CANADA WEST
VETERINARY SPECIALISTS

Approach to Immune-Mediated Hemolytic Anemia

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Immune-mediated hemolytic anemia (IMHA) is a common cause of anemia in dogs and cats. IMHA can be either primary (idiopathic or autoimmune) or secondary. Primary IMHA, a classic autoimmune disorder with no recognised underlying cause, is the most frequent form of IMHA in dogs and also occurs, less frequently, in cats. The condition typically affects young adult and middle-aged animals, and is most common in cocker spaniels, English springer spaniels, poodles, and old English sheepdogs, although there may also be geographic clusters in other breeds (in Mississippi, for example, we see a bad form of IMHA in Dachshunds). Breed predispositions strongly suggest that there is an inherited susceptibility to IMHA and, in fact, there has been shown to be a strong association between several DLA-79 mutations and multiple immune-mediated diseases, including IMHA, in dogs. Neutered dogs (both male and female) appear to be more prone to IMHA than intact dogs of either sex.

IMHA can also occur secondary to a wide range of infectious, inflammatory or neoplastic processes. Important causes of secondary IMHA in small animals include Feline Leukemia Virus (FeLV) or hemobartonellosis (mycoplasmosis or hemoplasmosis) in cats, and or neoplasia (particularly lymphosarcoma) in dogs. Various medications have also been reported to trigger IMHA. Modified live vaccines have also been implicated, but it is hard to establish causation and, even if vaccines may trigger the occasional case of IMHA, this appears to be an uncommon phenomenon. Secondary IMHA affects animals of any age or breed, and should be strongly suspected in patients with a signalment atypical for primary IMHA, such as geriatric animals. Interestingly, seasonality has been found for IMHA in dogs in some areas (spring and summer in San Diego, for example), potentially suggesting some as yet identified environmental or infectious factor. Unlike the dog, IMHA in the cat is (arguably) more commonly secondary, although this varies with location and incidence of infectious diseases that cause secondary IMHA. Distinction between primary and secondary IMHA is therapeutically important because secondary IMHA will often respond poorly to treatment, or recur, unless the underlying cause is recognized and eliminated.

POTENTIAL CAUSES OF SECONDARY IMHA

Medications

- Trimethoprim/sulphonamide
- Penicillins
- Cephalosporins
- Levamisole (dogs)
- Propylthiouracil/methimazole (cats)
- Non-steroidal antiinflammatories (phenylbutazone)
- Dipyrone
- Quinidine
- Chlorpromazine

Immunological

- SLE
- Transfusion reactions
- Neonatal isoerythrolysis (esp. cats)
- Antilymphocyte globulin (transplantation patients)

Infectious/Parasitic

- Feline leukemia virus infection
- Hemobartonellosis (mycoplasmosis), esp. in cats
- Babesiosis
- Bartonellosis
- Ehrlichiosis
- Dirofilariasis

Neoplastic

- Lymphoproliferative disease (esp. lymphosarcoma)
- Hemangiosarcoma

Miscellaneous

- Post-vaccinal
- Elapid snake bites (dogs)
- Bee stings (dogs)
- Pancreatitis (cats)

