was dose-dependent and sometimes submaximal, but other times full-scale aggregation was induced (Figure 4.2b). The presence of 5 mM EGTA or 5 mM EDTA prevented full aggregation induced by these "activated" aggregates but submaximal clumping still occurred (Figure 4.2b). When HAIgG was incubated with heat-inactivated plasma (5 min 56°C), the washed HAIgG did not induce platelet clumping (Figure 4.2b). When HAFabs were incubated with plasma and then washed, they induced immediate clumping of GFP in the absence of plasma (Figure 4.2b). Therefore, a complement protein or a product of complement activation either became associated with the HAIgG (Fab) or altered it in such a way as to induce platelet clumping.

Figure 4.2. Aggregometry curves for GFP incubated with washed HAIgG (a; b1,3,4) or HAFabs (2) previously incubated in plasma (a; b2-4) or heat-inactivated plasma (1). "Activated" HAIgG generated dose-response curves (a) and occasional full-scale aggregation (b4) inhibitable with 5 mM EGTA (b3).

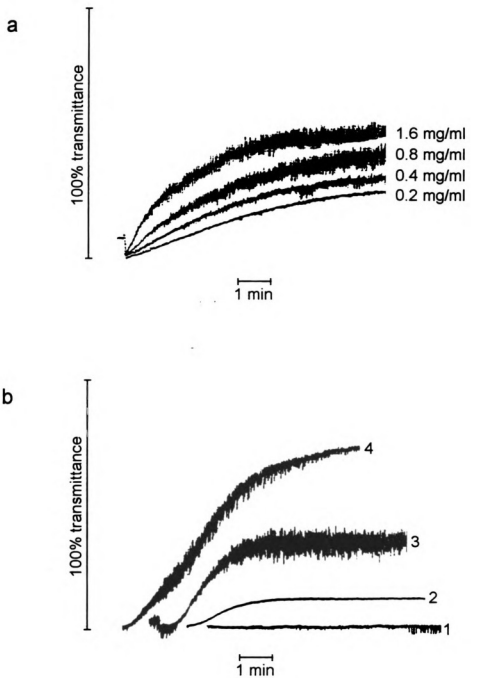

a
100% transmittance
1.6 mg/ml
0.8 mg/ml
0.4 mg/ml
0.2 mg/ml
1 min
b
100% transmittance
4
3
2
1
1 min

Figure 4.2

To investigate whether or not C3 was associated with the "activated" aggregates of IgG, they were probed with a ^{125}I-labeled polyclonal anti-[canine C3] antibody. Compared to HAIgG incubated with heat-inactivated plasma, there was a significant but relatively weak signal from the "activated" aggregates using the anti-C3 antibody (Table 4.1). Nonspecifically bound or trapped ^{125}I-labeled control antibody, HB43, was not increased with the "activated" aggregates. Therefore, C3 appeared to be fixed to the HAIgG that had been incubated with plasma.

Table 4.1. Detection of C3 on HAIgG incubated with unheated plasma and with heat-inactivated plasma.

	% bound ± S.D.	
	heated plasma HAIgG	unheated plasma HAIgG
anti-C3	9.1 ± 1.2	14.6 ± 1.6
HB43	2.5 ± 0.2	1.6 ± 0.1

To determine if this fixed C3 was instrumental in clumping the platelets together, C3-HAIgG was incubated with goat anti-[canine C3] or control goat IgG prior to aggregometry. Platelet clumping still occurred with the control sample, but no clumping occurred when C3 proteins were blocked by the anti-C3 pretreatment (Figure 4.3).

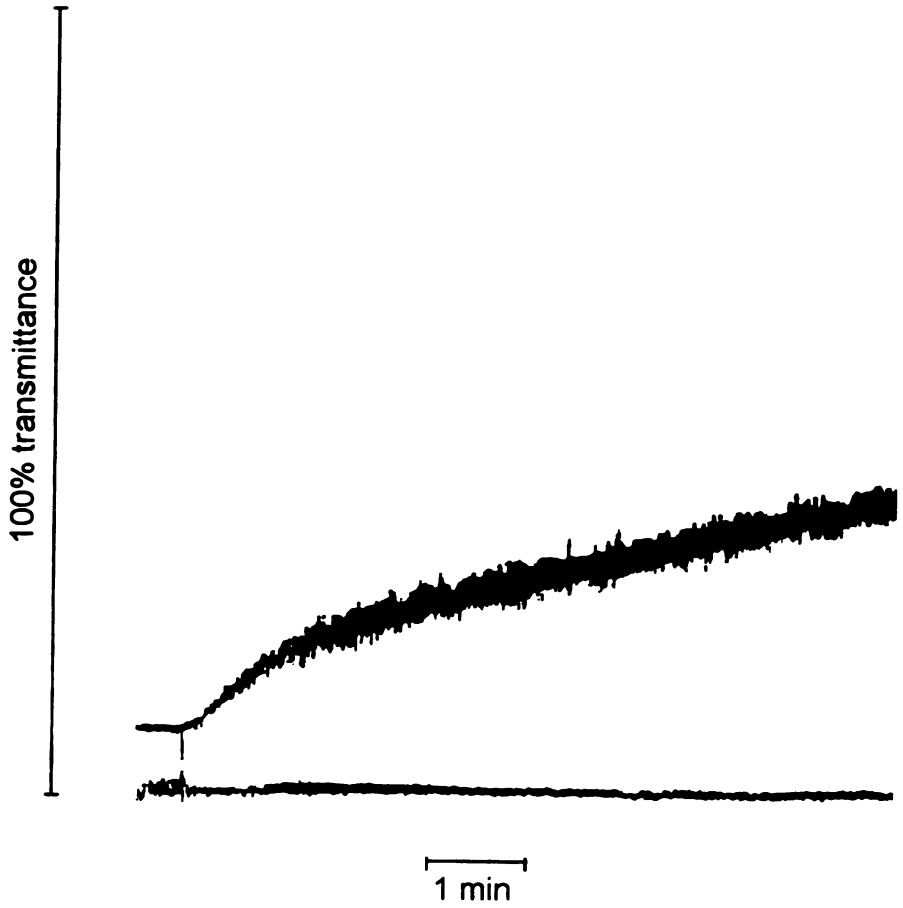


Figure 4.3. Effect of goat anti-[canine C3] antibodies on HAIgG-induced clumping of GFP. HAIgG was first incubated with plasma to form C3-HAIgG and then with 1) goat anti-[canine C3] to block HAIgG-associated C3, or 2) control goat IgG. The same results were obtained with GFP from a second dog.

While human and canine HAIgG (K9HAIgG) were capable of inducing canine platelet clumping, canine plasma appeared to be specifically required. K9HAIgG did not induce platelet clumping when human, guinea pig, or rabbit plasma or serum were substituted for canine plasma or serum. This was true even when GFP were treated with 1 μM PgE_1, pelleted, and completely resuspended in test plasma, a treatment that did not affect the response of GFP to K9HAIgG in canine plasma. Human HAIgG also failed to induce clumping of canine GFP when human plasma was used in place of canine plasma. In the reverse experiments, human platelets did not clump when incubated with canine plasma and K9HAIgG, despite the fact that "activated" HAIgG formed (data not shown). Also, "activated" K9HAIgG did not agglutinate human erythrocytes which are known to have CR1 receptors (data not shown).

Further studies of the mechanism of HAIgG-induced platelet clumping excluded a role for fibrinogen binding to $\alpha_{IIb}\beta_3$. RGDS peptides did not inhibit HAIgG-induced clumping at concentrations that markedly inhibited aggregations induced by collagen and ADP; a nonsense peptide was uniformly uninhibitory (Figure 4.4).

Figure 4.4. Effects of RGDS (lower curves) and nonsense peptides (upper curves) on aggregometry using PRP with a) 0.25 mg/ml HAIgG, b) 10 μM ADP, and c) 5 μg/ml collagen.

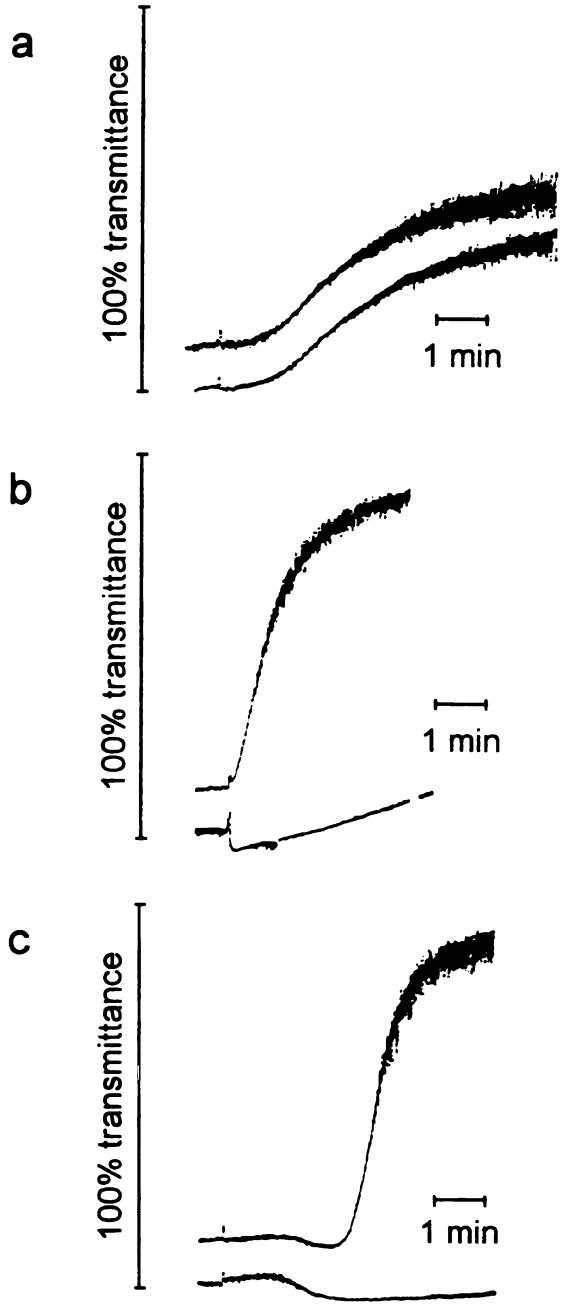

a
100% transmittance
1 min
b
100% transmittance
1 min
c
100% transmittance
1 min

Figure 4.4

The observation that clumped platelets were still responsive to collagen, ADP, and the weak effects of ATP indicated that these had neither fully aggregated nor become refractory to these agonists (Figure 4.5). Though TEM findings also suggested that HAIgG-induced platelet clumping was not associated with the platelet release reaction, ATP release was specifically evaluated during aggregometry. No significant ATP release occurred with HAIgG, while it was marked with calcium ionophore and collagen, moderate with PMA, and minimal with ADP. ATP release was quantitated only for ADP, HAIgG, and collagen, for which the results were respectively 0.5, 0.1, and 3.4 μM.

