Differential Diagnosis of Hemolytic Anemia

Hemolytic anemia reflects shortened RBC life span due to intravascular, extravascular, or fragmentation hemolysis. Intravascular hemolysis is primarily associated with type 1 hypersensitivity reactions, either idiopathic or secondary to another primary disease. In some cases an infectious intraerythrocytic or epierythrocytic agent or miscellaneous disorders (e.g., severe hypophosphatemia, zinc toxicity, or severe heinz body anemia) are underlying causes. Extravascular hemolysis is usually caused by RBC removal by the mononuclear phagocyte system and often reflects idiopathic primary or secondary immune-mediated mechanisms. In some cases, hemolysis is secondary to heinz bodies, membrane abnormalities, or enzymatic defects, limiting cell energy production (e.g., pyruvate kinase, phosphofructokinase after exercise induced respiratory alkalosis) and deranging membrane flexibility. Fragmentation hemolysis is associated with shearing effects that damage RBC flexibility crucial for navigating microcirculatory pathways (e.g., vasculitis, hemangiosarcoma, other neoplasia, splenic disease, vena caval syndrome [Dirofilaria tarsalis], and DIC).

Pathomechanism

Immune-mediated hemolytic anemia (IMHA) is an immunohematologic disorder caused by antibody-mediated RBC destruction. This is the most common form of hemolytic anemia in dogs. Antibody binding to RBCs causes premature cell removal by mononuclear-phagocytes (spleen and liver). The antibody can reflect a 1) primary immune disorder or 2) a secondary disorder induced by a) modification of RBC membrane antigens (e.g., absorbed drugs, microorganisms or products, exposure of normally hidden epitopes, formation of haptons or neo-antigens), b) cross-reacting antibodies (e.g., microorganisms sharing antigenic determinants), c) failure of autoregulatory immune functions, d) genetic predisposition (e.g., cocker spaniels), or e) innocent bystander reactions. Secondary IMHA reflects underlying disorders, including but not limited to, neoplasia, chronic infectious diseases (e.g., Borrelia, Leptospira, Anaplasma, Ehrlichia, Rocky Mountain Spotted Fever, Bartonella, abscesses, discospondylitis, pyometra, bacterial cystitis, and pyelonephritis), and exposure to drugs, toxins, or vaccines that can provoke immunoglobulin attachment to RBCs. While 60% to 75% of canine IMHA is considered primary or “idiopathic,” this may reflect diagnostic ignorance in identifying causal conditions.

Antibody Involvement

Until recently, the direct antiglobulin Coombs’ test was the only clinical method for detecting anti-RBC antibodies. Done either using an EDTA or heparin anticoagulated whole blood sample, the test is species specific and relatively insensitive (compared to flow cytometry detection). Laboratories employ a polyclonal anti-canine antibody (IgG, IgM, and C’). Recent work (Piek et al. 2008) described canine IMHA antibodies in 143 dogs: 14/143 (10%) positive for IgM; 27/143 (19%) positive for IgG; 15/143 (10.5%) positive for IgG and IgM; 4/143 (3%) positive for IgM and complement; 27/143 (19%) positive for IgG and complement; and 55/143 (38.5%) dogs positive for combined IgG, IgM, and complement. Testing antibodies at different temperatures: 4°C and at body temperature 37°C is recommended. Even with testing at 4EC and 37EC, the Coombs’ test may be negative in a dog with IMHA. See table 1.

Table 1. Reasons for false-positive and false-negative Coombs’ tests

<table>
<thead>
<tr>
<th>False-Positive</th>
<th>False-Negative</th>
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<tbody>
<tr>
<td>1. Antibody on RBCs from other reasons.*</td>
<td>1. Antiserum inadequate in strength.</td>
</tr>
<tr>
<td>a. Drugs</td>
<td></td>
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<tr>
<td>b. Parasites</td>
<td></td>
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<tr>
<td>3. Some malignancies.</td>
<td>3. Rx with corticosteroids.</td>
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<tr>
<td>4. Antiserum not adsorbed with RBCs.</td>
<td>4. Disease in remission.</td>
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<tr>
<td>5. Antiserum with activity against transferrin.</td>
<td>5. Small amount of autoantibody on RBCs below detection with antiserum.</td>
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*This can still be pathogenic.
Flow Cytometry
Several investigations have demonstrated the higher sensitivity of flow cytometry for detection of anti-RBC antibodies compared to the direct Coombs’ test. High sensitivity allows detection of antibodies on RBC not initiating an immunohemolytic response. A recent study (Morley et al. 2008) demonstrated RBC-antibody binding in a variety of disorders associated with anemia (chronic disease) and greater IgG & IgM initiated IMHA versus IgG alone. False negative responses also are possible.