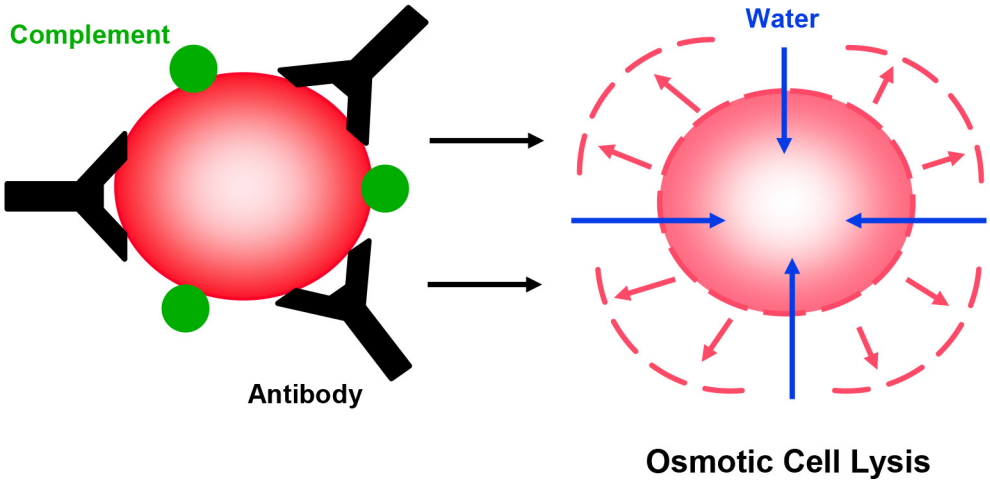
Mechanisms of Red Cell Destruction

The mechanism underlying typical cases of IMHA is antibody-mediated cytotoxic (Type II) destruction of circulating red blood cells (RBCs). Although most cases share this common mechanism, the disease is otherwise very heterogenous: in primary IMHA, the most studied form of IMHA, both the pattern of immunoglobulin and complement involvement in RBC destruction and the site of antibody attachment to RBC membranes varies widely between patients. Although the most common immunoglobulin type involved in primary IMHA is IgG, less commonly IgM may also be implicated, along with variable involvement of complement, and both IgG and IgM combined. Dogs with a combination of both IgG and IgM anti-RBC autoantibodies tend to have lower hematocrits and more autoagglutination. Antibodies have been reported to attach to various components of the RBC membrane, particularly glycophorins and the band 3 anion transport protein, but also a range of other proteins, including calpain, complement component 3, and peroxiredoxin 2. Given the range of different immunoglobins and antigenic targets involved, it is probably overly simplistic to think of IMHA as a single disease syndrome: rather, it is a actually a heterogenous mix of different diseases with different presentations, different prognoses, and different responses to therapy.

Antibody attachment to cell membranes triggers RBC destruction by a number of different mechanisms. With high levels of antibody attachment and, particularly, complement

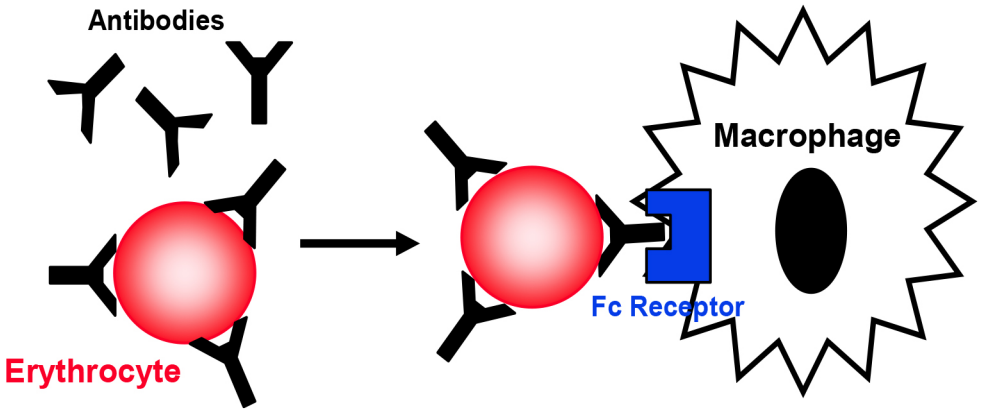
fixation (with involvement of the membrane attack complex), membranes may be so damaged that extracellular water leaks into the cytoplasm, causing swelling and rupture of the RBC while it is still in the circulation, so-called intravascular hemolysis.

MECHANISM OF INTRAVASCULAR HEMOLYSIS



In the absence of direct RBC lysis, antibody attachment and subsequent cell membrane damage can still lead to an accelerated rate of destruction of affected RBCs by tissue macrophages within the mononuclear phagocytic system (MPS), a process that occurs outside of the circulation (extravascular hemolysis). MPS destruction of RBCs is mediated by Fc receptors on the macrophage surface, receptors which bind the Fc component of the antibodies attached to the RBC membranes. Since the MPS is located throughout the body, extravascular hemolysis can occur in many organs, but typically is most pronounced in the liver and, particularly, the spleen.