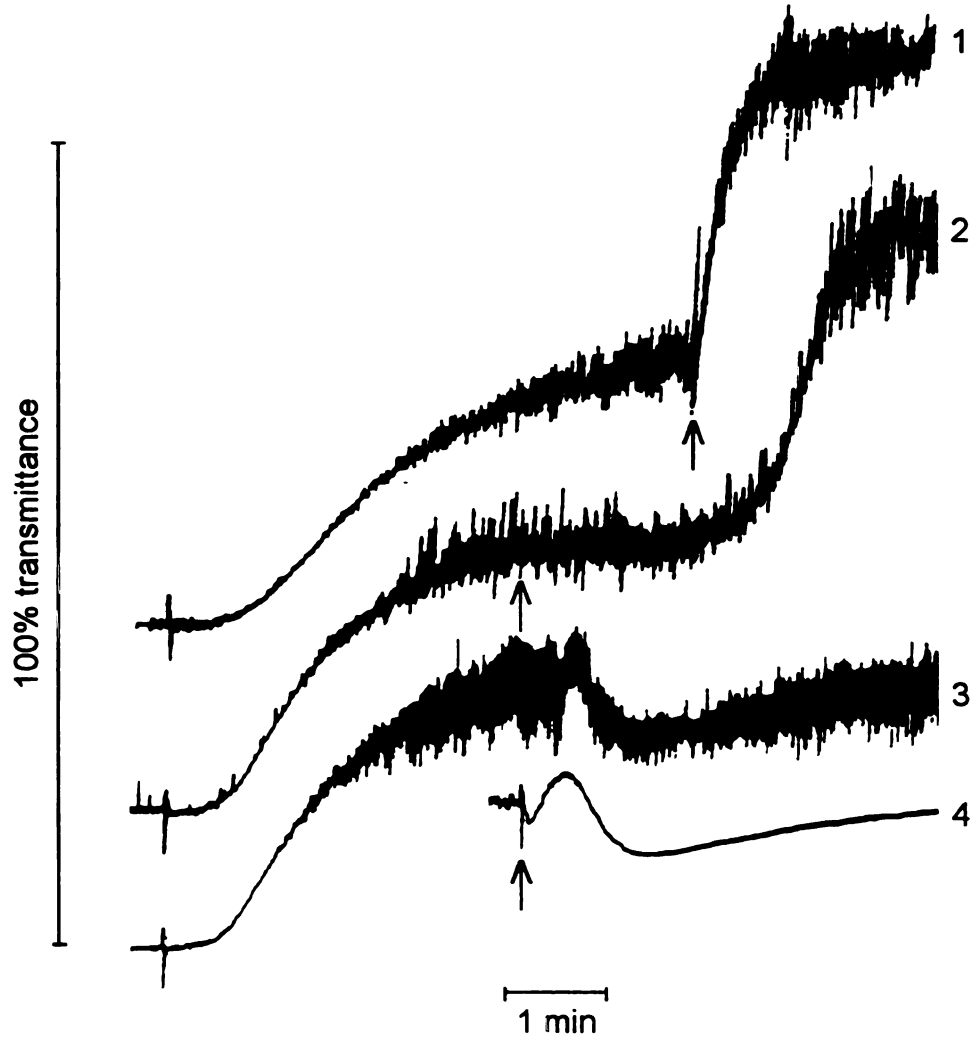
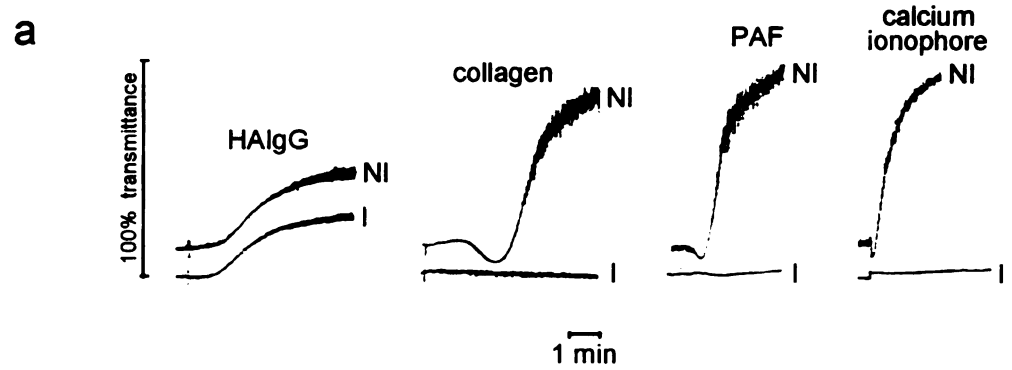
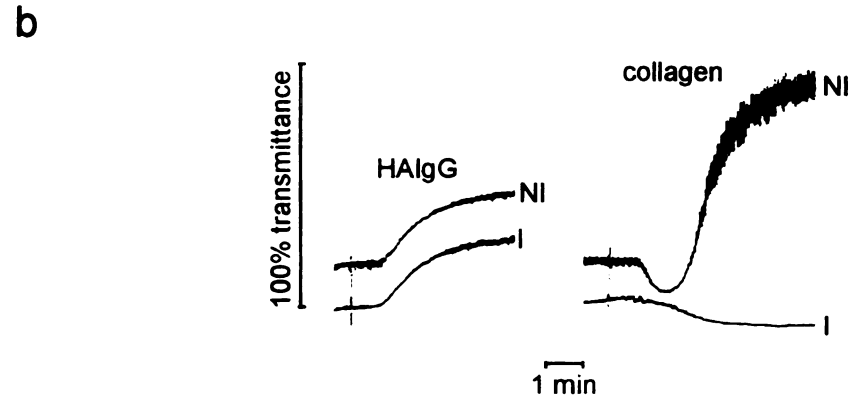


Figure 4.5. Responsiveness of HAIgG-clumped platelets to 1) 10 μM ADP, 2) 2.5 μg/ml collagen, and 3) 5 μM ATP. The effect of ATP on unclumped platelets is shown for comparison (4). Arrows indicate addition of platelet agonists.

The role of platelet metabolism in HAIgG-induced platelet clumping was further evaluated by metabolic inhibitors and use of fixed platelets. HAIgG-induced clumping was minimally affected by PgE_1, sphingosine, or a combination of antimycin A and 2-deoxy-D-glucose, but aggregations induced by other agonists were markedly inhibited or prevented. Qualitatively, PgE_1 mildly, but consistently, lowered the maximal amplitude of light transmittance for HAIgG (Figure 4.6a). Sphingosine prevented collagen-induced aggregation at concentrations which did not inhibit even low concentrations of HAIgG (Figure 4.6b). Antimycin A and 2-deoxy-D-glucose completely inhibited ADP, collagen, PAF, calcium ionophore, and PMA, but they did not affect HAIgG (Figure 4.6c).

Figure 4.6. Effects of a) 1 μM PgE_1, b) 100 μM sphingosine, and c) 5 μg/ml antimycin A plus 10 mM 2-deoxy-D-glucose on HAIgG-induced platelet clumping. In (c), the inhibition of ADP, collagen, PAF, calcium ionophore, and PMA are represented by a single tracing. I=inhibitor, NI=no inhibitor


a
100% transmittance
HAIgG
NI
I
collagen
NI
I
PAF
NI
I
calcium ionophore
NI
I
1 min


b
100% transmittance
HAIgG
NI
I
collagen
NI
I
1 min

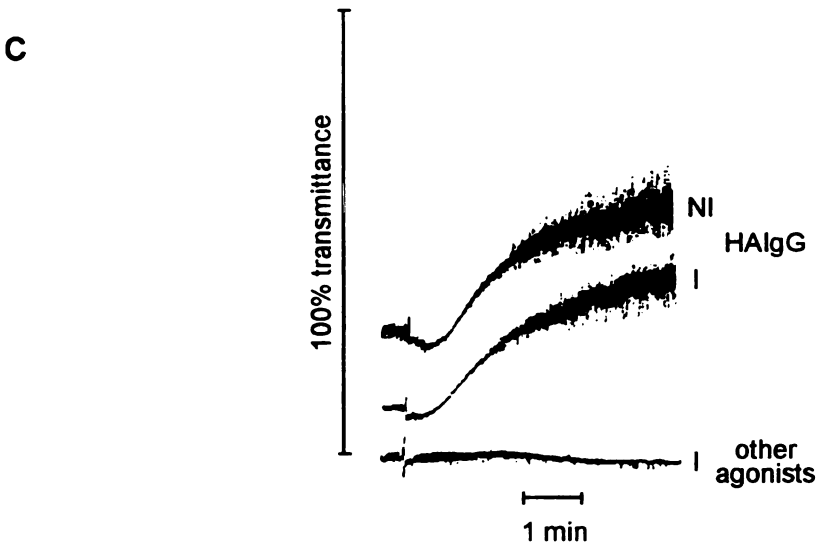

c
100% transmittance
NI
HAIgG
I
other agonists
I
1 min

Figure 4.6

The addition of HAIgG to 0.01% glutaraldehyde-fixed platelets resuspended in plasma resulted in a visible but relatively mild degree of platelet clumping (Figure 4.7). Platelet clumping was much more evident when platelets were fixed in 0.001% glutaraldehyde. HAIgG did not induce visible clumping of platelets in similar experiments using 4% paraformaldehyde-fixed platelets (Figure 4.7). Fixed platelets did not aggregate in response to ADP, collagen, or PAF.

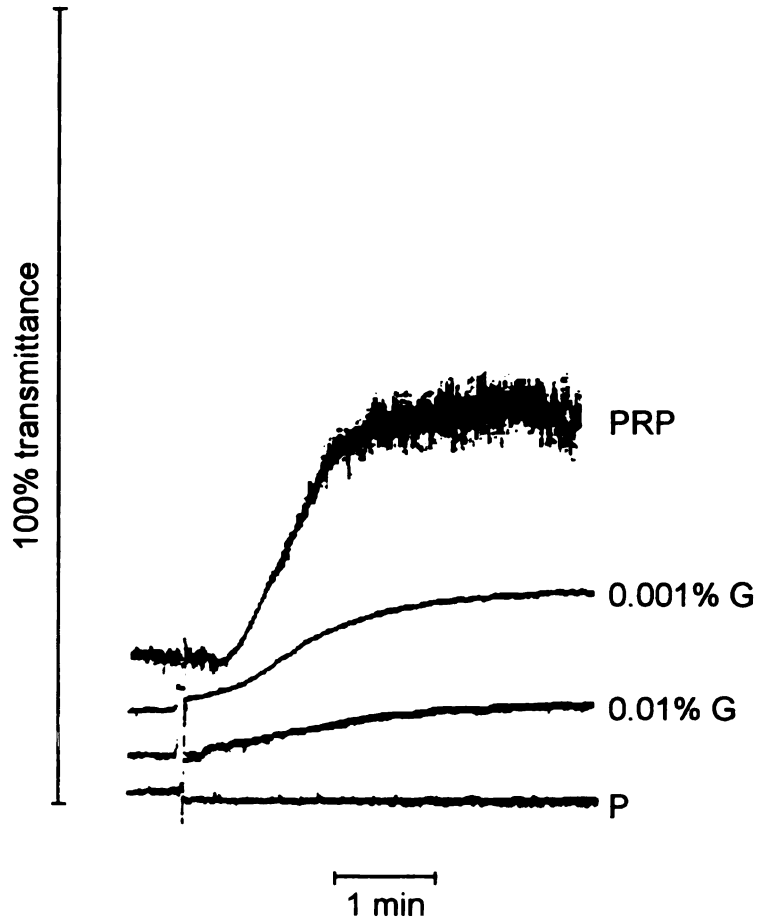


Figure 4.7. Effects of HAIgG on PRP compared to platelets fixed with 4% paraformaldehyde (P), 0.01% glutaraldehyde (G), or 0.001% G. Fixed platelets were resuspended in fresh plasma. HAIgG was added simultaneously to the test cuvette and to plasma in the blank cuvette to cancel out absorbance changes due to the interaction of HAIgG with plasma.

In contrast to the usual nonmetabolic agglutination described above, under certain conditions, HAIgG inconsistently induced full-scale aggregation with platelet activation and release (Figures 4.2 and 4.8). This occurred under the following four sets of conditions. First, it occurred when serum with hirudin was used to support HAIgG in the GFP system (Figure 4.8). In this case, aggregation was sometimes biphasic, and granule release was apparent by TEM. The second phase could be prevented or inhibited by 1 μM PgE_1, and both phases were eliminated by heating the serum. Second, when PRP was harvested from blood drawn into hirudin (500 U/ml), the amplitude of light transmittance reached with K9HAIgG was similar to that of full aggregations with ADP; 1 μM PgE_1 inhibited the effect to that seen with citrated samples. Third, when activated aggregates of K9HAIgG were added to GFP, full aggregation sometimes occurred (Figures 4.2 and 4.8). Fourth, the platelets of one particular dog usually aggregated fully in response to HAIgG, even in citrated systems (PRP or GFP plus citrated plasma) (Figure 4.8). ATP was released, and the aggregation tracings were biphasic. ATP release and the second phase of aggregation were eliminated by PgE_1. Full aggregation of this dog's GFP did not require autologous plasma; citrated plasma from a dog whose platelets never aggregated fully in citrated systems was also supportive. In the reverse experiments, when plasma from the dog whose platelets fully aggregated was used with GFP from a dog whose platelets responded submaximally to HAIgG, aggregation was complete and biphasic.