Clinical Signs
In most dogs, clinical signs of IMHA exist for 1–5 days prior to presentation. Signs may include pallor, hepatosplenomegaly, fever (RBC stroma is a pyrogen), anorexia, vomiting, lethargy, weakness, and peripheral lymphadenopathy. Depending on the rate of hemolysis, dogs may demonstrate jaundice, orange feces, dark orange urine (bilirubinuria), or port wine urine (hemoglobinuria and hemoglobinemia if acute severe IV hemolysis). Concurrent thrombocytopenia (Evan’s Syndrome) can cause petechia, ecchymosis, epistaxis, hematemesis, melena, and hematochezia.

Acrocyanosis, gangrenous skin lesions due to cold agglutinins, may be a primary presenting sign. Lesions involve extremities (toenails, feet, tail, ear tips) and reflect ischemic necrosis secondary to RBC agglutination obstructing microvasculature. Affected dogs may have lesions aggravated in cold weather.

Thromboembolic (TE) complications comprise a primary lethal sequella of IMHA. Splenic vasculature TE is most commonly detected ante mortem. Severe lethal TE involves pulmonary and mesenteric vasculature. Initially, dogs with pulmonary TE may have normal or hyperlucent thoracic radiographs (occluded circulation). Later on pulmonary consolidation and large truncated vessels may be obvious. Arterial blood gases initially reflect respiratory alkalosis due to hyperventilation (pain, hypoxia). In dogs, TE is reduced by mini-dose aspirin (0.5 mg/kg PO SID) administration. Mixed molecular weight heparin may augment TE. The influence of small molecular weight heparin (e.g., Fragmin, Enoxaparin) has not been evaluated. While pulse oximetry may have limited utility in jaundiced dogs, nevertheless, this method of assessing hypoxemia is preferable to arterial blood punctures for blood gas sampling, as these patients are both prone to TE and may be thrombocytopenic. Phlebotomy and catheter associated vascular injury may augment TE. Treatment for suspected pulmonary TE includes an oxygen-enriched environment, minimizing stress and vascular trauma (limited sampling, establish a sampling/treatment catheter). While TE is serious and may lead to death, it also may resolve. Owners must be informed of the high risk for and severe nature of this complication during the first 14 days of treatment.

Clinicopathologic features: A markedly low PCV is the hallmark of IMHA. However, the PCV can be widely variable at initial diagnosis. Most dogs demonstrate a marked regenerative erythrocyte response typified by an increased MCV, RDW, polychromasia, and a high reticulocyte count. While increased nucleated RBCs are common, these do not provide direct evidence of regeneration as they also occur in other disorders (e.g., BM infiltration, reduced splenic function, lead poisoning). In some dogs, IMHA is focused at the BM. These dogs lack an appropriate regenerative response. A left-shifted neutrophilic leukocytosis is common, and leukemoid response may be observed. Concurrent thrombocytopenia exists in 50–75% of dogs.

Spherocytes, small spherical RBCs (lacking biconcave shape) reflect surface membrane loss and are found in > 90% of dogs with IMHA. Spherocytes also occur in dogs with a negative Coombs’ test; these dogs usually have anti-RBC antibodies demonstrable by flow cytometry. Serum biochemical findings may include prerenal azotemia, increased liver enzyme activity, and variable hyperbilirubinemia (direct [conjugated and indirect [unconjugated moieties). Bilirubinuria is common. Hemoglobinuria reflects intravascular hemolysis. Pathologic proteinuria may be difficult to detect in the presence of marked hemoglobinemia or hematuria.

In saline-autoagglutination: Autoagglutination can be demonstrated using EDTA anticoagulated blood. A drop of blood is mixed with a larger volume of 0.9% NaCl on a microscope slide, cells dispersed, and cover-slipped for microscopic examination. Evaluation at room temperature and 4°C (refrigerated) is recommended. True autoagglutination must be distinguished from rouleau (rouleau disperses). A positive slide agglutination supports a diagnosis of IMHA, and an unambiguous response can be used in lieu of a direct Coombs’ test (end point is agglutination).
Osmotic fragility test: Indirectly detects spherocytosis by evaluation of RBC endurance to severe “hypotonic” conditions (dilutions of 0.9% NaCl from 0.85% to 0.50%). The fragility curve is interpreted in light of a “normal curve” (historical and concurrently evaluated samples from normal animals). The test may be difficult to interpret (e.g., indeterminate if “slightly” abnormal). In one report, osmotic RBC fragility was positive in 117/142 (82%) dogs with IMHA.