MECHANISM OF EXTRAVASCULAR HEMOLYSIS



In some patients with high levels of anti-RBC antibodies, many individual antibodies can each bind to two different RBCs, a process that causes the cells to clump together (agglutinate). Patients that exhibit significant RBC agglutination at body temperature typically have an increased rate of extravascular hemolysis, since clumping of RBC slows their passage through vessels and facilitates their removal by the MPS.

Typically, IMHA is caused by antibodies directed against circulating, mature RBC, with the marrow mounting a healthy regenerative response to the resultant anemia. However, in some small animal patients (perhaps up to about one third), antibodies may also be directed against marrow RBC precursors at any stage in their development. Hemolytic anemia with an inappropriately poor regenerative response ("non-regenerative IMHA") will develop if antibodies are directed against cell membrane components that are present both on mature RBC and their marrow precursors. In contrast, if antibodies are directed against membrane components that are present only on marrow precursors, and not on mature RBC, non-regenerative anemia will develop without peripheral hemolysis. Pure red cell aplasia (PRCA), in which all stages of marrow RBC precursor are reduced or absent, may be the most extreme form of this process. Interestingly, in some dogs with IMHA, lack of regeneration appears to be due to a functional iron deficiency (a mechanism comparable to anemia of chronic disease). Acute phase proteins such as C-reactive protein are markedly elevated in some dogs with IMHA and, since anemia of chronic disease is thought to be a functional iron deficiency mediated by acute phase proteins (hepcidin in particular), it should perhaps not be surprising that this mechanism may affect erythropoiesis in IMHA patients.

In primary IMHA, autoantibodies are directed against components of the patient's own RBC membrane. Although the same process can occur with secondary IMHA, antibodies may alternatively be directed against a foreign antigen (such as a drug or virus) that is attached to the RBC membrane, against normal RBC membrane components that are antigenically similar to non-RBC antigens that are associated with the underlying disease process, or against membrane components that are normally hidden but are exposed by the underlying disease.

Categories of IMHA

Typical IMHA is caused by antibodies that exert their effects at body temperature, so-called warm reactive antibodies. Some animals, however, have anti-RBC antibodies that are only reactive at much lower temperatures. Although such cold reactive antibodies usually cause minimal harmful effects, their presence can potentially cause specific clinical syndromes, and can also lead to a false positive diagnosis of IMHA if tests such as slide agglutination are performed at cold temperatures. Classically, IMHA has been sub-divided into five main categories based on the thermal reactivity of the anti-RBC antibodies, and their major clinical effects at optimal temperature:

1. ***Warm Antibody Type, Agglutination:***

   High levels of antibody lead to detectable autoagglutination of RBC. Agglutination is often associated with acute severe extravascular hemolysis.

2. ***Warm Antibody Type, Intravascular Hemolysis:***

   Intravascular hemolysis, usually associated with high levels of antibody and complement fixation, causing severe anemia with detectable hemoglobinemia and hemoglobinuria.

3. ***Warm Antibody Type, Incomplete Antibody:***

   Anti-RBC antibodies cause extravascular hemolysis, without autoagglutination or hemoglobinemia. Disease onset can be chronic or sub-acute, and resultant anemia varies from mild to severe.

4. ***Cold Antibody Type, Agglutination:***

   Anti-RBC antibodies are only reactive at cold temperatures, and agglutination does not occur at body temperature. Agglutination can however occur within the vasculature of the extremities, particularly in colder weather. Obstruction of the blood supply to the peripheral vasculature due to agglutination can lead to ischemic necrosis of the ear or tail tips, the end of the nose, and the feet.

5. ***Cold Antibody Type, Non-agglutinating Hemolysis:***

   Antibodies are again only reactive at cold temperatures, and hemolysis does not occur at body temperature. In cold weather, however, some degree of hemolysis may occur within the extremities, which manifests clinically as transient hemoglobinemia and hemoglobinuria.

Although the above categorization system is derived by extrapolation from people, all five categories of IMHA have been reported in small animals. Severe agglutinating and (especially) hemolysing cold antibody types of IMHA are however uncommon to rare in both dogs and cats. Intravascular warm antibody type IMHA is also relatively uncommon.

