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DIAGNOSTIC METHODS IN IMMUNOLOGY

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INTRODUCTION

Laboratory diagnostic methodology applicable to the diagnosis of immune-mediated disease (allergy, autoimmunity, primary immunodeficiency and immune system neoplasia) has traditionally been limited to relatively simple serological tests. Although it is possible to perform relatively complex assessment of cellular aspects of the canine and feline immune systems, this type of testing remains largely unavailable due to the impracticalities of establishing and running these rarely-requested tests in a general diagnostic laboratory. In recent years, there have however, been a number of advances in terms of the introduction of new tests and in more refined interpretation of existing methodology. These changes are the subject of this lecture.

FLOW CYTOMETRIC TESTING

One of the major advances has been in greater accessibility to flow cytometry which has a range of applications to general diagnosis. Although this apparatus remains expensive, larger laboratories or those associated with a veterinary school will often have access to such testing. The flow cytometer assesses fluorescence labelling of individual cells (lymphocytes, monocytes or granulocytes) within a population, by exciting each cell with an interrogating laser beam as it passes drop-wise in suspension through the machine. Positively labelled cells are recorded as an 'event' and the proportion of labelled cells (typically in a counted population of 10,000 cells) is determined.

Flow cytometry can thus be utilized to assess the relative number and proportion of lymphocytes within a population – and provides a measure of change in any one animal due perhaps to an inherited immunodeficiency, or transient immune responsiveness during disease. Classically, the machine is used to enumerate the key populations of blood lymphocyte (B cells, CD4 and $CD8^+$ T cells) and to determine the CD4:CD8 ratio. The normal canine blood CD4:CD8 ratio is approximately 2:1, and this may elevate during autoimmune disease, or decrease during infections such as deep pyoderma or leishmaniosis. Flow cytometry has also been used to analyse suspensions of lymphocytes obtained from tissue – such as disaggregated lymph node or intestinal mucosa.

The flow cytometer has also been applied to studies of immune cell function. Dual labelling with antibodies tagged with fluorochromes that are excited by light at different wavelengths allows, for example, the detection of intracellular cytokine of a specific type within the cytoplasm of $CD4^+$ T lymphocytes. Phagocytic cell function may also be assessed by flow cytometry. Purified canine neutrophils co-incubated with fluorescently-labelled microspheres or bacteria will engulf these targets, and the success of phagocytosis can be recorded by measuring fluorescence intensity.

Flow cytometry has found ready applicability to the diagnosis of leukaemia and lymphoma. The presence of leukaemia can be detected by routine haematology, and subsequent labelling of the circulating neoplastic population can be used to determine the immunophenotype. Similarly, the use of multiple labels on cells aspirated from lymphoma tissue has been used to define subsets of $CD4^+$ T cell lymphoma with variable prognostic outcome.

Finally, flow cytometry has been adapted for the detection of erythrocyte- or platelet-bound antibody in immune-mediated haemolytic anaemia (IMHA), thrombocytopenia (IMTP) or neutropenia (IMNP). Comparison with the Coombs test has, in some studies, shown flow cytometry to have greater sensitivity, but less specificity, so the Coombs test is still most widely used in the diagnosis of IMHA. By contrast, flow cytometry is the method of choice for the diagnosis of IMTP or IMNP, where other test modalities are cumbersome and less accurate.

THE COOMBS TEST

The Coombs test (or direct antiglobulin test; DAT) is the test of choice for diagnosis of IMHA and has been used in veterinary medicine for over 30 years. The fundamental basis of the test has remained largely unaltered, but refinements in methodology and interpretation have occurred. It is clear that the

occurrence of occasional false positive and negative reactions in this test can be minimized by using both polyvalent and individual antisera (for individual immunoglobulin classes and complement) which are fully titrated. The test should also be performed at both 4 and 37°C as erythrocyte-specific autoantibodies sometimes show preferential thermal reactivity *in vitro*. A modification of the test, employing an ELISA-based detection system proved to provide greater specificity, but was not amenable to being used for routine diagnostic purposes.

Recent studies have confirmed the utility of the feline Coombs test. Early investigations suggested that this may be invalid in cats, due to the proposed frequent presence of IgM cold haemagglutinins in normal cats. However, these early data were flawed and it is now clear that the Coombs test is useful in the confirmation of feline IMHA. In one study, we performed a full Coombs test on blood samples from 60 anaemic and 60 non-anaemic cats. Eighteen of the 60 anaemic cats were Coombs positive, and a high proportion (n=13) of these had IMHA as defined by a series of specific criteria. Only 2/60 non-anaemic cats were significantly Coombs positive, and these animals both had pancreatitis – which is a recognized factor underlying IMHA in the cat.

BLOOD TYPING AND CROSS MATCHING

The importance of blood typing and cross matching dogs and cats before transfusion is now widely accepted, as is the determination of feline blood groups to avoid neonatal isoerythrolysis. Traditional agglutination assays using specific polyclonal antisera (or lectins) have been available only through specialist Immunohaematology laboratories. However, the generation of reagents specific for the major canine (DEA-1) and feline (A, B) blood group antigens has led to the generation of new rapid, in-practice tests for determination of blood type. The original card-based system has now been expanded by the availability of a tube-based or immunochromatographic technologies. In-practice cross-matching tests are now also available.