Coombs test and flow cytometry: See above

Other immune tests: ANA & LE prep. An antinuclear antibody test (ANA) investigates for the presence of antibodies to “nuclear components” (there are many). This relatively crude test has an end point determined by immunofluorescence (or other marking system) that detects antibodies against a nuclear substrate (a commercial protozoal prep [Crithidia luciliae] or neonatal mouse liver slices). A low positive titer can be observed in geriatric animals; the titer required for positivity varies among laboratories. In the author’s hospital, a titer ≥ 1:80 has pathologic significance. However, in a young animal with signs of immune-mediated disease, a low positive titer may be important.

LE cell test: This is a tedious test with low sensitivity but high specificity, done either with whole clotted blood or heparinized blood. LE cells are phagocytes containing engulfed lymphocyte nucleoprotein (amorphous pink “globoid” material); these must be differentiated from “tart” cells containing nucleoprotein showing chromatin clumps. LE cells also may be found in joint fluid or CSF samples. Positive concurrent ANA and LE tests suggest SLE. An LE prep should not be requested unless a positive ANA is confirmed.

Additional clinicopathologic tests: Blood cultures should be considered in dogs with IMHA to rule out septicemic hemolysis (suggested by the left-shifted leukocytosis, pyrexia, splenomegaly, lymphadenopathy). Immunosuppressive therapy is usually pursued before culture results are obtained. Prophylactic antimicrobials are advised for possible infectious disorders, especially tick-borne diseases (doxycycline). It is important to consider relevant historical antimicrobial exposure as a causal factor (e.g., penicillins, cephalosporins, sulfa drugs).

Bone marrow aspiration often discloses increased RBC and granulocyte precursors with a normal M:E ratio or a slightly decreased ratio due to RBC regeneration. Animals with a BM RBC maturation arrest (BM mediated IMHA “aplastic anemia”) will show erythroid hypoplasia. Erythrophagocytosis and an increased plasma cell population may be notable in bone marrow, spleen, or liver aspirates.

Titers for relevant logical infectious disorders should be submitted, including Babesia, Ehrlichia sp, Rocky Mountain Spotted Fever, Lyme Disease, Bartonella, Dirofilariasis, and Mycoplasma Many cats with hemolytic anemia are FeLV positive. Furthermore, cats with regenerative anemia should be tested for mycoplasma haemofelis and candidatus mycoplasma haemominutum.

Supportive Care and Ancillary Therapy

Blood Transfusion/Blood Substitute
Dogs with severe symptomatic anemia should be cage rested to reduce oxygen demand and receive whole blood or blood substitute (polymerized bovine hemoglobin; Oxyglobin®, Biopure Corp). Blood transfusion may precipitate or accelerate hemolysis and TE and must always be accompanied by immunosuppression. Blood transfusion may enhance antibody production, facilitate antibody consumption or FC receptor blockade, may initiate a graft-vs.-host response (prolonged fever, malaise, joint pain), and may suppress BM response. Oxyglobin is administered by slow IV infusion (maximum rate 10 ml/kg/hr/ treatment), delivering a dose of 10–30 ml/kg. Infusions may be required every 2–3 days in symptomatic dogs with continued RBC hemolysis or delayed BM response. While an opened Oxyglobin bag may be retained for 4 days refrigerated (do not freeze), it should be disposed of if brown discolored. Oxyglobin administration makes it impossible to monitor biochemical indices, as the pigment interferes with test end point determinations. Initial reports suggested that Oxyglobin imposed a negative survival influence in dogs with IMHA, but findings of a broad retrospective study in the author’s hospital contradict that observation.

Anti-thrombotic Therapy
Ultra low-dose aspirin (0.5 mg/kg/day) improves survival in dogs receiving prednisolone and Azathioprine. This treatment also salvaged dogs receiving mixed molecular weight heparin that worsens survival.
**Immunosuppressive Therapy**