Clinical Signs

Signs typically associated with IMHA reflect the presence of both anemia (lethargy, weakness, pale mucous membranes, and a hemic heart murmur) and compensatory responses caused by tissue hypoxia and stimulation of the sympathetic nervous system (tachypnea, tachycardia, and bounding pulses). Some patients may also show clinical signs of an ongoing immunological or inflammatory process, such as pyrexia, anorexia and, uncommonly, lymphadenopathy. Surprisingly, since the MPS within the spleen and liver is usually the main site of RBC destruction, organomegaly is only variably present in

animals with IMHA. Patients with IMHA of acute onset tend to be very severely affected by their anemia, and are often very depressed, weak or even collapsed.

Hyperbilirubinemia, bilirubinuria and tissue jaundice are often seen during acute severe episodes of IMHA. Since intravascular hemolysis is relatively uncommon, hemoglobinemia and hemoglobinuria are observed infrequently. Hemoglobinemia can lead to falsely elevated MCHC, because even hemoglobin that is not inside RBCs is measured, leading to falsely increased estimates of intracellular hemoglobin in animals with low RBC counts. Patients with extravascular hemolysis due to sub-acute or chronic IMHA can compensate to some extent for their lack of erythrocytes, and may be remarkably bright despite the presence of severe anemia. In these patients, the liver can often cope with the extra bilirubin released by RBC breakdown, and jaundice does not occur.

Pulmonary thromboembolism (PTE) is a well-recognised complication of IMHA, and is particularly common in those animals with acute severe anemia that are receiving high dose glucocorticoids. Many dogs with IMHA appear to be prothrombotic, as evidenced by high levels of activated platelets (particularly in thrombocytopenic IMHA patients), increased levels of tissue factor and phosphatidylserine positive microparticles, and a range of altered thromboelastographic markers suggestive of hypercoagulability. Pulmonary thromboembolism should always be suspected in those anemic patients that suddenly develop severe and persistent dyspnea, although other causes of dyspnea such as cardiogenic pulmonary edema or acute bacterial pneumonia should also be considered, especially in dogs already receiving glucocorticoid and immunosuppressive therapy. Transient ascites has also been reported, possibly due to cor pulmonale secondary to PTE, or alternatively due to serositis. Disseminated intravascular coagulation (DIC) can also complicate IMHA, but clinically significant DIC is probably uncommon to rare.

Diagnosis of IMHA

Hematology in patients with IMHA typically reveals a moderate to severe anemia, which is most commonly regenerative, with anisocytosis, polychromasia, a high corrected reticulocyte count and, sometimes, increased numbers of nucleated RBCs. Reticulocyte counts can however sometimes be inappropriately low, either because antibodies are also directed against RBC precursors, or because anemia is peracute (since it takes about 5 days for the marrow to mount a strong regenerative response and, before that, reticulocyte counts will be normal or “pre-regenerative”). White cell and neutrophil counts are often moderately to markedly increased, probably in response to both non-specific marrow stimulation and the inflammatory process associated with RBC breakdown. Occasionally, white cell counts can be high enough to mimic myelogenous leukemia, a reaction sometimes called a “leukemoid response”. Platelet counts are usually normal unless the animal also has immune-mediated thrombocytopenia (IMT) or platelet consumption secondary to PTE or DIC. Concurrent IMHA and IMT, a condition known as Evan’s syndrome, may affect up to approximately 10% of dogs with IMHA, but

the frequency of Evan's may be overestimated if the effects of PTE or DIC on platelet counts are not considered as an alternative diagnosis to IMT.

Hematology can often also reveal clues that suggest a specific etiological diagnosis:

1. ***Spherocytosis:***

   Spherocytes are small spherical erythrocytes that, when present in high numbers, strongly suggest a diagnosis of either primary or secondary IMHA. The absence of spherocytes, however, does not absolutely exclude a diagnosis of IMHA. Spherocytes are formed when tissue macrophages remove a piece of RBC membrane without cell destruction or a significant loss of cytoplasm. Since cytoplasm is not lost, RBC volume (as indicated by MCV) remains normal. Spherocytes can be difficult to recognize in cats, because normal feline RBCs tend to be smaller and less discoid than canine RBCs. Experienced veterinary clinical pathologists, however, may be able to recognize the presence of spherocytes in the cat.