SERUM ANTINUCLEAR ANTIBODY TESTING

The detection of serum antinuclear antibody (ANA) has not changed in terms of methodology employed for many years. Indirect immunofluoresence or immunoperoxidase labelling are used to identify serum ANA that bind the nuclei of substrate cells – either cultured cells grown in monolayers, or tissue sections of nucleated cells (e.g. hepatocytes). Although a variety of ELISA-based or immunoprecipitation tests have also been employed to detect the fine specificity of nuclear antigens – these latter are largely utilized in a research setting. Interpretation remains an important part of ANA testing. Patient sera should be fully titrated to an end point and clinical significance should be determined by reference to a normal reference population. It is clear that approximately 10% of clinically normal dogs, cats and horses will have low-titred serum ANA, and this autoantibody is also not uncommon in animals with a wide range of chronic inflammatory, infectious or neoplastic diseases. A high titre more closely approximates the presence of an autoimmune reaction.

Limited studies have shown that the pattern of nuclear labelling might have clinical relevance in dogs, as it does in man. ANA giving speckled nuclear labelling is more often associated with the presence of musculoskeletal disease and the absence of precipitating antibody. By contrast, homogenous nuclear labelling correlates with the presence of precipitins, and multisystemic clinical disease. A further investigation revealed that homogenous labelling was linked to the presence of antibodies specific for dsDNA and nucleosome DNA, while speckled labelling was associated with antibodies specific for RNP and the Sm antigen.

AUTOANTIBODY SPECIFICITY

Research has defined the fine specificity of autoantibodies in diseases such as pemphigus, bullous pemphigoid (and its many subtypes), diabetes mellitus, myasthenia gravis, masticatory muscle myositis, IMHA and IMTP. This type of testing is largely restricted to the research setting although one laboratory is the global reference centre for measuring AChR autoantibodies in myasthenia.

ALLERGY TESTING

Determination of the specific allergens to which an animal may be sensitized has traditionally relied on intradermal provocation with an antigen panel. Although the intradermal test remains the method of choice for most specialist dermatologists, for the past 20 years there has been increasing availability of serologically-based tests that detect circulating allergen-specific IgE or IgG antibodies. The correlation between these test methods is not strong, as the tests are designed to specifically

measure different aspects of the hypersensitivity response. A variety of test platforms (solid or liquid phase, antibody or labelled receptor detection) are available, and the tests have improved in sensitivity and specificity since they were first introduced; however, a recent study still shows poor correlation between tests.

A possible reason for this variability lies in the presence of cross-reactive carbohydrate antigens which are widely shared between different plants and insects. Normal dogs often make IgE and IgG immune responses to these antigens. Blocking reactions to such epitopes *in vitro* enhances the specificity of serological tests for detection of clinically relevant allergens.

Increasingly available is a range of serological tests for food allergy – although unlike the tests for aeroallergen sensitization, these are not recommended for diagnostic use of diet-related hypersensitivity. A 'food reaction test' may, however, be used to identify exposure to dietary antigens and to define the optimum elimination diet for diagnostic purposes.

IMMUNOHISTOCHEMISTRY

In recent years the application of immunohistochemistry to pathological diagnosis has become routine, with the availability of extensive panels of reagents and automation of the process in many laboratories. The most common use of this technique is in phenotyping or subtyping tumours of a wide histological spectrum.

CLONALITY TESTING

Clonality testing (or PARR testing) is used to distinguish between a polyclonal and monoclonal population of lymphocytes. This has relevance in the diagnosis of lymphoma – particularly in situations where it may be difficult to differentiate between a reactive or neoplastic infiltrate (e.g. in the feline intestinal mucosa). Clonality testing uses the polymerase chain reaction to assess variation in the T- and B-cell receptors expressed by the population. Primers are designed to react with sequences within the V and J regions of the immunoglobulin heavy chain and T cell receptor (TCR) β or γ chains. In a reactive population, numerous bands visible in the end product gel indicate multiple specificities, whereas a restricted (clonal) pattern associates with neoplasia.

More recently, the ability to screen tumours for the expression of genes related to phenotype and function has begun to refine the diagnosis and prognostication for neoplastic disease. In lymphoma, the measurement of serum biomarkers has also been used to track response to therapy of different tumour subtypes.

IMMUNODEFICIENCY TESTING

The most inaccessible of immunodiagnostic procedures are those that are designed to test immune function. Those based on flow cytometric testing are described above. Measurement of serum immunoglobulin (Ig) G, M and A concentration is also fairly widely available for the dog and horse. By contrast, tests able to evaluate the function of lymphocytes (e.g. mitogen- or antigen-driven proliferation, cytokine or chemokine production, cytotoxicity) or phagocytes (e.g. chemotaxis, phagocytosis and intracellular killing) are rarely available outside research institutions. For those canine immunodeficiency diseases with a defined molecular pathogenesis (e.g. X-linked severe combined immunodeficiency, the canine leucocyte adhesion deficiency, cyclic haematopoiesis of the grey collie, trapped neutrophil syndrome of the border collie and lethal acrodermatitis of the bull terrier) specific molecular tests are available for detection of the genetic mutation or identification of heterozygous carriers of the traits.

VACCINAL ANTIBODY TESTING

In recent years it has become possible to test for the presence of antibodies specific to the three canine core viral vaccine antigens (CDV, CPV-2 and CAV) and to feline parvovirus. A range of in-practice test kits enable such testing in a relatively rapid and qualitative or semiquantitative fashion. There are several applications for such tests: (1) determining seroconversion of puppies and kittens after the primary vaccination series, (2) determining the need for core vaccination in animals of unknown vaccination status or in place of routine adult revaccination, (3) determining the need for core vaccination in dogs that have previously suffered a suspected post-vaccinal adverse event, and (4) managing disease outbreaks in shelters. Such screening might also be part of a work-up for suspected primary immunodeficiency disease.

FURTHER READING

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