**Glucocorticoids—Prednisolone:** Starting dose for dogs is 1–2 mg/kg PO BID and for cats is 2–8 mg/kg SID to BID, titrated to response using a slow judicious 25% downward adjustment. Some clinicians start with Dexamethasone: 0.1–0.6 mg/kg SID x several days and than switch to oral prednisolone. Experimental evidence suggests that dexamethasone may decrease macrophage Fc receptor expression to a greater extent (controversial). High dose glucocorticoids are ideally continued until IMHA is in remission and anemia controlled before gradual dose taper. Optimal taper remains controversial and is best individualized; slow reductions are essential as disease recrudescence may be more difficult to control. Using 25% dose reductions, initial reassessments are done at 7–10 day intervals. When a dose of 0.125 to 0.25 mg/kg/day prednisolone is achieved, EOD therapy (double the daily dose for first EOD treatment) is used to preserve the pituitary-adrenal axis. Dexamethasone requires adjustment to 72–96 hour intervals. Glucocorticoids are usually continued for 6 months or more UNLESS an underlying cause for IMHA is found and eliminated or a vaccination is the suspected cause. Glucocorticoid mechanisms: "paralysis" of macrophage Fc receptors is responsible for removal of antibody-bound RBCs and alteration of antibody avidity for RBC surface antigens. They DO NOT greatly suppress antibody synthesis.

**Azathioprine:** Starting dose (dogs) is 1–2 mg/kg (50 mg/m²) PO SID daily for 4–7 days then transitioned to EOD. Is not advised for cats. Weekly monitoring the first month (CBC, chemistry profile) detects side effects of BM suppression (thrombocytopenia, granulocytopenia, suppressed RBC regeneration), liver injury, and pancreatitis. Acute adverse hematologic effects necessitate drug withdrawal until recovery and then institution of a 25–50% dose reduction. Chronic adverse hematologic effects, liver injury or pancreatitis, necessitate permanent drug withdrawal. Azathioprine inhibits cell-mediated immunity and T lymphocyte-dependent antibody synthesis, and reduces macrophage recruitment into inflammation. While it interferes with DNA and RNA synthesis and modifies humoral and cell-mediated immune functions, it has greater inhibitory effects on T-lymphocytes than B-lymphocytes, (differs from cyclophosphamide in this way).

**Mycophenolate Mofetil:** Starting dose (dogs) 10–20 mg/kg PO BID, with chronic dosing reduced by 50%. Mycophenolate is proposed to achieve immunosuppression before azathioprine (not proven in dogs). Mycophenolate mofetil is a pro-drug transformed to mycophenolic acid (MPA); MPA is glucuronidated (liver) to the inactive MPA-glucuronide. MPA is a selective potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), critical for *de novo* synthesis of guanosine triphosphate (GTP). Lack of GTP impairs synthesis of DNA, RNA, proteins, and glycoproteins. MPA is relatively selective for lymphocytes, which are solely dependent on the *de novo* purine synthetic pathway for DNA synthesis. (Two isoforms of IMPDH exist, one form prominent in rapidly proliferating lymphocytes is most sensitive to MPA suppression). Lymphocytes are dependent on a single IMPDH isoform inhibited by MPA. MPA targets lymphocytes, inhibiting clonal expansion of stimulated B and T lymphocytes, antibody production, and expression of cellular adhesion molecules. It also potentiates apoptosis in activated lymphocytes. It has been well researched as an immunosuppressant in organ transplant rejection in dogs. Side effects are bone marrow suppression and gastroenteric toxicity (vomiting, bloody diarrhea).

**Cyclosporine:** A 5 mg/kg PO SID to BID dose for dogs is suggested; dose adjustment is based on trough whole blood drug concentrations (goal 500 – 600 ng/mL). Neoral or Atopica (microemulsion forms) are used concurrent with glucocorticoids. Mechanism: blockade of IL-2 transcription which impairs proliferation of activated T-helper and T-cytotoxic lymphocytes, suppresses generation of antigen specific cytotoxic T-lymphocytes and IL-2 (post-receptor level), and inhibits α-interferon transcription (amplifies signals for macrophage/monocyte activation). B cells are not affected and myelosuppression does not occur. Best responses are achieved when cyclosporine is given before T-cell proliferation. Care must be given to avoid drug interactions that may alter cyclosporine blood concentrations; ketokonazole remarkably reduces dose requirements by as much as 90%. A 2.5 to 10 mg/kg dose per day of Ketaconazole allows a 1 mg/kg PO BID Neoral dose to achieve therapeutic concentrations. Side effects are anorexia, nausea, vomiting, diarrhea, weight loss, gingival hyperplasia, and papillomatosis.