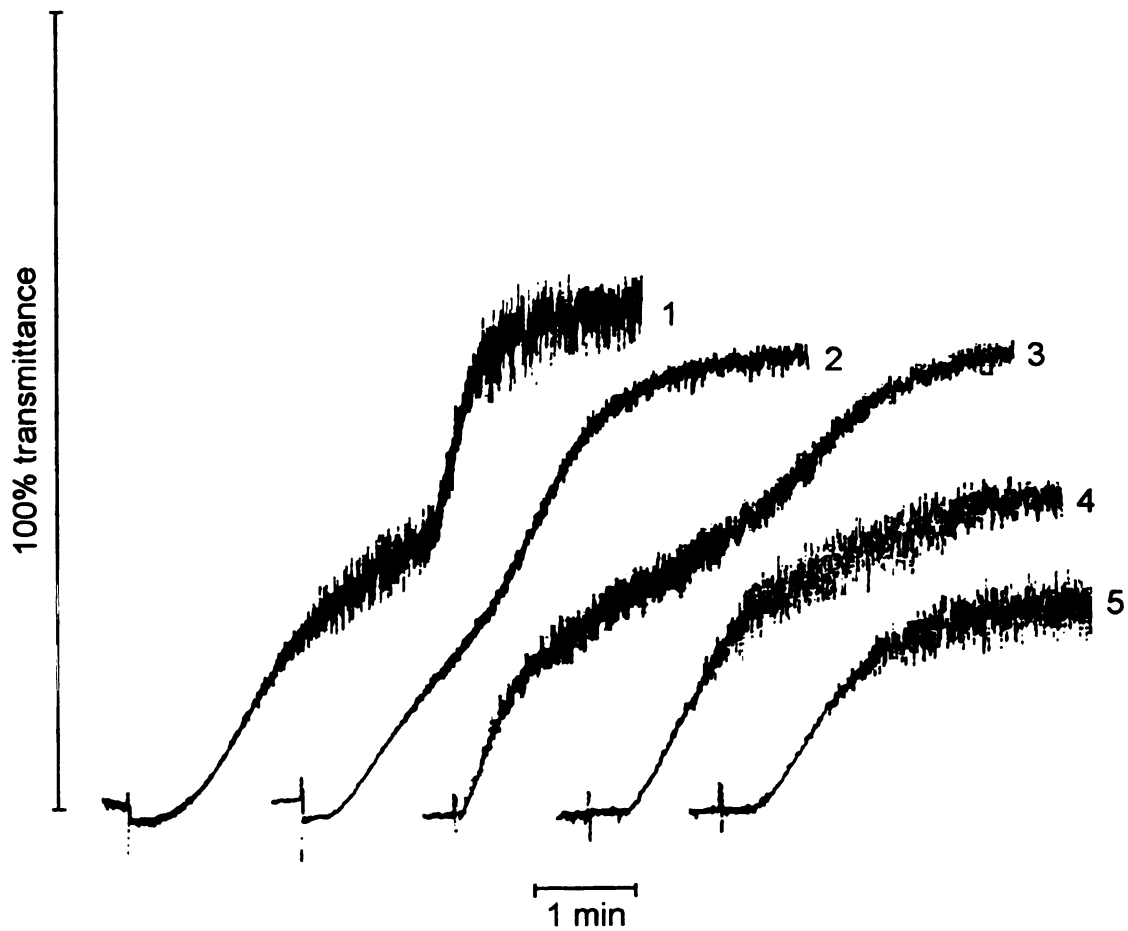


Figure 4.8. HAIgG-induced aggregation occurring with 1) GFP plus citrated plasma from one particular dog, 2) GFP plus serum (with hirudin), 3) the addition of "activated" HAIgG (preincubated in plasma) to GFP, and 4) PRP from hirudin-anticoagulated blood. Addition of PgE_1 limited these responses to agglutination (5). These tracings were recorded on different days.

To evaluate the possibility that occasional leukocyte contamination was responsible for the full aggregation, leukocytes were harvested from canine blood and added to platelets in the aggregometer. The addition of HAIgG caused typical submaximal aggregation tracings, except that the clumps that formed were clearly larger than usual, presumably because of incorporation of leukocytes (data not shown). The addition of HAIgG to leukocytes in plasma did not induce their clumping (data not shown).

Discussion

HAIgG induced passive agglutination of canine platelets through a complement-mediated bridging known as immune adherence. The complement activation induced by HAIgG[1] resulted in C3 incorporation within the IgG aggregates. These C3-HAIgG aggregates then appeared to form interplatelet bridges, presumably via specific C3 receptors on canine platelets.

Immune adherence was implicated as the mechanism of HAIgG-induced platelet clumping by a series of six major findings. First, complement was required for clumping to occur.[1] More specifically, a C3 requirement was identified by the failure of C3-deficient canine plasma to support HAIgG-induced clumping.[1] Second, the incubation of HAIgG with plasma caused C3 to be deposited on the IgG aggregates as detected by a ^{125}I-labeled anti-[canine C3] antibody. Third, these C3-HAIgG complexes clumped canine GFP in the absence of plasma and without a delay, while control HAIgG incubated with heat-inactivated plasma did not. C3-HAIgG complexes were active even in the presence of 5 mM EDTA or EGTA, indicating cation independence of the

reaction. The binding of human C3b to human CR1 is independent of calcium and magnesium.[10] Fourth, complement-activated HAFabs also clumped canine GFP in the absence of plasma, indicating that the Fab portion of the IgG was instrumental in mediating the effect. This is supportive of an immune adherence mechanism because the covalent binding of C3b and C4b to human IgG occurs at domains on the Fab portion of the IgG molecule.[11] Fifth, TEM of platelets clumped by HAIgG showed that HAIgG was associated with platelet surfaces, sometimes forming apparent interplatelet bridges. In contrast, HAIgG was not associated with platelet surfaces when complement was inactivated by heating plasma for 5 min at 56°C. And sixth, incubation of C3-HAIgG complexes with an affinity-purified goat anti-[canine C3] antibody prevented subsequent platelet clumping, but it still occurred after incubation of C3-HAIgG with control goat IgG. Fibrinogen was excluded as a platelet bridging protein because RGDS peptides were not inhibitory.

The activation of the human alternative complement pathway by immune complexes reportedly leads to larger aggregates than are formed by the classical pathway alone, and the ICs bind more efficiently to CR1.[12] This may partially explain the primary importance of alternative pathway activation in HAIgG-induced platelet clumping in dogs.[1]

The slow reversal of platelet clumping seen in these canine studies may have been due to ongoing C3b degradation causing HAIgG to dissociate from platelet C3 receptors. Human erythrocyte CR1 receptors participate in the degradation of C3b to C3d and C3dg such that the ICs detach from the CR1 receptors and cannot rebind without further

fixation by new C3b.[12] A similar degradation of platelet-associated, HAIgG-bound C3b may occur in dogs.

Human, guinea pig, and rabbit plasma or serum did not support immune adherence with canine platelets. Similarly, equine and porcine complement did not support rabbit ICs in immune adherence reactions.[13] This likely relates to the species specificity of complement receptor interactions.[14] While human CR1 can bind human, rabbit, and porcine C3,[15] the rabbit platelet complement receptor does not efficiently bind human C3b.[5] The failure of canine C3-HAIgG complexes to agglutinate human erythrocytes suggests that human CR1 also does not bind canine C3b. Similarly, the analogous canine platelet receptor for C3b may also have some species specificity for C3b binding. Alternatively, K9HAIgG may not have the capacity to activate complement from some species.

Platelet metabolism was not required for C3-HAIgG or HAIgG to induce platelet clumping. This was evidenced by the agglutination of glutaraldehyde-fixed platelets exposed to HAIgG in the presence of plasma, and by the failure of several metabolic inhibitors to prevent clumping of fresh platelets. A consistent but very mild suppression of clumping by PgE_1 suggests that arachidonate metabolism may have mildly accentuated the platelet response. However, clumping was not reduced by simultaneous inhibition of mitochondrial oxidative phosphorylation and glycolysis by antimycin A and 2-deoxy-D-glucose, respectively.

Several other findings were consistent with a largely passive role for platelets in HAIgG-induced clumping. During aggregometry, the recorder deflection typical of shape change did not occur, and the maximal amplitude of light transmittance corresponded

only to that of primary aggregations induced by subthreshold concentrations of ADP. When clumped, platelets still had normal responses to ADP, collagen, and ATP, and they had not released detectable ATP. There was also no morphological evidence of granule secretion by TEM, and the platelets appeared quiescent with no shape change or granule centralization. Therefore, HAIgG caused passive agglutination of canine platelets, just as bacterial LPS has been reported to cause passive agglutination of canine platelets.[16]

However, under certain conditions, HAIgG and LPS also induced full-scale aggregation with ATP release. PgE_1 and RGDS independently limited these responses to agglutination, indicating that aggregation required arachidonic acid metabolism and fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$. Because complement proteins can activate leukocytes which in turn can produce platelet activating factors,[3] sporadic leukocyte contamination of platelet suspensions may have caused aggregation. However, we excluded this possibility by showing that marked leukocyte contamination did not convert agglutination responses to aggregation responses.

In three of the four situations where metabolism-dependent aggregation occurred, cation concentrations were not reduced by the presence of citrate. This was true when: 1) serum (with hirudin) was used as the complement source, 2) PRP was obtained from blood anticoagulated with hirudin, and 3) aggregation was induced by C3-HAIgG complexes washed and resuspended in Ca-Tyrode's buffer. It is unknown if greater platelet reactivity under these conditions was due to the more nearly physiologic calcium concentrations or to other factors.

HAIgG and LPS also induced aggregation, in addition to agglutination, in citrated systems with the platelets from one particular dog (dog R). These platelets were also the

only platelets tested that aggregated in response to arachidonic acid (1.4 mM); aggregation was full-scale but delayed about 5 min. Studies with GFP and plasma from dog R and from a dog (dog N) whose platelets only agglutinated in these systems showed that dog R's plasma contained the necessary factor(s) to impart greater reactivity to dog N's platelets, but dog R's platelets remained aggregable even after plasma was removed by gel filtration. The nature of the plasma factor(s) responsible for this phenomenon is unknown, as is the relationship, if any, to the arachidonic acid and thromboxane-sensitivity of some dogs' platelets.[17-19] However, the variable in vitro response to HAIgG among dogs suggests that in vivo platelet responses to immunologic and complement stimuli may also be varied. This heterogeneity of response may be important in explaining individual predispositions to certain diseases.

The potential significance of immune adherence by canine platelets is great. As in other species, immune adherence in dogs may provide a means of IC clearance by phagocytic cells bearing Fcγ receptors for IgG[12,20] and CR3 receptors for inactivated C3b.[10] The infusion of ICs into rabbits leads to their rapid immune adherence to platelets with subsequent clearance.[7,8] A similar result would be expected in dogs, but it is unknown if platelet phagocytosis would occur and cause or contribute to thrombocytopenia. If complement binding to canine platelets activates platelet signal transduction pathways under physiologic conditions, immune adherence may further contribute to thrombocytopenia due to platelet activation. The common association of thrombocytopenia with complement activators such as neoplastic cells[21] and polyions,[22-24] and with many infectious agents[25-27] and foreign materials[28] capable of initiating in vitro platelet immune

adherence in dogs[2], suggests that immune adherence may contribute to the thrombocytopenia of many canine conditions.

The potential for canine platelets to participate in immune adherence also has important ramifications for the detection of platelet-associated immunoglobulin (PAIg) in dogs. If a canine platelet complement receptor can bind IC-associated immunoglobulins such that they are not easily removed by washing, positivity for PAIg will not differentiate ICs from specific antiplatelet antibodies. The finding that acid eluates of canine platelets positive for PAIg rebound to allogeneic normal platelets[29] does not confirm Fab-mediated binding until it is shown that immune adhered immunoglobulin reacts differently under the same conditions. Therefore, the contribution of immune adhered immunoglobulin to detectable PAIg and thrombocytopenia in dogs should be evaluated.

In summary, HAIgG induced passive agglutination of canine platelets by a C3-mediated mechanism that appears to be immune adherence. That is, HAIgG activated complement leading to the binding of C3 to HAIgG such that the C3-HAIgG complexes formed interplatelet bridges, presumably by binding to as yet unidentified C3 receptors on canine platelets. Under some conditions, and with some dogs, agglutination progressed to aggregation, suggesting that immune adherence may activate signal transduction pathways in canine platelets. These findings may have considerable in vivo significance in conditions associated with circulating complement activators. The contribution of immune complexes, complement, and immune adherence to the thrombocytopenia of many neoplastic, infectious, drug-induced, and immune-mediated canine diseases deserves further investigation.

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CHAPTER 5

EVALUATION OF HUMORAL IMMUNE MECHANISMS IN THE PATHOGENESIS OF CYTOPENIAS IN DOGS ADMINISTERED DRUG X

Abstract

Pancytopenia and isolated cytopenias occurred in some dogs receiving a proprietary compound, drug X (DX), during a 1-year toxicity study. The bone marrows of selected affected dogs were cellular with myeloid hyperplasia, and coagulation profiles were not indicative of disseminated intravascular coagulation. Preliminary testing showed that platelet surface-associated immunoglobulin (PSAIg) was increased in 2 affected dogs as compared to controls. To further investigate the hypothesis that humoral antibodies or immune complexes were causing or contributing to the cytopenias, immunoradiometric methods to detect erythrocyte surface-associated immunoglobulin (ESAIg) and circulating immune complexes (ICs) were developed. These assays and the assay for PSAIg were used to measure PSAIg, ESAIg, and ICs in other treated and control dogs (phase I studies), and in two recovered dogs during rechallenge with DX (phase II). In the phase I studies, one dog had mild increases in circulating ICs and PSAIg, but in most dogs, the administration of high doses of DX was not associated with increased PSAIg, ESAIg, or C1q-bindable ICs. In the phase II studies, one of the two rechallenged dogs developed only mild and inconsistent increases in PSAIg. Humoral

immune mechanisms therefore did not seem to contribute to the noted clinical toxicity in these rechallenged dogs. In conclusion, while immune mechanisms may have contributed to the sporadic cytopenias that occurred in some DX-treated dogs, the cytopenias did not appear to be mediated principally by humoral immunity to the parent drug. Instead, DX may have interfered with some maturation or regulation mechanism that was not assessed by these studies.