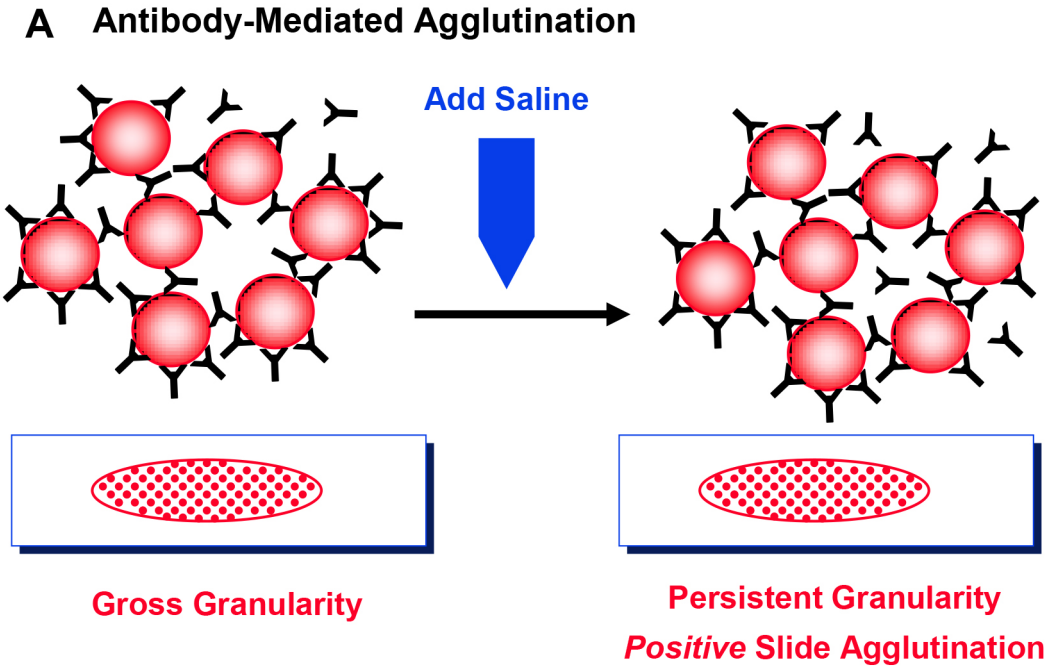
2. ***Agglutination:***

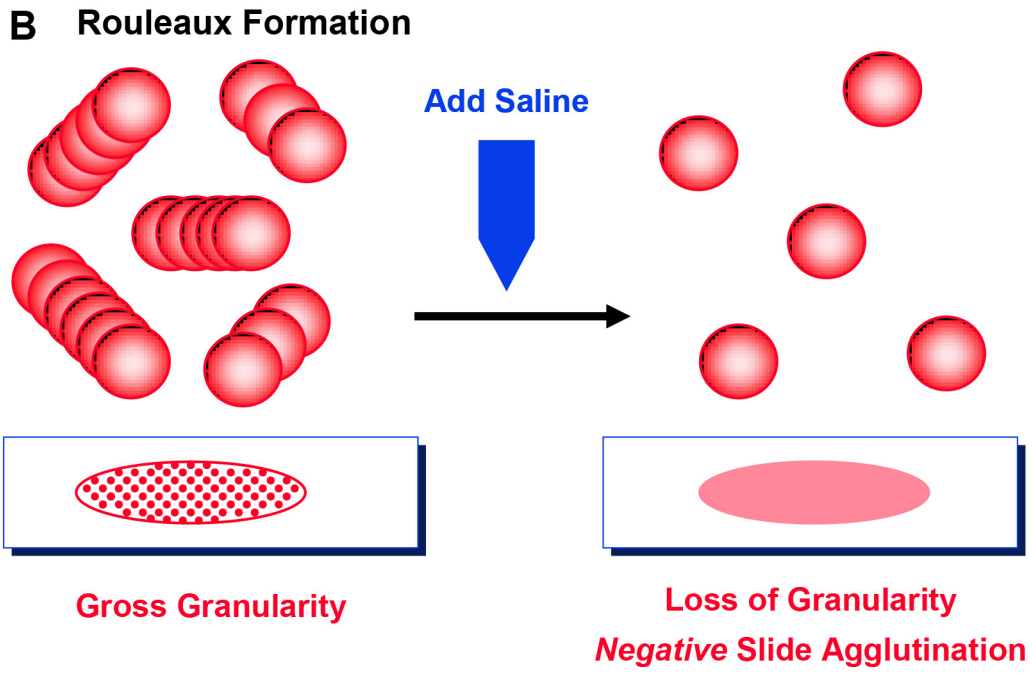
   Examination of blood smears may reveal microscopic autoagglutination (clumping) of RBCs. Such agglutination can form large rafts of RBC that, when a collection tube containing anticoagulated blood is closely inspected, are visible to the naked eye as multiple red speckles. Similar speckles can however be created by rouleaux formation, a phenomenon that can occur in normal animals, especially cats. Clinicians should therefore perform a saline dilution (one drop of RBCs to one or two drops of saline in dogs, one drop of RBCs to two or more drops of saline in cats) slide agglutination test to differentiate rouleaux from genuine autoagglutination. True agglutination can be seen grossly as persistent speckles despite dilution with saline, and microscopically as non-linear clumps of RBCs.

