HEMOLYTIC ANEMIA: IS IT IMMUNE-MEDIATED?

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Introduction

Immune-mediated hemolytic anemia (IMHA) is one of the most common and serious hemolytic anemias in dogs, but occurs rarely in other animal species. In IMHA an immune response, including anti-erythrocytic antibodies, complement and macrophages, targets directly or indirectly erythrocytes and a hemolytic anemia ensues. There are many triggers for IMHA such as infections, drugs and other agents, and cancer leading to secondary IMHA, but in many dogs no cause is identified (so-called idiopathic, autoimmune or primary IMHA) or a genetic predisposition has been proposed (Cocker spaniels). Furthermore, alloimmune hemolytic anemias, such as hemolytic transfusion reactions, both acute and delayed, and neonatal isoerythrolysis (only litters from transfused bitches), are caused by specific anti-erythrocytic alloantibodies. In contrast to other species, dogs with IMHA also develop an often overwhelming inflammatory response resulting in thrombosis and necrosis of various organs. And while the anemia can be corrected with transfusions, these complications in dogs are causing severe morbidity and mortality despite aggressive immunosuppression and antithrombotic interventions.

This session will review the diagnostic approach to IMHA with special emphasis on the Coombs’ test including the authors’ comparative prospective study on different in-clinic and laboratory Coombs’ tests. Other hemolytic anemias such as infectious, inherited and toxic causes need to be carefully excluded.

Immune Destruction of Erythrocytes

Regardless of the underlying cause, IMHA results from a breakdown in immune self-tolerance or from a deficit in the control mechanism that regulates B and T lymphocyte activity as well as macrophage reactivity. Immune destruction of erythrocytes is initiated by the binding of IgG or IgM antibodies to the surface of erythrocytes. Under most clinical circumstances, immune destruction is an extravascular process that depends on recognition of erythrocytes opsonized with IgG, IgM and/or complement by specific receptors on reticuloendothelial cells. Macrophages with engulfed erythrocytes may be noted on cytological examination of blood and tissue aspirates as erythrophagocytosis, but this is not definitive proof of an immune-mediated process. Antibody-coated erythrocytes may also be lysed by complement fixation and the membrane attack complex, which is clinically noted as intravascular hemolysis.

A diagnosis of IMHA must demonstrate accelerated immune destruction of erythrocytes. Evidence of a hemolytic anemia is suggested clinically by icterus and a regenerative anemia with hyperbilirubinuria, and hemoglobinemia and hemoglobinuria refers to an intravascular process. However, the erythroid response in the bone marrow may be blunted by the immune and inflammatory process or the underlying disease thereby leading to non-regenerative anemias. Besides documenting a hemolytic anemia, one or more of the following three hallmarks must be present to support a diagnosis of immune-mediated hemolysis: persistent
autoagglutination, marked spherocytosis and a positive direct Coombs’ test result. As in human medicine, the Coombs’ test should be considered the best test to definitively diagnose IMHA, although marked spherocytosis and persistent/true autoagglutination (after 3x washing of EDTA blood with saline) are other important parameters indicating immune-destruction of erythrocytes.

**Autoagglutination**

Anti-erythrocytic IgM and in large quantities IgG antibodies may cause direct erythrocyte autoagglutination. The autoagglutination may be seen by naked eye in an EDTA tube or on a glass slide or may become apparent as small clumps of erythrocytes on blood smears. For yet unexplained reasons, canine erythrocytes have a tendency to unspecifically agglutinate in the presence of plasma and colder temperatures as well as possibly with excessive EDTA anticoagulant. Mixing blood with one drop of saline may break up rouleaux formation but not other forms of unspecific red cell agglutination. It is, therefore, important to determine whether the agglutination persists after “saline washing”, which has been coined persistent or true autoagglutination. This is accomplished by adding physiologic saline to the tube containing a small amount of EDTA-anticoagulated blood, mixing, centrifuging and removing the supernatant including the plasma and repeating this saline washing 3 times. True or persistent autoagglutination is indicative of an immune process, but precludes the performance of Coombs’ test or blood typing and crossmatching procedures which are based upon an agglutination reaction as result. Those based upon chromatographic techniques do not seem to be affected by autoagglutination as free red cells can move along the strip. If the agglutination breaks up after washing, the Coombs’ test is expected to be positive, if it is a case of IMHA. There is no evidence for washing away red cell bound antibodies in dogs.