Introduction

During a 1-year toxicologic study of a proprietary pharmacologic agent, drug X (DX), several of the test dogs became thrombocytopenic, leukopenic, and/or mildly anemic. The bone marrow from affected dogs was cellular with increased, and sometimes markedly increased, myeloid to erythroid ratios. Prothrombin and partial thromboplastin coagulation times were normal, and there was no other evidence of disseminated intravascular coagulation. These findings suggested the possibility that humoral immunity may have been involved in the pathogenesis of the cytopenias. Furthermore, other dogs administered high doses of DX consistently developed a dramatic arteritis, suggesting a possible role for immune complexes in the pathogenesis of the vasculitis.

In a preliminary study, we assessed two affected dogs (34 and 65) for platelet surface-associated immunoglobulin (PSAIg). The methods for this immunoradiometric assay are desribed below. The samples were shipped overnight at ambient temperature along with samples from two untreated control dogs and two treated but unaffected dogs. Both cytopenic dogs had increased platelet binding of radiolabeled staphylococcal protein

A (SpA) in comparison to the four control dogs and in comparison to ten normal dogs tested under equivalent conditions (Table 5.1). More platelets were harvested from the same samples so that they could be assessed for PSAIg with radiolabeled antibodies to canine IgG and IgM. Again, there was increased SpA binding, and in addition, increased binding of the anti-M antibody for both dogs and increased binding of the anti-G antibody for dog 65. These data indicated that PSAIg was increased in the samples from the thrombocytopenic dogs treated with DX.

Table 5.1. Initial assay for platelet-associated immunoglobulin in thrombocytopenic and control dogs.

Subject			% bound		
treated	cytopenic	dog #	SpA	anti-γ	anti-μ
		1	5.9	2.0	7.5
		36	1.9	1.3	5.4
x		35	2.9	1.1	5.5
x		70	2.6	1.0	3.8
x	x	34	**16.9***	-	-
x	x	65	**44.8***	-	-
x	x	34*	8.6	3.7	**11.0**
x	x	65*	**19.1**	**11.0**	**15.8**
			3.0±1.6**	2.2±1.0**	5.9±2.4**

Bold values exceed mean + 2 S.D. of control dogs.

* 2nd harvest of additional platelets from resuspended samples for testing with protein A (SpA) and antibodies to canine IgG and IgM.

** Mean ± S.D. for 10 normal dogs using 24 hour-old samples.

The preliminary positive data for PSAIg supported the hypothesis that humoral antibodies or immune complexes were causing or contributing to the cytopenias in affected dogs. In order to investigate this possibility further, we developed methods to detect erythrocyte surface-associated immunoglobulin (ESAIg) and circulating immune complexes (ICs). We used these assays and the assay for PSAIg to measure PSAIg, ESAIg, and ICs in other high-dose treated and control dogs (phase I studies), and in dogs 34 and 65 after rechallenge with DX (phase II). Because some drug-induced antibodies require the presence of the drug to bind to their target epitopes,[1] each assay for cell-associated immunoglobulin was done in the presence and in the absence of DX.

Methods

Blood collection

Blood was collected by jugular venipuncture into plastic syringes containing 1/10 part 3.2% citrate, to give a final concentration of 0.32% citrate. Blood was transferred to plastic tubes which were placed directly into a 37°C incubator for transport to the laboratory. All samples arrived within 3 hr of collection.

Radiolabeling

The following antiglobulin reagents were radiolabeled by modifications of the method of Fraker and Speck:[2]: staphylococcal protein A (SpA) (Sigma Chemical Company, St. Louis, MO), a polyclonal goat anti-[canine IgG(γ)] (anti-γ), and a polyclonal goat anti-[canine IgM(μ)] (anti-μ) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Briefly, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma) was

dissolved in dimethyl chloride and 0.6 μg were deposited in the base of a glass test tube by evaporation. To this were added 25 μg of protein (1 mg/ml) and 0.3-0.5 μCi iodine-125 (NaI, NEN Dupont). After 10-15 minutes of incubation with mixing, radiolabeled protein was separated from free iodide by filtration through a 10 ml Sephadex G25 filtration column (Pharmacia Biotech, Inc., Piscataway, NJ). The labeled protein was pooled, diluted with assay buffer (phosphate buffered saline (PBS) with 3% bovine serum albumin (BSA) and 0.025% sodium azide, pH7.1) to stock concentrations of 1.2 μg/ml, and frozen in aliquots until use.

Immunoglobulin purification

Canine IgG and IgM were purified from normal citrated plasma by staphylococcal protein A-agarose (Sigma) affinity chromatography followed by exclusion chromatography with Sephacryl-300 HR (Pharmacia) to separate the immunoglobulins. The pooled fractions were dialyzed against water, lyophylized, and resuspended in PBS, pH 7.2. The final products were assessed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions using Coomassie Blue to visualize the bands. The IgG appeared pure, and the IgM was slightly contaminated by IgG.

Erythrocyte surface-associated immunoglobulin

Erythrocytes (RBCs) were sedimented from 37°C citrated blood samples by centrifugation for 10 min at 2000 x g in a Beckman J-6B floor centrifuge. After the buffy coats and plasma were removed by aspiration, RBCs were washed 3 times in assay

buffer, plus and minus DX. After the final wash and resuspension, the hematocrits of each sample were determined, and each sample was diluted with assay buffer (plus and minus DX) to a 4% suspension. For each sample, triplicate determinations were made with each of the 3 radiolabeled immunoglobulin detectors: SpA, anti-γ, and anti-μ. Fifty microliters of each 4% RBC suspension were added to test wells of U-bottomed polypropylene microtitration plates, and to each well was added 50 μl (10ng) of the appropriate antiglobulin reagent, plus and minus DX. After a 1 hr incubation at room temperature, the RBCs were pelleted by centrifugation for 5 min at 850 x g in the Beckman centrifuge. The pellets were washed 3 times by alternating centrifugation and resuspension in assay buffer, plus and minus DX. After the final resuspension, the samples were transferred to a TmAnalytic gamma (Bensenville, IL) counter for measuring RBC-associated radioactivity. Results were reported as percentages of added radioactivity that remained associated with the RBCs (% bound).

The positive control for all 3 antiglobulin reagents was canine serum containing IgG and IgM alloantibodies to DEA1.1 (dog erythrocyte antigen 1.1). This serum was generously provided by Dr. Robert Bull, Michigan State University. Positive control serum was incubated with normal canine DEA1.1-positive RBCs for 1 hr at room temperature. The pellets for these positive controls were then washed 3 times and antiglobulin reagents were added as described for the test samples. A dose-response curve of positive control serum was generated with each assay using SpA. Anti-γ and anti-μ were tested with the highest concentration of the positive control serum.

Platelet surface-associated immunoglobulin

Platelets were harvested by repetitive centrifugation for 2-3 min at 1300 x g. Aliquots of platelet-rich plasma (PRP) were removed after each centrifugation. Prostaglandin E_1 (1 μM final) was added to the pooled PRP sample of each dog before washing three times with 37°C assay buffer (same as ESAIg assay buffer), plus and minus DX. Contaminating RBCs, when present, were removed by differential centrifugation in a microfuge. Washed platelets were resuspended in assay buffer, plus and minus DX, and the platelet concentration of each sample was determined with a Neubauer hemocytometer. For each sample, triplicate determinations were made with each of the three radiolabeled immunoglobulin detectors: SpA, anti-γ, and anti-μ.

Five million platelets from each sample were added to each test well of U-bottom polypropylene microtitration plates and pelleted by centrifugation at 850 x g for 8 minutes in a Beckman J6B floor centrifuge. The buffer was removed and the platelets were resuspended in 50 μl assay buffer containing 10 ng of ^{125}I-labeled antiglobulin reagents, plus and minus DX. After incubation at room temperature for 1 hr, the platelets were washed 3 times in assay buffer, plus and minus DX, with alternating resuspensions and centrifugations for 8 min at 850 x g. The test platelets were resuspended after the last wash, and aliquots were removed from each well and assessed for platelet-associated radioactivity in a gamma counter. Results were reported as percentages of the added radioactivity that remained associated with the platelets (% bound).

The assay positive control for SpA and anti-γ was citrated plasma from a dog with naturally occurring immune-mediated thrombocytopenia and a high concentration

of antibody that immunoprecipitated platelet glycoprotein Ib. Normal plasma and fixed normal platelets served as negative controls. A dose-response curve of positive control plasma was generated with each assay by incubating increasing concentrations of the positive control plasma with 5 million glutaraldehyde-fixed normal platelets for 1 hr at room temperature (plus and minus DX). These platelets were then washed 3 times in assay buffer (plus and minus DX), and incubated with SpA as for the test samples. Anti-γ was used only with the highest concentration of positive control plasma. The positive controls for the ESAIg and C1q assays verified continued function of the anti-μ antibody.

Solid phase radiometric C1q assay for immune complexes

Plasma was collected from citrated blood samples after centrifugation for 10 min at 2000 x g. When samples were not assayed immediately, they were frozen at -65°C until used. Human C1q (Sigma) was adsorbed onto the wells of Immulon II Removawell microtitration plates (Dynatech Laboratories, Inc., Chantilly, VA) by incubating each well with 1 μg C1q in 200 μl 50 mM carbonate buffer, pH 8.6, overnight at 4°C. The wells were then washed twice with 300 μl C1q assay buffer (PBS with 1.5% BSA and 0.01% Tween, pH 7.2). Each well was then blocked by incubation with 300 μl of C1q assay buffer for 2 hr. After removal of the buffer, samples or standards were added with C1q assay buffer to a total volume of 200 μl per well. The volume of citrated plasma used for the test samples was 1.0 and 0.2 μl, except on one date when 0.4 μl replaced 1.0 μl. Each sample was tested in triplicate with 0.2 μl, but some samples were tested in duplicate at the higher sample volumes. After a 1 hr incubation at room temperature, the wells were washed 3 times in C1q assay buffer. Each sample was

assessed with each of the 3 antiglobulin reagents: SpA, anti-γ, and anti-μ. Ten nanograms of each reagent were added to the appropriate wells in 200 μl C1q assay buffer and incubated for 1 hr at room temperature. After incubation, the wells were washed 3 times, removed from the plates, and assessed for radioactivity in a gamma counter. Results were reported as "% bound", the percentage of the added radioactivity that was ultimately bound to each well.

A standard curve was generated with each C1q assay by the addition of 0.0, 0.125, 0.25, and 1.0 μg of heat-aggregated canine IgG (HAIgG) to successive wells in place of test samples. IgG was aggregated by heating a 30 mg/ml solution for 60 min at 56°C in a water bath. For the standard curve, the bound HAIgG was detected with SpA. HAIgG was also used at 1 μg/well as a positive control for the anti-γ antibody. Individual aliquots of a single preparation of HAIgG were frozen at -65°C, and a different aliquot was used for each assay.

Canine heat-aggregated IgM (HAIgM) was added to C1q wells at 1 μg/well as a positive control for the anti-μ antibody. A single preparation of HAIgM was used for all assays, and it was stored at 4°C.

Phase I studies

The first phase of the toxicologic investigations focused on dogs in the high and medium-high dose groups. Each dog was tested for PSAIg and ESAIg, plus and minus DX in the assay buffers. The plasma of each dog was also assessed for C1q-bindable ICs. Treated dogs and untreated controls were assessed on each of 5 sample dates, but the treatment status of the samples was not revealed until the study was complete. For

each assay, values greater than the mean of control dogs plus 2 standard deviations were considered increased.

Phase II studies

Phase II studies focused on dogs 34 and 65 who were initially found to have increased PSAIg as compared to control samples. These dogs were rechallenged with DX, and baseline and serial samples were assessed over 6 wk for increases in PSAIg, ESAIg, and ICs. DX dosing began on the day baseline samples were tested. As for phase I studies, samples from treated dogs were assessed concurrently with samples from untreated controls. For each assay, values greater than the mean of control dogs plus 2 standard deviations were considered increased.