   A positive slide agglutination result is strongly suggestive of a diagnosis of IMHA, and also suggests that the condition is likely to be acute and severe. Non-immune autoagglutination (sometimes triggered by anticoagulants such as EDTA) has, however, also been uncommonly reported in dogs and cats. A negative slide agglutination does not rule out IMHA, since in fact a negative result has been reported in some studies to be the most common result in small animals with IMHA because most actually have non-agglutinating antibodies. Recent clinical studies of canine IMHA, however, report a much higher incidence of positive slide agglutination, perhaps reflecting a referral bias as a result of practitioners tending to refer only the more severe cases of IMHA. Cell washing techniques using repeated centrifugation and saline washes have been reported to decrease the diagnostic sensitivity and increase the specificity of slide agglutination.

   Automated hematology analyzers sometimes register a clump of agglutinated RBCs as a single cell, often of a size too large to even be recorded as a RBC at all. Resultant erroneous results may include an artifactually high MCV or, if clumped

cells are not recognised as erythrocytes, lowering of the calculated hematocrit. Since the hemoglobin within all RBCs is still measured by the analyzer, this leads to an erroneously high estimation of mean corpuscular hemoglobin concentration (MCHC). When agglutination is suspected to be the cause of a lower than expected hematocrit, packed cell volume (PCV), which is not affected by RBC clumping, should be monitored using microhematocrit tube centrifugation rather than an automated analyzer.





3. ***Ghost Cells:***

   RBC ghosts are cells that have ruptured in the circulation as part of intravascular hemolysis, losing their hemoglobin. Residual RBC membranes linger as ghost cells. Ghost cells can be seen with intravascular IMHA, but also with non-immune causes of intravascular hemolysis such as zinc toxicity and, in small numbers, as an artefact.

4. ***Other RBC Abnormalities:***

   Careful examination of RBC morphology may suggest an underlying cause of either immunological or non-immunological hemolysis. Diagnostically useful RBC abnormalities include detection of parasites such as Mycoplasma haemofelis or haemocanis or Babesia species (which may cause secondary IMHA), Heinz bodies (suggesting hemolysis secondary to oxidative damage) and schistocytosis (suggesting a microangiopathic hemolytic process such as DIC).

Serum biochemistry and urinalysis are often normal in dogs with IMHA. Potential abnormalities that may be seen in some patients include mild to moderate elevation of liver enzymes (thought to indicate hepatic hypoxia secondary to severe anemia) and variable hyperglobulinemia. Since serum albumin is usually normal, hypoalbuminemia is an unexpected finding that may suggest that anemia is in fact due to occult blood loss rather than hemolysis, or that the patient also has another illness. Mild to moderate hyperbilirubinemia and bilirubinuria may be seen transiently in animals with acute severe anemia. Since the liver is usually able to cope with all but the transient overwhelming bilirubin loads produced by acute severe hemolysis, severe hyperbilirubinemia or persistence of jaundice for more than 3 to 5 days, even in the markedly anemic animal, usually indicates the presence of concurrent hepatic disease or biliary obstruction. Hemoglobinemia and hemoglobinuria are uncommon, transient events that indicate the presence of severe intravascular hemolysis. Dogs with IMHA, not surprisingly, have been shown to have increased RBC osmotic fragility, although osmotic fragility testing is laborious, and therefore unlikely to attain common usage in practice.

