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Immunological techniques in avian studies

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There is a wide spectrum of techniques available to evaluate the functional aspects of the immune system in both humans and animals, including avian species. Some of the immunological techniques are qualitative and others are quantitative. In this paper, these techniques will be reviewed starting with a description of the nature of the antibody-antigen interaction, followed by the immune techniques that are used to detect immune complexes (*i.e.* antigen-antibody responses) whether free or associated with cells, then the numerous immune markers or parameters that are used to measure the avian immune responses.

Keywords: avian; immunity; techniques

Introduction

Examples of immunological techniques involve investigations to measure the proportions of the differential leukocyte populations, the total number of leukocytes, phagocytosis, oxidative burst, chemotactic response, natural killer cell activity, cytotoxic T-lymphocyte activity, lymphocyte proliferation, production of cytokines, production of antibodies, delayed type hypersensitivity (DTH), inflammation responses and expression of molecules on cell membranes. These methods may involve cell purification in cultures or using whole blood to provide a biological environment which is more identical to the *in vivo* situation due to the fact that it retains all blood components and all physiological factors that may affect immune function in one way or another. Assessment of immune responses in avian species can be achieved by removing and weighing lymphoid organs such as the spleen, thymus, bursa of Fabricius, and lymphoid tissues and aggregates. Resistance to diseases in poultry is most frequently measured by introducing pathogenic challenge and measuring the immune status before and after the challenge. Of critical importance is the fact that these immune parameters and functions are in continuous dynamic change as affected by the general physiological status of the animal and the time of sampling. Moreover, they are affected by the concentration of the antigenic stimulant being used in a dose-dependent relationship.

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Antibody-antigen interactions

Antigen-antibody interaction involves specific recognition and linkage between an antigenic determinant or an epitope and the variable region of the antibody by four kinds of non-covalent bonds, namely, hydrogen, ionic, hydrophobic, and Van der Waals interactions. Aside from the well-established fact that antibodies are highly specific to antigens, cross reactivity sometimes exist if two antigens have identical or very similar epitopes (Lydyard *et al.*, 2000).

Antigen-antibody reactions can be detected qualitatively and/or quantitatively by measuring immune complexes formed of antigens and antibodies. The stability and detection of immune complexes depends on many factors such as the affinity of the antibody for the antigen, the multivalency of antibodies and antigens (*i.e.* avidity), antigen to antibody ratio, and the physical form of the antigen (Goldsby *et al.*, 2003; Wood, 2001). Most of the immunologic assays rely on detecting reactions of antibodies with free or cell-associated antigens. These assays are discussed below.

Flow cytometry

Flow cytometry allows analysis and separation of labelled cells in suspension. The principle of this technique depends on analysis, separation and sorting of different populations of cells based on their size and fluorescence profile.

Cells are first stained by fluorescent antibodies or dyes which bind specifically to antigens on the cell membranes or diffuse into the inside of cells, respectively. The stains are excited by a laser beam, and emit light at specific wavelengths that can be picked up by light detectors. These signals identify specific cells that are stained. Proportions of different cells can also be determined because this instrument can run more than one-colour analysis (Uchiyama *et al.*, 2005). There are numerous immunological applications of this technique in the field where information about the size of different cells can be gained from the light-scattering profile (Rocksen *et al.*, 2003; Goldsby *et al.*, 2003; Lydyard *et al.*, 2000). Some new versions of flow cytometers can even take digital images of individual cells. The data generated from flow cytometers can be analysed and presented as a histogram (*i.e.* one dimension) and as dot plots in two or three dimensions. Gating of cell populations is one of the main principles of flow cytometry, which is a process of selective visualisation of the cells of interest while eliminating results from unwanted particles (*e.g.* dead cells and debris). Gating is traditionally achieved according to physical characteristics of cells that is determined in forward scatter versus side scatter graphs (Watson, 2004).

Measures of avian immunological functions

In the field of avian immunology, there are numerous immune parameters and functions to which different immunological protocols and tests are evolved. Some of these parameters are used to monitor the health status of poultry, while others are measured under a challenged situation by introducing a pathogen. In both cases, some of the immune measures can be performed *ex vivo* or *in vitro*, while others are performed *in vivo*. The general measures of the avian immune response are discussed below, followed by a review of the more specialised markers of the avian functional immune responses.

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GENERAL MEASURES OF AVIAN IMMUNE SYSTEM

These measures are applied to provide a general indication of the strength of the immune system in birds. However, they do not provide a direct assessment of the functionality of the immune system against antigens and infectious diseases. Measurement of the general status of the avian immune system includes assessment of weights and cellularity of immune organs such as the thymus, spleen and bursa of Fabricius, peripheral leukocyte count, and assessment of plasma proteins concentration.

ASSESSMENT OF WEIGHTS AND CELLULARITY OF IMMUNE ORGANS

The main immune organs in poultry are the thymus, spleen and bursa of Fabricius. Upon immunological response, mature lymphocytes and other immune cells interact with antigens in these immune tissues. Consequently, the mass and cellular status of these organs can generally indicate an immune status (Smith and Hunt, 2004; Grasman, 2002; Swamy *et al.*, 2004).