Results

Phase I

ESAIg

The positive control results for phase I RBC experiments are shown in Tables 5.2a, 5.2b, and 5.2c, and in Figure 5.1. The correlation coefficients (r values) and slopes (m values) for the SpA dose-response curves with positive control anti-DEA1.1 plasma, plus and minus DX, are shown in Table 5.2a. The means and standard deviations for these curves are depicted in Figure 5.1. Positive control values for anti-γ and anti-μ are listed in Table 5.2b and Table 5.2c, respectively. The positive controls were positive with every assay.

Table 5.3 summarizes the results for ESAIg in the presence and absence of DX using RBCs from treated and control dogs. The data are stratified by each dog's specific study number. Increased anti-γ binding was present for dog 30 in the absence (0.8%) and in the presence (0.6%) of DX. Dog 32 had increased ESAIgM in the presence of DX (0.6%). In the main, ESAIg was not increased in dogs treated with DX.

Table 5.2a. Phase I correlation coefficients (r) and slopes (m) of the SpA standard curves, plus and minus DX, for the erythrocyte surface-associated immunoglobulin (ESAIg) assay.

DX-		DX+	
r	m	r	m
0.999	12.1	0.993	8.4
0.998	9.6	0.999	7.7
0.999	11.0	0.996	7.8
0.999	10.8	0.999	10.4
0.999	9.2	0.999	8.5

Table 5.2b. Phase I positive control results for anti-γ in the assay for ESAIgG, plus and minus DX (% bound).

DX -		DX+	
0 µl αDEA 1.1*	1.6 µl αDEA 1.1	0 µl αDEA 1.1	1.6 µl αDEA 1.1
1.2	22.9	1.1	17.1
1.4	17.9	1.2	18.4
1.4	21.2	1.1	18.7
1.3	16.8	1.0	13.7
0.2	11.0	1.6	20.4

Table 5.2c. Phase I positive control results for anti-µ in the assay for ESAIgM, plus and minus DX (% bound).

DX -		DX+	
0 µl αDEA 1.1*	1.6 µl αDEA 1.1	0 µl αDEA 1.1	1.6 µl αDEA 1.1
0.3	4.5	0.2	3.2
0.4	4.5	0.4	2.8
0.2	3.3	0.2	3.4
0.3	3.0	0.2	3.8
0.3	3.0	0.2	3.3

*αDEA1.1 = positive control antiserum to Dog Erythrocyte Antigen 1.1.

Table 5.3. Phase I results for erythrocyte surface-associated immunoglobulin in treated and control dogs, plus (+) and minus (-) DX (% bound).

	SpA		anti-γ		anti-μ	
Subject	-	+	-	+	-	+
Control Dogs						
3	0.3	0.4	0.6	0.5	0.4	0.4
4	0.2	0.2	0.4	0.3	0.2	0.2
5	0.1	0.1	0.4	0.3	0.1	0.2
6	0.1	0.2	0.3	0.4	0.3	0.2
7	0.2	0.2	0.5	0.3	0.2	0.2
38	0.4	0.2	0.4	0.4	0.4	0.3
39	0.3	0.2	0.4	0.2	0.1	0.2
40	0.1	0.2	0.4	0.3	0.1	0.2
41	0.1	0.1	0.5	0.3	0.2	0.2
42	0.1	0.2	0.3	0.2	0.2	0.2
mean ± S.D.	**0.2±0.1**	**0.2±0.1**	**0.4±0.1**	**0.3±0.1**	**0.2±0.1**	**0.2±0.1**
Treated Dogs						
19	0.3	0.3	0.4	0.5	0.3	0.2
19	0.2	0.4	0.3	0.6	0.2	0.5
26	0.1	0.3	0.3	0.4	0.3	0.3
27	0.2	0.2	0.3	0.3	0.4	0.4
28	0.2	0.2	0.5	0.4	0.3	0.2
29	0.2	0.1	0.3	0.2	0.2	0.2
30	0.4	0.3	**0.8***	**0.6***	0.2	0.3
31	0.1	0.1	0.4	0.4	0.2	0.3
32	0.1	0.2	0.2	0.4	0.3	**0.6***
35	0.2	0.1	0.3	0.3	0.1	0.2
58	0.3	0.3	0.4	0.4	0.2	0.1
62	0.2	0.2	0.4	0.2	0.1	0.2
62	0.2	0.2	0.4	0.3	0.2	0.3
63	0.1	0.1	0.3	0.2	0.1	0.1
64	0.3	0.1	0.3	0.4	0.2	0.2
66	0.2	0.1	0.5	0.3	0.4	0.2
67	0.2	0.2	0.3	0.4	0.3	0.5
68	0.1	0.1	0.4	0.4	0.2	0.1
69	0.2	0.2	0.3	0.2	0.2	0.2
70	0.1	0.1	0.4	0.3	0.1	0.2
mean	**0.2**	**0.2**	**0.4**	**0.3**	**0.2**	**0.2**

* Values exceed mean + 2 S.D. of control dogs.

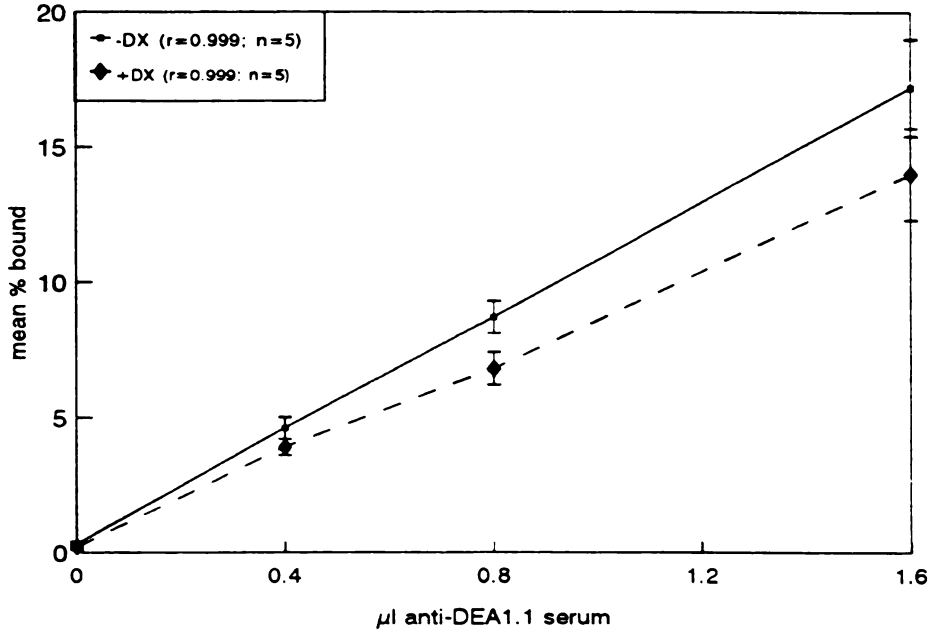

Figure 5.1. Phase I mean SpA standard curves, plus and minus DX, for the erythrocyte surface-associated immunoglobulin assay.

PSAIg

The correlation coefficients and slopes for the SpA dose-response curves with canine plasma containing anti-[canine platelet] antibodies, plus and minus DX, are shown in Table 5.4a. The means and standard deviations for these curves are depicted in Figure 2. Table 5.4b shows the percentage binding of positive control plasma, plus and minus DX, using anti-γ. The activity of the anti-μ antibody was verified using the C1q and ESAIg anti-μ positive controls. The positive controls were positive with every assay.

The PSAIg results for treated and untreated dogs, plus and minus DX, are shown in Table 5.5 where they are stratified by specific dog number. On initial testing, dog 19 had increased platelet binding of SpA without DX (1.9%), of anti-γ with (1.0%) and without (2.3%) DX, and of anti-μ with (4.8%) and without (6.8%) DX. A week later, dog 19 was again positive for PSAIg using SpA, both with (1.9%) and without (2.7%) DX. The binding of anti-γ was also increased without DX (0.9%), and the binding of anti-μ was increased with (4.2%) and without (5.3%) DX. Dog 29 had increased PSAIg using SpA with DX (2.4%) and using anti-γ with DX (2.3%). These data indicate that dog 19 had mildly increased PSAIgG and PSAIgM while being treated with DX. However, increased PSAIg was very mild and uncommon in DX-treated dogs.

Table 5.4a. Phase I correlation coefficients (r) and slopes (m) of the SpA standard curves, plus and minus DX, for the platelet surface-associated immunoglobulin (PSAIg) assay.

DX -		DX +	
r	m	r	m
0.996	31.1	0.998	27.9
0.999	33.3	0.997	25.5
0.998	34.8	0.995	27.6
0.999	33.7	0.999	29.3
0.999	31.5	0.999	32.8

Table 5.4b. Phase I positive control results for anti-γ in the assay for PSAIgG, plus and minus DX (% bound).

DX -		DX +	
0 µl PC*	1.0 µl PC	0 µl PC	1.0 µl PC
0.8	23.8	0.6	26.4
0.9	23.1	1.0	27.4
0.9	25.0	0.9	25.3
1.0	25.0	1.2	24.0
0.8	22.3	0.8	24.3

*PC = positive control plasma

Table 5.5. Phase I results for platelet surface-associated immunoglobulin in treated and control dogs, plus (+) and minus (-) DX (% bound).

	SpA		anti-γ		anti-μ	
Subject	-	+	-	+	-	+
Control Dogs						
3	0.3	0.4	0.6	0.5	1.5	1.0
4	0.7	0.5	0.9	0.7	2.8	2.3
5	0.4	1.0	0.6	0.6	1.0	0.8
6	0.7	0.6	0.6	0.3	1.6	1.1
7	0.5	0.2	0.4	0.9	2.4	1.4
38	0.6	0.3	0.6	0.5	1.5	1.1
39	0.6	0.6	0.6	0.5	1.1	1.1
40	0.8	2.0	0.6	1.4	2.1	1.8
41	0.6	0.3	0.7	0.5	2.7	1.8
42	0.3	0.3	0.5	0.9	2.2	1.3
mean ± S.D.	**0.6±0.2**	**0.6±0.5**	**0.6±0.1**	**0.7±0.3**	**1.9±0.6**	**1.4±0.5**
Treated Dogs						
19	**1.9***	1.3	**1.0***	**2.3***	**6.8***	**4.8***
19	**2.7***	**1.9***	**0.9***	0.8	**5.3***	**4.2***
26	0.5	0.2	0.4	0.4	1.2	0.9
27	0.3	0.3	0.7	0.3	0.8	0.8
28	0.2	0.2	0.3	0.8	0.9	0.8
29	0.2	0.5	0.4	0.4	2.8	**3.4***
30	0.6	1.1	0.4	0.4	0.8	0.7
31	0.3	0.9	0.5	0.7	1.2	1.0
32	0.2	0.2	0.5	0.4	1.3	1.3
35	0.4	0.3	0.4	0.3	1.9	1.4
58	0.5	0.3	0.6	0.5	1.7	1.4
62	0.2	**2.4***	0.5	**2.3***	2.0	1.7
62	0.4	0.3	0.5	0.4	1.4	1.8
63	0.7	0.5	0.4	1.2	2.3	1.7
64	0.5	0.5	0.6	0.4	2.1	1.8
66	0.4	0.3	0.5	0.4	1.0	1.2
67	0.6	0.8	0.7	0.6	2.0	1.7
68	0.4	0.7	0.5	0.4	1.3	0.8
69	0.4	0.4	0.4	0.4	1.5	1.8
70	0.4	0.3	0.5	1.1	2.8	1.3
mean	**0.6**	**0.7**	**0.5**	**0.6**	**2.0**	**1.7**

* Values exceed mean + 2 S.D. of control dogs.

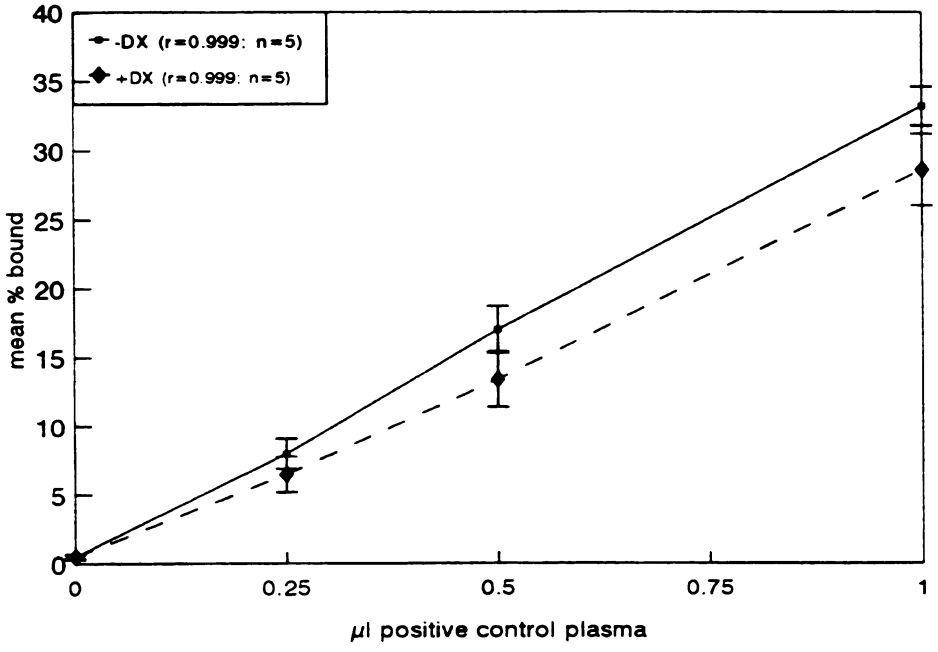

Figure 5.2. Phase I mean SpA standard curves, plus and minus DX, for the platelet surface-associated immunoglobulin assay.