Many laboratory parameters have been found, in individual canine IMHA studies, to be suggestive of poor prognosis, including hyperbilirubinemia, autoagglutination, azotemia, and high serum lactate levels, amongst others. None, however, have as yet been consistently shown to reflect prognosis in multiple different studies in dogs with IMHA.

Immunological Testing

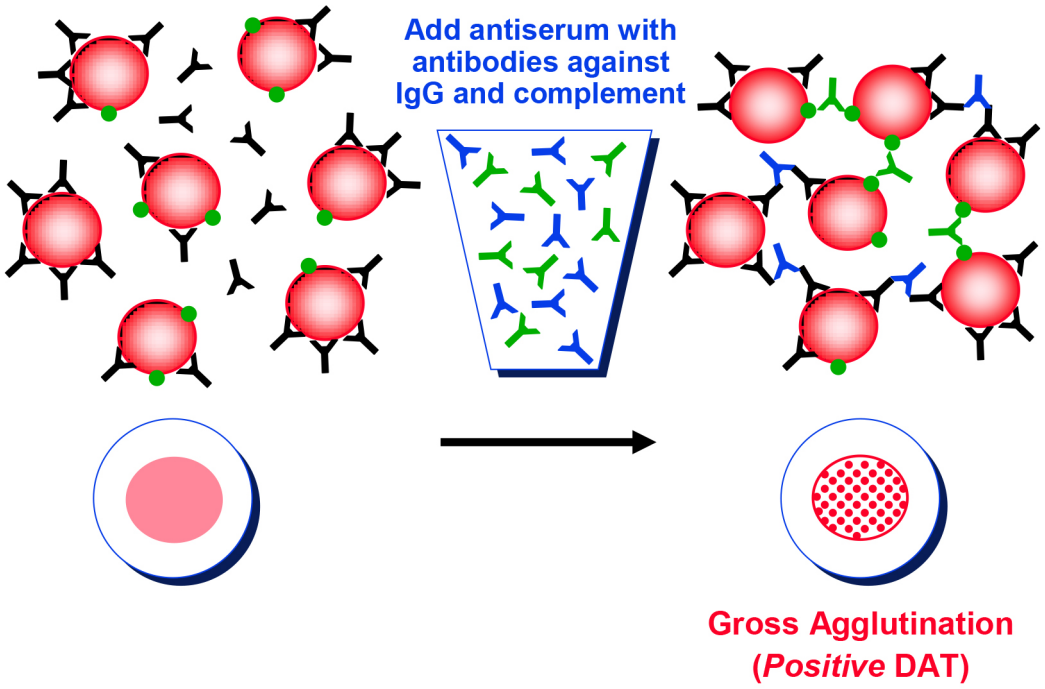
Specific immunological testing can be used to support a tentative diagnosis of IMHA. The most widely used test is the direct antiglobulin test (DAT) or Coombs' test, which detects antibodies and/or complement bound to RBC membranes. A standard DAT as provided by most laboratories typically uses a mix of antibodies directed against IgG, IgM (to a variable extent) and complement, and is performed at body temperature. Modifications

of the routine screening DAT that have been shown to increase its diagnostic value include running the test at different temperatures and titers, and using individual monovalent antibodies against IgG, IgM, IgA and complement as well as the standard polyvalent antibody/complement mix. Positive DAT results at 4° Celsius, however, may be of dubious diagnostic significance unless the patient has clinical signs consistent with cold antibody type agglutination or intravascular hemolysis.