There is no evidence for efficacy of chlorambucil being greater than azathioprine in dogs.
**Danazol:** A 5.0–10 mg/kg PO BID dose in dogs has been concurrently used with glucocorticoids and/or other immunosuppressives. Danazol is an impeded synthetic androgen: 2,3 isoxazol derivative of 17α-ethynyl testosterone synergistic with glucocorticoids by displacing cortisol from its glucocorticoid-binding globulin. It reduces concentrations of circulating IgG and expression of macrophage Fc-receptors; suppresses type II hypersensitivity responses; inhibits complement activation and binding of complement to RBC or platelet cell membranes; and reduces soluble IL-2 receptor expression and elaboration of IL-6 and IL-1. Danzaol also increases plasma AT III concentrations and Protein C and Protein S activities, which may help thwart TE. However, danazol also reduces RBC osmotic fragility. Side effects in humans are mild reversible seborrhea, acne, increased hair growth, masculinization, weight gain, fatigue, nausea, irritability, rashes, cholestatic jaundice, hepatic necrosis, hepatocellular adenoma, and pancreatitis. Few side effects (cholestatic injury) have been reported in animals, but use has been low.

**High-Dose Pulsed Glucocorticoid Therapy**

**Methylprednisolone sodium succinate (Solu-Medrol):** Used to overwhelm glucocorticoid receptor binding. Reduces glucocorticoid side effects by limiting exposure chronicity. The human dose is 1 g/day for 3 consecutive days. Limited experience in animals, where dosing has been extrapolated. Remains controversial in humans, where it is used in patients failing to respond to conventional oral glucocorticoids. Most often used to secure acute remission in ITP to allow splenectomy. Utility in dogs has not been determined.

**Intravenous Human Immunoglobulin Therapy (hIVIG)**

A 0.5–1.5 g/kg. IV infusion over 6–12 hours has been used in dogs. Pretreatment with aspirin is advised owing to heightened risk of TE. May stall clinical deterioration while awaiting response to conventional immunomodulation. Is a proposed preferred treatment for myelofibrosis (in humans), where it may provide a longer term response. Safety of repeated treatments in dogs is not established. hIVIG is a polyspecific human IgG obtained from plasma of healthy donors (~1000 humans). It contains 90% intact, biologically active IgG with a normal subclass distribution. It is free of aggregates, prekallikrein activator, kinins, plasmin, preservatives, infectious agents, and other potentially harmful contaminants (except possibly prions), as a WHO quality requirement. The plasma is screened for antibodies to HIV, hepatitis B & C, but it contains antibody titers to common infectious agents such as polio, tetanus, diphtheria, and measles. At the prescribed dose, hIVIG is costly: $1,000–3,000 per treatment for a medium-sized Labrador. In humans hIVIG has been effectively used to treat IMHA, immune-mediated neutropenia, pure red cell aplasia, ITP, SLE, SLE- immune-mediated vasculitis, demyelinating polyneuropathies, recurrent pregnancy loss, myasthenia gravis, polymyositis, and childhood asthma. Mechanism(s): mononuclear phagocyte Fc receptor blockade; reduced autoantibody production by B cells; F(ab)2 fragments of hIVIG inhibit autoantibody binding to autoantigens in vitro and neutralizing functional activity of autoantibodies in a dose-dependent manner. Anti-idiotypic antibodies in hIVIG may decrease autoantibody production by modulating function of the idiotype-anti-idiotypic network. Modulation of T lymphocytes includes decreasing natural killer cell activity, blocking complement mediated cell damage, and blocking release/function of proinflammatory cytokines.

**Plasmapheresis**

Removal of plasma components (disease-provoking antibodies, circulating immune complexes, other harmful humoral products) from whole blood and while salvaging RBCs. Concurrent treatment with glucocorticoids and immunomodulators is imperative to blunt rebound antibody production. Manual plasmapheresis involves sequential phlebotomy of large blood volumes (10% blood volume) followed by plasma removal after low-speed centrifugation and reinfusion of RBCs are suspended in saline or donor plasma. The process is time and labor intensive, requiring up to 15 collections for effective plasma removal (36-hour interval). Plasmapheresis to selectively extract immunoglobulins involves centrifugal blood cell separators or filtration instruments containing sieves or special adsorption columns.

**Splenectomy**

Controversial, but some propose its utility as an early intervention for IMHA and ITP to thwart development of hypersplenism. Some human and veterinary patients achieve IMHA remission after splenectomy. One report described efficacy in dogs unresponsive to cortisone (n = 15), with an 80% permanent remission (6–17 month follow-up). IgG nonagglutinating IMHA RBC removal is proposed to occur largely in the spleen; as such, therapeutic splenectomy would be appropriate for nonresponsive nonagglutinating IgG-mediated IMHA. However, splenectomy is not inconsequential considering that the spleen is responsible for initial IgM production, provides extramedullary hematopoiesis, stores 30% of platelets at a given time, and can function as a RBC reservoir.
References