ICs

The third component of the phase I investigations was analysis of plasma samples for soluble immune complexes using the solid phase C1q assay. The correlation coefficients and the slopes for the SpA dose-response curves with C1q-bound HAIgG are shown in Table 5.6a. The means and standard deviations for these curves are depicted in Figure 3. The positive control results for anti-γ detecting C1q-bound HAIgG are shown in Table 5.6b. Similarly, Table 5.6c is a summary of the positive control results obtained using HAIgM with the anti-μ antibody. The positive controls were positive with every assay.

C1q assay results for the control and treated dogs are shown in Table 5.7. The data are stratified by specific dog number. On initial testing, dog 19 had increased ICs as detected by SpA (1.6%) and anti-μ (3.2%). Repeat determinations for dog 19 one week later again showed increased binding of SpA (3.9%). Dog 64 had increased binding of anti-γ (1.7%) and of SpA (4.0%) at one of the two plasma volumes tested. Plasma from dog 67 produced increased binding of anti-γ (2.2%) and anti-μ (3.6%). The positive values for dogs 19, 64, and 67 suggest that these dogs had increased circulating immune complexes when tested. However, increases in ICs in DX-treated dogs were neither consistent nor marked.

Table 5.6a. Phase I correlation coefficients (r) and slopes (m) of the SpA standard curves for the C1q assay.

r	m
0.980	12.0
0.997	9.0
0.960	8.5
0.988	11.1
0.997	21.3

Table 5.6b. Phase I positive control results (% bound) for C1q-bindable IgG using heat-aggregated IgG.

0 μg HAIgG	2.0 μg HAIgG
0.2	11.9*
0.2	12.1
0.1	7.6
0.2	11.4
0.2	9.4

* in duplicate

Table 5.6c. Phase I positive control results (% bound) for C1q-bindable IgM using heat-aggregated IgM.

0 μg HAIgM	1.0 μg HAIgM
0.4	5.9
0.6	8.0
0.3	4.4
0.6	7.5
0.5	6.3

Table 5.7. Phase I results for C1q-bindable immune complexes in treated and control dogs (% bound).

	SpA		anti-γ	anti-μ
Subject	0.2 μl	1.0 μl**	0.2 μl	0.2 μl
Control dogs				
3	0.6	1.8	0.6	1.1
4	0.9	2.0	0.5	1.4
5	0.7	2.5	0.6	0.9
6	1.5	3.9	0.8	2.2
7	1.2	1.8#	0.2	2.7
38	0.8	2.1	0.5	1.9
39	0.6	1.6	0.4	1.0
40	0.9	3.0	0.6	1.2
41	0.9	2.6	0.7	1.9
42	1.1	1.6#	0.6	2.2
Mean ± S.D.	**0.9±0.3**	**2.4±0.7**	**0.6±0.2**	**1.6±0.6**
Treated dogs				
19	**1.6***	2.5#	0.9	**3.2***
19	1.3	**3.9***	0.9	2.5
26	0.6	2.3	0.5	1.5
27	1.0	3.8	0.8	1.4
28	1.2	1.6#	**1.1***	2.2
29	0.8	2.2	0.5	1.3
30	0.9	2.0	0.4	1.0
31	1.3	3.1	0.5	1.5
32	1.2	3.1	0.6	2.0
35	1.0	2.4	0.7	1.9
58	1.0	2.9	1.5	1.9
62	1.2	2.8	0.4	1.4
62	0.8	2.1	0.5	1.5
63	0.8	1.3#	0.6	1.8
64	1.2	**4.0***	**1.7***	2.0
66	0.6	1.7	0.5	1.1
67	1.1	0.8	**2.2***	**3.6***
68	1.1	3.2	0.5	1.5
69	0.6	1.8	0.4	1.2
70	0.6	1.8†	0.2	1.2

* Values exceed mean + 2 S.D. of control dogs.

\# Samples were run at 0.4 μl instead of 1.0 μl. and were not included in the mean.

** Values for dogs numbered 28 through 69 are means of duplicate determinations.

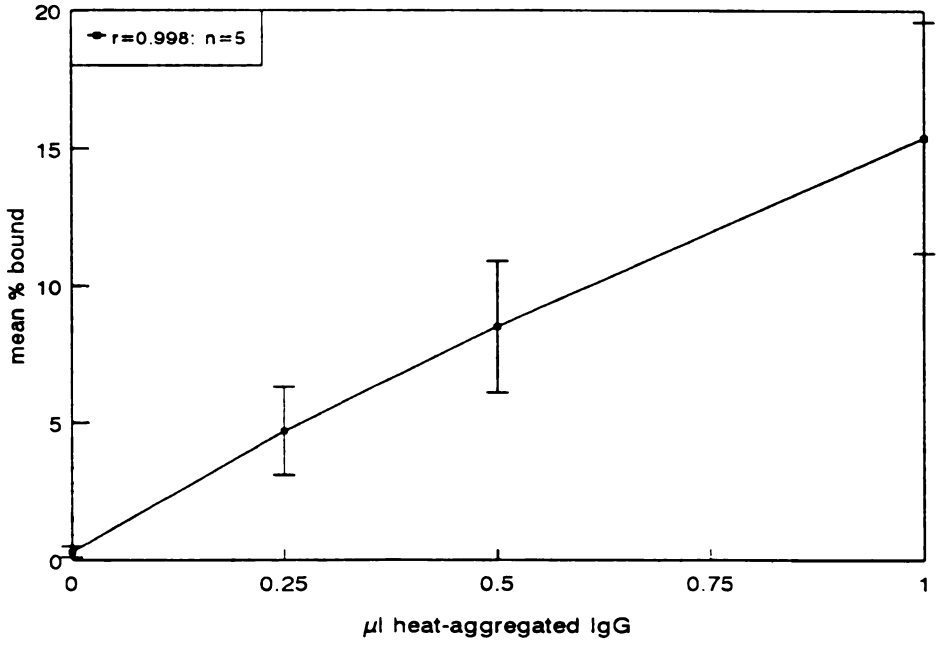

Figure 5.3. Phase I standard curve for the C1q assay for immune complexes.

Phase II

Phase II studies were used to evaluate dogs 34 and 65 for the development of PSAIg, ESAIg, or ICs with DX rechallenge beginning after sampling on day 1.

ESAIg

Table 5.8a lists the correlation coefficients and slopes for the SpA dose-response curves using positive control anti-DEA1.1 plasma, plus and minus DX. The means and standard deviations for these curves are graphed in Figure 4. Positive controls, plus and minus DX, are shown for anti-γ in Table 5.8b, and for anti-μ in Table 5.8c. The positive controls were positive with every assay.

ESAIg results for dogs 34, 65, and the control dogs are summarized in Table 5.9, where they are stratified by specific dog number. Increased binding of SPA without DX occurred with RBCs obtained from dog 34 on day 30 (0.5%). On day 16, RBCs from dog 65 had increased binding of anti-γ (0.7%) and anti-μ (0.5%) without DX. Increases in ESAIg were sporadic and very mild.

Table 5.8a. Phase II correlation coefficients (r) and slopes (m) of the SpA standard curves, plus and minus DX, for the erythrocyte surface-associated immunoglobulin (ESAIg) assay.

	DX -		DX +	
Day	r	m	r	m
1	0.999	8.7	0.992	7.6
10	0.999	10.9	0.999	10.8
16	0.999	11.0	0.999	8.8
24	0.998	8.0	0.999	8.6
30	0.999	7.7	0.995	9.4
42	0.999	9.9	0.998	8.8

Table 5.8b. Phase I I positive control results for anti-γ in the assay for ESAIgG, plus and minus DX (% bound).

	DX-		DX+	
Day	0 µl αDEA 1.1*	1.6 µl αDEA 1.1	0 µl αDEA 1.1	1.6 µl αDEA 1.1
1	1.2	19.1	1.0	18.9
10	1.1	16.3	1.1	18.0
16#	1.7	27.4	1.7	31.2
24	1.6	28.7	1.2	23.4
30	2.1	24.8	2.0	23.4
42	1.9	23.8	1.6	24.2

Table 5.8c. Phase II positive control results for anti-µ in the assay for ESAIgM, plus and minus DX (% bound).

	DX-		DX+	
Day	0 µl αDEA 1.1	1.6 µl αDEA 1.1	0 µl αDEA 1.1	1.6 µl αDEA 1.1
1	0.2	3.1	0.2	2.7
10	0.3	3.6	0.2	3.1
16#	0.3	6.4	0.4	6.0
24	0.2	5.0	0.4	4.6
30	0.4	3.2	0.4	3.1
42	0.2	4.4	0.2	3.7

* αDEA 1.1 = positive control antiserum to Dog Erythrocyte Antigen 1.1.

\# New labeling of anti-γ and anti-µ (same lot numbers).

Table 5.9. Phase II results for erythrocyte surface-associated immunoglobulin in treated and control dogs, plus (+) and minus (-) DX (% bound).

		SpA		anti-γ		anti- μ	
Subject	Day	-	+	-	+	-	+
Control Dogs							
5		0.2	0.3	0.4	0.4	0.1	0.2
5		0.2	0.3	0.6	0.5	0.3	0.2
7		0.3	0.4	0.5	0.3	0.2	0.1
40		0.2	0.2	0.4	0.4	0.2	0.2
40		0.2	0.2	0.5	0.7	0.3	0.3
41		0.3	0.3	0.4	0.3	0.2	0.1
43		0.4	0.6	0.6	0.5	0.3	0.2
43		0.2	0.4	0.2	0.3	0.2	0.3
84		0.4	0.4	0.5	0.5	0.3	0.3
84		0.2	0.2	0.4	0.2	0.2	0.2
16		0.3	0.2	0.3	*	0.2	*
60		0.1	0.4	0.2	*	0.2	*
mean ± S.D.		**0.2±0.1**	**0.3±0.1**	**0.4±0.1**	**0.4±0.1**	**0.2±0.1**	**0.2±0.1**
Treated Dog							
34	1	0.1	0.2	0.3	0.4	0.2	0.2
34	10	0.1	0.1	0.3	0.3	0.1	0.1
34	16	0.3	0.2	0.5	0.5	0.3	0.2
34	24	0.2	0.2	0.2	*	0.2	*
34	30	**0.5#**	0.2	0.4	0.4	0.2	0.3
34	42	0.2	0.1	0.3	0.2	0.2	0.1
Treated Dog							
65	1	0.4	0.3	0.4	0.4	0.3	0.2
65	10	0.2	0.2	0.3	0.4	0.2	0.1
65	16	0.3	0.3	**0.7#**	0.4	**0.5#**	0.2
65	24	0.2	0.3	0.2	*	0.2	*
65	30	0.3	0.4	0.4	0.4	0.3	0.2
65	42	0.1	0.1	0.3	0.2	0.2	0.3

Values exceed mean + 2 S.D. of control dogs.

* Gamma counter problem; data were not retrievable.