**Spherocytosis**

If erythrocytes are only partially phagocytized or lysed by complement in circulation, erythrocytes with reduced surface area to volume ratio, known as spherocytes, are formed. They appear spherical and microcytic with no central pallor and are considered fragile. Note proper areas on the blood smear needs to be reviewed to find spherocytes in between single regular discoid red cells. Large numbers of spherocytes (>20/microscopic high power field) are nearly diagnostic for IMHA, whereas small numbers may be seen with other conditions including DIC, endotoxemia and zinc intoxication. In our experience all dogs with marked spherocytosis and suspected to have IMHA also had a positive Coombs’ test. However, only 60-80% of dogs with a positive Coombs’ test or clinically diagnosed with IMHA had marked spherocytosis. Hereditary spherocytosis due to genetic membrane defects has rarely been seen in dogs, but should be considered as a differential diagnosis in dogs with negative Coombs’ test results.

Because of the difficulties with the Coombs’ test (see below), Slappendale had proposed to use the erythrocytic osmotic fragility test at specific saline concentrations as a mean to diagnose IMHA and this test is currently used in various clinics in Europe. However, there are many other reasons for increased fragility of erythrocytes beside IMHA including hereditary red cell defects. This test is not used in human medicine and has not been shown to be superior to determination of marked spherocytosis and a positive Coombs’ test in dogs with IMHA. The osmotic fragility test is also a cumbersome and not well standardized technique.
Positive Direct Coombs’ Test Result

The direct Coombs’ test is also known as direct antiglobulin test (DAT) and is used to detect antibodies and complement on the surface of erythrocytes when the anti-erythrocyte antibody strength or concentration is too low to cause spontaneous agglutination (subagglutinating titer). Separate canine-specific IgG, IgM, and C3b antibodies as well as polyvalent antiglobulin reagents are available. They are added at various concentrations after washing the patient’s erythrocytes free of plasma (3x as shown above) and mixtures are generally incubated at room temperature or 37°C (cold agglutinins appear to be rarely of clinical importance and rarely cause hemolysis). The strength of the Coombs’ reaction does not necessarily predict the severity of hemolysis, but reaction changes are useful in monitoring the disease.

Typically tube or microtiter methods have been used exclusively in the reference or teaching laboratory setting, but a flow cytometric method has also been introduced in a couple of places. A standardized, sensitive, and simple gel column method was available by DiaMed (Switzerland), but unfortunately the company was sold to another company which decided to not pursue the veterinary market. A novel standardized antiglobulin test method has just been developed by Alvedia (France) similar to the immunochromatographic strip technique for blood typing of dogs and cats (see updates on blood typing and crossmatching). Although many commercial laboratories offer Coombs’ testing for dogs, clinicians have questioned the tests sensitivity and specificity and often forgo the test and/or use response to therapy as a diagnostic. However, negative Coombs’ test results may be seen because of technical reasons, insufficient quantities of bound antibodies, the presence of weakly bound antibodies, or the disease in remission. The Coombs’ test stays positive for days to months after initiating treatment. A few days of immunosuppressive therapy will likely not reverse the Coombs’ test result, as unlikely a transfusion would cause a positive Coombs’ test result. Thus, dogs with negative Coombs’ test results should be reevaluated for other causes of hemolytic anemia.

In a recent prospective study of anemic and non-anemic dogs we compared various direct Coombs’ test methods including microtiter plate assays, gel column, capillary, and immunochromatographic techniques using polyvalent antiglobulins in a laboratory setting and found excellent correlations between tests and with spherocytosis and without noticeable interference by immunosuppressive or transfusion therapy in anemic dogs.

In conclusion, a diagnosis of IMHA requires the documentation of red blood cell destruction and an immune process. While regenerative anemia, icterus, and hyperbilirubinuria are suggesting a hemolytic anemia, evidence of true autoagglutination, spherocytosis, and/or a positive direct Coombs’ test are required to document immune destruction. The authors also recommend monitoring IMHA patients for the disappearance of these immunological parameters to adjust and taper therapy.

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