Strictly interpreted, a positive DAT result would support a diagnosis of IMHA, while a negative test result would suggest a non-immunological cause of hemolysis. Numerous studies, however, have shown that a DAT, particularly using polyvalent antibodies, can often be of only mediocre diagnostic accuracy: although sensitivity and specificity undoubtedly improve with meticulous attention to test methodology, the fact remains that both false positive and false negative results do occur relatively commonly. Veterinarians should therefore be aware that since IMHA can occur in the presence of a negative DAT and, conversely, a positive test does not absolutely prove the presence of IMHA, sometimes a diagnosis must be made based on clinical judgement despite the presence of an apparently discrepant DAT result. Performing a DAT is however still recommended by some hematologists in all patients with suspected IMHA even if criteria such as spherocytosis or a positive slide agglutination already strongly suggest a diagnosis, since a positive DAT will add support to the diagnosis and characterize the disease further by determining the involvement of various immunoglobulin types and complement. Various other immunological tests for detecting anti-RBC antibody have been reported, including an enzyme-linked immunosorbent assay, and a direct enzyme-linked antiglobulin test but, although some of these tests may arguably be more sensitive than the DAT, they have not as yet become commonly available. Regardless of whether a DAT or an alternative test for ant-RBC antibody is used, however, clinicians should be aware that a positive result merely records the presence of antibody, and does not determine whether IMHA is primary (AIHA) or secondary.

Uncommonly, IMHA (with or without IMT) will be merely one component of systemic lupus erythematosus (SLE), a multisystemic immunological disturbance. Measurement of serum anti-nuclear antibody (ANA) is therefore indicated in those patients displaying evidence of the concurrent involvement of more than one body system, such as IMT, glomerulonephritis, polyarthritis, polymyositis or immune-mediated skin disease. In contrast, ANA is not indicated (and is usually negative) in those patients suspected to have uncomplicated IMHA. A high incidence of perinuclear antineutrophil cytoplasmic autoantibodies has been observed in canine IMHA patients, although the prognostic significance of this finding is unknown.

MECHANISM UNDERLYING COOMB'S TEST (DAT)



Identification of Underlying Disease

Since IMHA is often secondary, particularly in cats and in dogs with an atypical signalment, confirmation of a diagnosis of IMHA is not necessarily the end of the diagnostic trail. Primary IMHA can only be diagnosed with absolute certainty once potential underlying causes have been thoroughly investigated. Unfortunately, this presents practitioners with a dilemma: although IMHA is unlikely to be treated effectively unless underlying causes have been eliminated, a complete search for such causes can be expensive, time-consuming, invasive and, in the case of primary IMHA, ultimately fruitless. Standard screening tests for underlying disease which ideally should be performed in all animals with IMHA include hematology (including careful examination of a blood smear), serum biochemistry, urinalysis, thoracic and abdominal radiography and, in cats, testing for retroviruses (particularly FeLV). Serologic and/or PCR testing for RBC parasites such as hemobartonellosis, now more correctly termed mycoplasmosis (*Mycoplasma haemofelis* in cats, *Mycoplasma haemocanis* in splenectomized dogs), *Babesia canis* (particularly in greyhounds) or *Babesia gibsoni* (particularly in pit bull terriers, or dogs that have been bitten by pit bull terriers) is also often indicated. Since arguably rickettsial diseases may also predispose to secondary IMHA, testing for *Ehrlichia* species may also be indicated in endemic areas, as may, in dogs, testing for bartonellosis. Further tests that might be considered in some patients, particularly in older animals in which underlying occult neoplasia (especially lymphoproliferative disease) is a real possibility, include abdominal ultrasonography, lymph node aspiration cytology, and bone marrow analysis.

Bone Marrow Analysis

Bone marrow analysis (aspiration cytology and/or core biopsy histopathology) is also indicated in all patients suspected to have the non-regenerative forms of IMHA. Pure red cell aplasia is characterised by a relative or complete lack of RBC precursors within the marrow, whereas cytological or histopathological evidence of an erythroid "maturation arrest" (preponderance of immature precursors, with an absence of more mature RBC precursors) suggests that, rather than being directed against very early stem cells, antibodies are directed against a later stage of marrow RBC development. Marrow cytology and/or histopathology may also reveal macrophages phagocytosing erythrocytes or RBC precursors. In such patients, when available, techniques such as immunofluorescent or immunoperoxidase staining of marrow samples may confirm the presence of antibodies directed against RBC precursors.