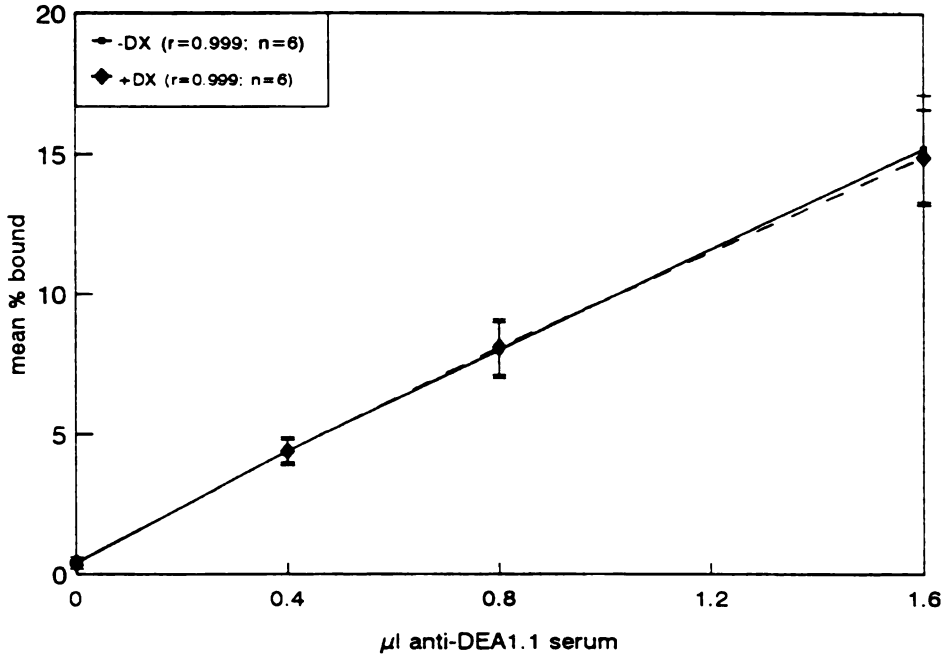

Figure 5.4. Phase II SpA standard curves, plus and minus DX, for the erythrocyte surface-associated immunoglobulin assay.

PSAIg

Table 5.10a lists the correlation coefficients and slopes for the SpA dose-response curves using plasma containing anti-[canine platelet] antibodies, plus and minus DX. The means and standard deviations for these curves are graphed in Figure 5. Positive control values for PSAIgG, plus and minus DX, are listed in Table 5.10b. The positive controls were positive with every assay.

PSAIg results for dogs 34, 65, and the control dogs are summarized in Table 5.11 and stratified by specific dog number. Increased SpA binding occurred on day 16 with platelets from dog 34, both with (1.1%) and without (1.2%) DX. On the same date, anti-γ binding was increased with DX (2.4%), and anti-μ binding was increased with (5.3%) and without (6.3%) DX. Repeat determinations on dog 34 1 wk later (day 24) failed to show increased PSAIg. However, 2 wk later, increased SpA binding was again observed with (1.1%) and without (1.1%) DX. Anti-μ binding was also increased with (3.4%) and without (4.3%) DX. No increases in PSAIg were detected in dog 65 throughout the rechallenge period.

Table 5.10a. Phase II correlation coefficients (r) and slopes (m) of the SpA standard curves, plus and minus DX, for the platelet surface-associated immunoglobulin (PSAIg) assay.

	DX -		DX +	
Day	r	m	r	m
1	0.999	30.8	0.999	31.2
10	0.999	33.6	0.998	31.4
16	0.999	34.7	0.999	36.7
24	0.999	35.7	0.996	37.6
30	0.998	32.4	0.998	38.4
42	0.999	38.3	0.999	35.1

Table 5.10b. Phase II positive control results for anti-γ in the assay for PSAIgG, plus and minus DX (% bound).

	DX -		DX+	
Day	0 µl PC	1.0 µl PC	0 µl PC	1.0 µl PC
1	0.8	23.4	0.9	23.2
10	0.8	24.0	0.7	21.6
16*	1.1	43.4	1.0	40.1
24	1.1	39.1	1.1	38.4
30	1.0	36.7	1.0	36.1
42	1.1	33.0	1.1	32.7

* New labeling of anti-γ and anti-µ (same lot numbers).

Table 5.11. Phase II results for platelet surface-associated immunoglobulin in treated and control dogs, plus (+) and minus (-) DX (% bound).

		SpA		anti-γ		anti-μ	
Subject	Day	-	+	-	+	-	+
Control Dogs							
5		0.3	0.3	0.5	0.4	1.1	0.7
5		0.6	0.3	0.9	0.8	1.4	1.2
7		0.9	0.8	0.5	0.5	2.6	2.2
40		1.0	0.5	0.6	0.6	1.7	1.2
40		0.7	0.8	1.1	0.8	3.0	2.6
41		0.6	0.5	0.8	0.6	2.2	1.9
43		0.4	0.7	0.7	0.7	0.8	0.7
43		0.2	0.5	0.6	0.8	0.6	0.8
84		0.8	0.5	1.2	1.0	2.6	2.6
84		0.4	0.2	0.7	0.6	1.2	0.9
16		0.4	0.4	0.7	0.6	1.3	1.1
60		0.3	0.5	1.0	1.0	1.2	1.5
mean ± S.D.		**0.6±0.2**	**0.6±0.2**	**0.8±0.2**	**0.8±0.2**	**1.6±0.8**	**1.4±0.7**
Treated Dog							
34	1	0.5	0.3	0.6	0.5	2.0	1.0
34	10	0.6	0.4	0.5	0.5	1.6	1.4
34	16	**1.2***	**1.1***	1.1	**2.4***	**6.3***	**5.3***
34	24	0.7	0.5	0.7	0.7	1.8	1.7
34	30	**1.1***	**1.1***	0.9	0.7	**4.3***	**3.4***
34	42	0.5	0.3	0.7	0.6	2.2	1.9
Treated Dog							
65	1	0.8	0.4	0.4	0.5	1.8	1.0
65	10	0.3	0.4	0.4	0.4	1.2	1.0
65	16	0.6	0.4	0.9	0.8	3.0	2.6
65	24	0.4	0.6	0.6	0.6	1.8	1.9
65	30	0.4	0.6	0.8	0.7	3.0	2.5
65	42	0.3	0.2	0.6	0.5	1.5	1.7

* Values exceed mean + 2 S.D. of control dogs.

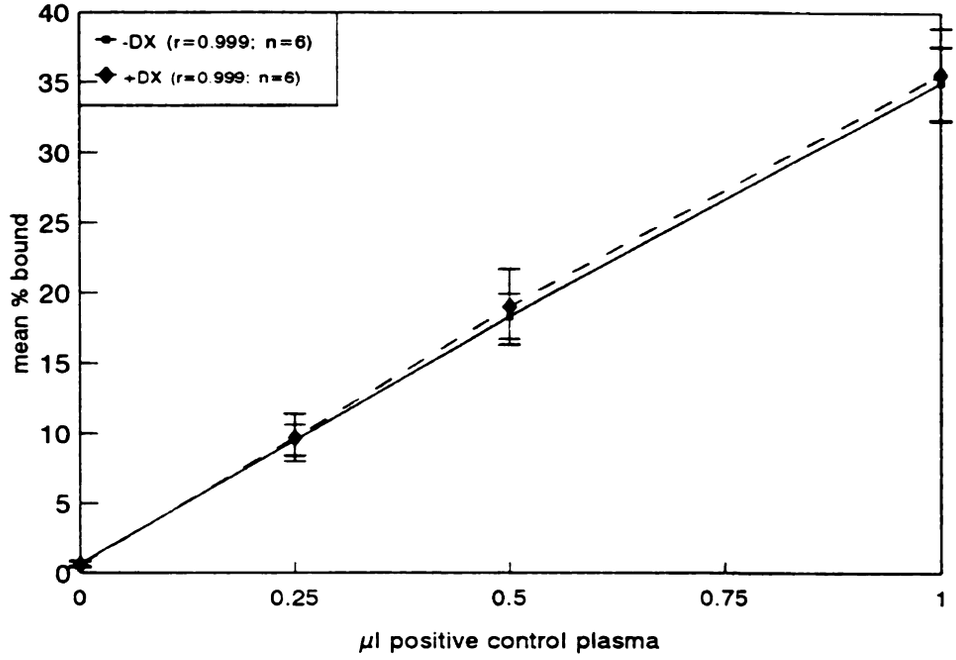

Figure 5.5. Phase II mean SpA standard curves, plus and minus DX, for the platelet surface-associated immunoglobulin assay.

ICs

The same blood samples were used for analysis of ICs. The correlation coefficients and slopes for the SpA dose-response curves using HAIgG are listed in Table 5.12a. The means and standard deviations for these curves are graphed in Figure 6. Table 5.12b lists the results for the positive controls using anti-γ, and Table 5.12c lists those obtained with anti-μ. The positive controls were positive with every assay.

C1q results for dogs 34, 65, and the control dogs are listed in Table 5.13. The data are organized by specific dog number. Increased SpA binding was present on day 16 for dog 65 (2.3%). All other values were within 2 standard deviations of the control group.

Table 5.12a. Phase II correlation coefficients (r) and slopes (m) of the SpA standard curves for the C1q assay.

Day	r	m
1	0.995	10.2
10	0.999	10.4
16	0.998	13.1
24	0.998	9.4
30	0.994	17.6
42	0.988	16.3

Table 5.12b. Phase II positive control results (% bound) for C1q-bindable IgG using heat-aggregated IgG.

Day	0 μg HAIgG	2.0 μg HAIgG
1	0.2	7.6
10	0.2	8.6
16*	0.3	18.4
24	0.3	14.0
30	0.4	13.7
42	0.3	12.1

Table 5.12c. Phase II positive control results (% bound) for C1q-bindable IgM using heat-aggregated IgM.

Day	0 μg HAIgM	1.0 μg HAIgM
1	0.3	4.6
10	0.3	4.9
16*	0.6	9.2
24	0.6	5.1
30	0.4	5.5
42	0.4	6.6

* New labeling of anti-γ and anti-μ (same lot numbers)

Table 5.13. Phase II results for C1q-bindable immune complexes in treated and control dogs (% bound).

		SpA		anti-γ		anti-μ	
Subject	Day	0.2 μl	1.0 μl	0.2 μl	1.0 μl	0.2 μl	1.0 μl
Control Dogs							
5		0.7	1.5	0.4	1.2	0.7	1.8
5		1.6	2.2	1.4	4.0	1.5	3.7
7		0.9	2.7	0.6	1.5	1.0	2.9
40		0.8	1.6	0.4	1.1	0.8	2.1
40		1.3	2.2	1.2	3.3	1.9	4.7
41		0.8	1.7	0.4	0.8	0.9	2.3
16		1.8	3.8	1.0	2.8	1.4	3.4
60		1.3	4.0	0.9	2.4	2.2	4.8
43		0.9	2.4	1.4	3.9	1.1	2.6
43		1.0	2.8	1.0	2.9	1.2	3.0
84		1.1	3.0	1.1	3.0	1.4	3.4
84		1.2	3.0	0.8	2.5	1.6	3.3
mean ± S.D.		**1.1±0.3**	**2.6±0.8**	**0.9±0.4**	**2.4±1.1**	**1.3±0.4**	**3.2±0.9**
Treated Dog							
34	1	0.7	1.8	0.3	0.9	0.8	2.0
34	10	1.0	1.8	0.3	0.9	0.9	2.2
34	16	1.5	2.0	1.0	2.5	1.6	4.1
34	24	1.4	4.0	1.1	3.0	1.7	3.8
34	30	0.9	2.2	1.0	2.4	1.2	3.0
34	42	1.0	2.9	0.6	1.9	1.3	3.3
Treated Dog							
65	1	0.8	1.4	0.4	0.9	0.8	2.0
65	10	0.9	1.6	0.3	0.8	0.8	2.3
65	16	**2.3***	2.1	0.9	2.5	1.8	4.7
65	24	1.3	3.4	1.0	2.7	1.7	3.9
65	30	0.6	1.3	0.8	1.9	1.1	2.8
65	42	0.8	1.6	0.7	1.6	1.0	2.4

* Value exceeds mean + 2 S.D. of control dogs.

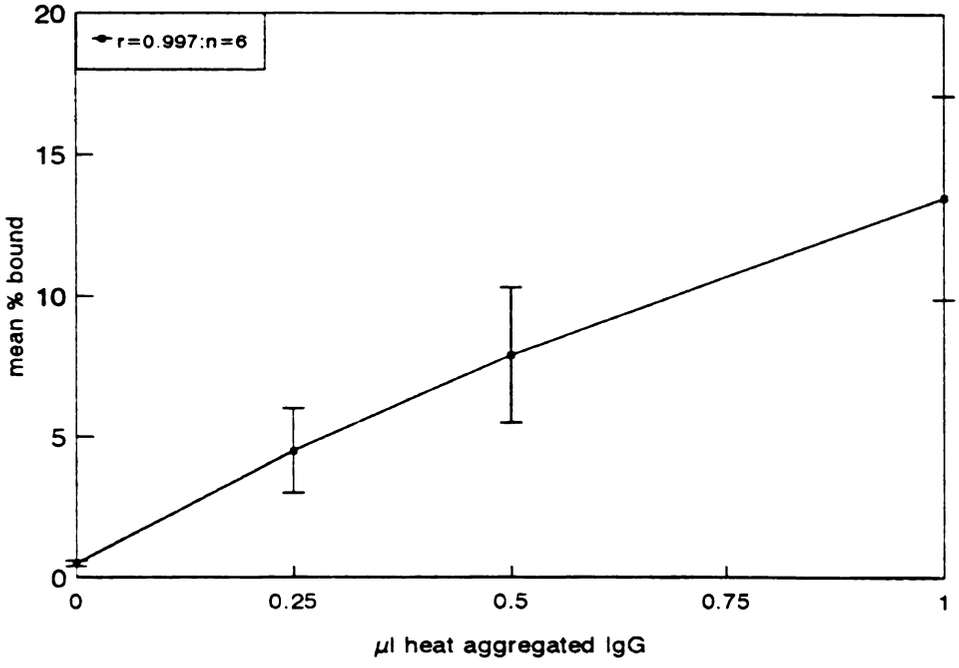

Figure 5.6. Phase II SpA standard curve for the C1q assay for immune complexes.

Discussion

The strong initial positive results for PSAIg in dogs 34 (16.9%) and 65 (44.8%) (reference mean $\pm$ S.D. = 3.0 $\pm$ 1.6) suggested that at that time of thrombocytopenia and exposure to DX, PSAIg may have caused or contributed to the thrombocytopenias. It is also possible that sample shipment caused increases in nonspecific PSAIg in the affected dogs but not in the controls. However, this possibility lacks a clear explanation.

The cumulative results of the phase I studies indicated that dog 19, who was in the medium-high dose group, did have increased PSAIg and increased circulating C1q-bindable immunoglobulins. Samples from dog 19 on initial testing had increased PSAIg in the absence of DX as detected by SpA, anti-γ, and anti-μ; PSAIg was also elevated in the presence of DX as detected by anti-γ and anti-μ. On recheck of dog 19 one week later, PSAIg was still increased with all 3 detectors in the absence of DX; it was also increased in the presence of DX using SpA and anti-μ. On both dates, increased C1q-bindable immune complexes were also detected. Later, at necropsy, the bone marrow of this dog was remarkable because it was hypercellular and similar to those of dogs 34 and 65 on initial evaluation. The dog also had notable splenic hematopoiesis. This indicated that hematopoietic stimulation was occurring in the presence of mildly increased PSAIg and circulating ICs. This suggests that humoral immunoglobulins may have contributed to the DX-associated thrombocytopenia in dog 19. However, in human patients with regenerative thrombocytopenias, mild increases in PSAIg may be detectable when immunological contributions are presumed to be absent.[3]

Phase I studies also identified other dogs with mildly elevated results, but these increases did not form a consistent pattern as they did for dog 19. It is therefore likely

that these results were not biologically significant. The overall conclusion from the phase I studies was that the administration of high doses of DX was not associated with antibody-mediated destruction of RBCs or platelets or with the formation of C1q-bindable immunoglobulins in most dogs.

In the phase II studies, dogs 34 and 65 were rechallenged with DX. While they showed clinical signs of toxicity quite rapidly, neither dog rapidly developed thrombocytopenia as would be expected from an anamnestic immune response. Instead, each developed a slowly progressive thrombocytopenia with nadirs of about 50-100,000/μl after 2-4 wk of dosing. The thrombocytopenia of dog 34 resolved spontaneously in the face of continued DX administration. This was also not typical of a drug-induced immune-mediated thrombocytopenia. While mild increases in PSAIg were present for dog 34 on days 16 and 30, they were not detected on day 24. Certainly, rechallenge of these dogs with DX did not produce a strong immune response with the formation of antibodies to RBCs or platelets, or with the formation of C1q-bindable immune complexes. Humoral immune mechanisms therefore did not seem to contribute to the noted clinical toxicity.

Taken together, the data from the initial studies as well as from phase I and phase II studies suggested that the sporadic cytopenias with increased marrow cellularity that occurred in DX-treated dogs were not principally mediated by humoral immunity. Some of the data suggested the presence of increased antibodies, but the data viewed as a whole strongly argue for an alternative pathogenic mechanism. With the methods used, we cannot exclude the possibility that the cytopenias were due to an immune response to a DX metabolite that became associated with the cells.[4] However, the lack of an

anamnestic response and the reversibility of the cytopenias while still receiving DX argue against an immunological mechanism if the initial and later drug formulations were indeed identical. Additional possibilities include interference with cell maturation akin to that produced by deficiencies of vitamin B12 and/or folate in human patients.[5] The combination of marrow hypercellularity with pancytopenia, as occurred in some dogs, is also similar to a myelodysplastic process.[5] DX may also have interfered with some maturation or regulation mechanism that was not assessed by these studies.

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SUMMARY AND CONCLUSIONS

Thrombocytopenia commonly occurs in dogs, and it has often been considered immune-mediated when other causes have been ostensibly excluded. In people, immune-mediated thrombocytopenia (IMT) may be a primary entity without any apparent disease associations, or it may occur secondary to systemic immune-mediated diseases, infections, neoplasia, and drug exposures. Contributions of immune-mediated platelet destruction to canine thrombocytopenic conditions are often suspected but rarely proven. This is due to the lack of reliable assays for platelet surface-associated immunoglobulins (PSAIg), and to a general lack of understanding about the interactions of canine platelets with immunoglobulins.

To investigate the role of humoral immunity in canine thrombocytopenic diseases, a reliable assay for PSAIg first had to be developed. A sensitive and reproducible immunoradiometric assay for detecting canine PSAIg and platelet-bindable immunoglobulin (PBIg) circulating in patient plasma is desribed in chapter two of this dissertation. Platelet reactive autoantibodies, alloantibodies, and xenogeneic antibodies were reproducibly detected in a dose-response fashion with ^{125}I-labeled staphylococcal protein A (SpA). Radiolabeled antibodies to canine IgG(γ) and IgM(μ) were used in conjunction with SpA to define the class of platelet-reactive antibodies and to detect SpA-nonbindable immunoglobulins. Unlike other assays to date, an autoantibody from a dog with clinical

IMT was used as the positive control for the assay. A glutaraldehyde fixation procedure was developed to provide a source of platelets with consistent reactivity to the positive control plasma and other antibodies.

In the course of testing this assay on known positive plasma samples and on clinical samples from thrombocytopenic dogs, it became evident that consistent sample management was critical for obtaining reproducible results. In short, the exposure of blood samples to 4°C or room temperature (RT) led to progressive increases in PSAIg which could make some normal samples appear positive within hours. The storage of isolated plasma at 4°C or RT also led to progressive and dramatic increases in PBIg, mostly IgM. False positive results due to this nonspecific PSAIg and PBIg, mostly IgM, were avoided by using fresh 37°C blood samples and 37°C plasma incubation temperatures.

Clinically, the assay appeared to be useful for differentiating dogs with IMT from dogs with nonimmune thrombocytopenia. Of 24 dogs given a diagnosis of IMT, results for PSAIg (usually IgG) were positive in 23. Of the other 19 thrombocytopenic dogs tested, 5 had increases in PSAIg that were considered clinically significant, and it is likely that immune-mediated platelet destruction played a principal role in the pathogenesis of thrombocytopenia for 4 of these dogs.

Results using SpA in this assay and in other procedures led to the suspicion that, contrary to recent reports, SpA did not bind all of canine IgG and IgM. Experiments were designed to investigate this possibility and to provide an estimate of the percentage of canine IgG and IgM that do bind to Cowan I strain SpA. Approximately 20% of canine IgG and 33% of canine IgM did not bind to SpA under conditions of liquid

affinity chromatography or to ^{125}I-SpA using an immunoradiometric assay similar to the one for PSAIg. This incomplete reactivity prevents precise quantitation of PSAIg with SpA, but qualitative results may be affected little if platelet-reactive antibodies in dogs with clinical IMT are generally polyclonal in nature.

While the described assay was a critical step in being able to explore canine IMT, other basic information about canine platelets was essential. In human beings, immunoglobulins may bind to platelets by the platelet Fcγ receptor for IgG. This Fc receptor is important in some drug-induced thrombocytopenias such as heparin-induced thrombocytopenia, and it can mediate a signal to the cell interior resulting in platelet activation. To assess canine platelets for a functional Fcγ receptor analogous to that of human platelets, the responses of human and canine platelets to heat-aggregated IgG (HAIgG) were compared in a platelet aggregometer (chapter 3). Canine and human HAIgG caused Fc-mediated aggregation of human gel-filtered platelets (GFP) that was blocked by the anti-FcγRII monoclonal antibody IV.3. In contrast, canine GFP were unresponsive to HAIgG. Dogs therefore lack a functional analogue of the human Fcγ receptor.

However, in platelet-rich plasma and whole blood, HAIgG induced delayed and submaximal clumping of canine platelets. This suggested the possibility that immune complexes may affect canine platelets in vivo, despite the lack of a functional platelet Fcγ receptor. Studies were undertaken to investigate the mechanism of this potentially important effect of immunoglobulins on canine platelets. In chapter three is reported the series of studies that identified complement as the essential plasma cofactor for

HAIgG-induced platelet clumping. Complement protein C3 was shown to be specifically required by the failure of canine plasma naturally deficient in C3 to support clumping.

The mechanism of HAIgG-induced platelet clumping was further explored through the use of electron microscopy and platelet aggregometry with platelet metabolic inhibitors (chapter 4). In summary, HAIgG activates canine complement leading to the binding of C3 to the Fab portion of HAIgG and to an apparent platelet C3 receptor (presumably a C3b receptor). In this way, the platelets are passively held together by interplatelet bridges of C3-HAIgG. This phenomenon, known as immune adherence, may be important in binding immunoglobulins to canine platelets in vivo and in mediating thrombocytopenia. It may be significant that HAIgG-induced agglutination sometimes progressed to active aggregation. This suggests the possibility of signal transduction through a platelet C3 receptor. It is possible that the presumed canine platelet C3b receptor may play a role in immune complex thrombocytopenias that in people are mediated by platelet Fc receptors.

In the fifth chapter of this dissertation, the assay for PSAIg was applied to a toxicologic study of a proprietary drug to help explain the cause of thrombocytopenia occurring in some test dogs. While the results of this study suggested that immune mechanisms were not of principal importance, the studies demonstrate the potential for use of the developed assay in toxicologic studies. This assay can be used similarly in other toxicologic investigations and for clinical cases of suspected drug-induced IMT in dogs.

Together, the studies described in this dissertation have provided essential groundwork for further investigations of canine IMT. With a reliable assay for PSAIg,

the contribution of humoral immunity to any canine thrombocytopenic condition of interest can now be assessed. This includes studies of autoimmunity and alloimmunity, as well as investigations of infectious, neoplastic, and drug-induced thrombocytopenia. The assay can also be used experimentally to better define and characterize canine platelet/immunoglobulin interactions.

The described studies have also generated many new questions and the need for more research. Further work is necessary to explore the importance of immune adherence in infectious, neoplastic, and drug-induced canine thrombocytopenias. The presence of a canine platelet C3 receptor should be verified by its direct identification and characterization. In vitro studies similar to those reported here with HAIgG should be repeated with more physiologic immune complexes, and studies should be designed to assess the effects of immune complexes on canine platelets in vivo. Whether or not immune adhered immunoglobulin is detected as PSAIg must also be determined, as this has great relevance for interpreting the meaning of positive assay results. These are but a few of the major paths leading from this work.

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VITA

Michael Alan Scott was born in Northampton, MA, on September 23, 1957. He attended kindergarten through high school in the Ripon public school system in Ripon, WI, except for one year in third form in Norwich, England. He graduated from high school as valedictorian of his class in 1976. He then attended Ripon College where he was elected to Phi Beta Kappa in 1980. The same year, he graduated Summa Cum Laude with a Bachelor of Arts degree (biology major, mathematics minor). After marrying Catherine Jo Galica (registered occupational therapist) in 1982, he moved to St. Paul, MN, where he attended the College of Veterinary Medicine, University of Minnesota. He was elected to Phi Zeta in 1985, and received several awards including the Minnesota Veterinary Medical Association Award for the senior selected as the outstanding student in clinical veterinary medicine for 1986. He received the degree of Doctor of Veterinary Medicine in 1986 (third in class), the same year his daughter Kimberly was born. He then enjoyed private veterinary practice for two years in a predominantly dairy practice in Weyauwega, WI. His son David was born in 1987. In 1988, he returned to academia as a clinical pathology resident/instructor in the Department of Pathology at Michigan State University. His son Ian was born in 1990, the year he began a doctorate program as a Fellow in a National Institutes of Health Training Program in Pathology/Toxicology, also at Michigan State University. He

became a Diplomate in the American College of Veterinary Pathologists (clinical pathology) in 1991, and he received the Student Scholarship Award for the C.L. Davis D.V.M. Foundation in 1993. He completed his Ph.D. in Pathology/Environmental Toxicology in 1995, and accepted an appointment as Assistant Professor in pathology at the University of Missouri, Columbia.