PERIPHERAL LEUKOCYTE COUNT

Circulating blood leukocytes represent a pivotal measure of the immune system status. They possess an effective influence on foreign particles in immune responses. Consequently, they are considered to reflect immune strength. Thus, counting the total and differential numbers of leukocytes is a general parameter of the health status. Abnormal numbers of the specific types of leukocytes may indicate immunosuppression or immunocompetence in response to an immunomodulator or an infectious challenge (Brennan *et al.*, 2002; Eeva *et al.*, 2005; Swamy *et al.*, 2004; Mashaly *et al.*, 2004).

ASSESSMENT OF PLASMA PROTEIN CONCENTRATIONS

Immunological and health status in terms of infection and inflammation is clearly characterised by the production and release of specific plasma or serum proteins. Examples of such proteins are the complement system proteins and the acute phase proteins and their concentration in the plasma is strongly associated with an inflammatory response. Some proteins such as antitrypsin, haptoglobin, fibrinogen, and ferritin are increased in response to inflammation. On the other hand, the concentrations of other proteins such as pre-albumin, albumin, and transferrin are decreased during inflammatory responses. Moreover, antibody responses are commonly associated with the production of γ- globulins. Accordingly, monitoring the level of proteins that are associated with infection and inflammation reflects the health status of birds (Grasman, 2002; Takahashi *et al.*, 2002; Al-Mayah, 2006; Bunchasak *et al.*, 2005).

MARKERS OF FUNCTIONAL AVIAN IMMUNE RESPONSES

The various measures of avian immune response give general information about the health status of birds. Consequently, these measures are unreliable in decision making regarding vaccinations or analysing mechanisms with which immunomodulators affect responses. However, these general measures are useful in monitoring the health status and can be applied as a first step before confirming using other parameters or markers that are more reliable in studying the avian immune functions and responses. The latter measures give more information about the mechanisms with which the immune system responds to a specific challenge. These functional measures can be applied *in vivo* or *in vitro* and include numerous immune parameters as follows.

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MEASURES OF ANTIBODY-MEDIATED IMMUNITY

Immunisation of birds with antigens induces the production of specific antibodies. The pattern of antibody production reflects numerous immunological functions in response to different antigenic stimulants. Primary and secondary immunisation measures are often applied against specific antigens. This is because when a bird is challenged with an antigen, a low magnitude and short duration primary antibody will be produced. A secondary immunisation with the same antigen produces a secondary antibody response that lasts longer and is greater in magnitude (Poli *et al.*, 2000; Goldsby *et al.*, 2003; Sijben *et al.*, 2000b; Fritsche and Cassity, 1992; Bedecarrats and Leeson, 2006; Puthpongsiriporn and Scheideler, 2005).

There are numerous techniques that are used to measure antibody production in response to a specific antigen *in vitro* or *in vivo*. Paramount of all is the use of the enzyme-linked immunosorbent assay (ELISA) technique that utilises species-specific antibodies which bind to the antibodies of the species in question (Elsafty *et al.*, 2006; Kondo *et al.*, 2004). This technique makes it possible to measure the circulating concentrations of total and subclasses of Ig, specific Ig in response to antigenic challenge, concentration of immunoglobulin A (IgA) in secretions such as mucosa, saliva and tears, as well as the circulating concentrations of cytokines and cytokine receptors (Verhulst *et al.*, 2005; Friedman *et al.*, 1998; Abdukalykova and Ruiz-Feria, 2006; Christe, 2002). Interestingly, the humoral immune response can be induced by a wide range of immunomodulators. Sheep red blood cells (SRBC), Newcastle disease virus (NDV), Keyhole limpet haemocyanin (KLH), 2,4-Dinitrophenol, a cellular metabolic poison (DNP), *M. butyricum* and *Escherichia coli (E. coli)* are commonly used in avian immunology studies to induce antibody production (Wang *et al.*, 2000; Fritsche *et al.*, 1991; Praharaj *et al.*, 1997; Guo *et al.*, 2004; Bartlett and Smith, 2003; Muhammad *et al.*, 2006; Velhner *et al.*, 2001; Hangalapura *et al.*, 2003; Sijben *et al.*, 2000a; Parmentier *et al.*, 1997; Huang *et al.*, 2007). However, the limited availability of species-specific antibodies for poultry makes the use of ELISA techniques limited. In these cases, other alternatives such as SRBC haemagglutinatin test and Jerne plaque assays are often used successfully. SRBC haemagglutinatin test involves immunisation of birds with SRBC and blood is collected after a period of time, usually 1-3 weeks, and serial dilutions of the plasma are incubated with SRBC. Sufficient antibody response is reflected by agglutination of SRBC by antibodies (Grasman, 2002; Takahashi *et al.*, 1999). In the Jerne plaque test, birds are first immunised with SRBC and their spleen cells are then incubated with SRBC and complement. Splenic B-lymphocytes will lyse SRBC in its vicinity in the presence of complement system (Luster *et al.*, 1992) .

PHYTOHEMAGGLUTININ SKIN-TESTING TECHNIQUE (PHA SKIN TEST)

PHA skin test is commonly used to measure T- cell mediated immunity because PHA is considered a T-lymphocytes mitogen. It is a carbohydrate binding lectin extracted from red kidney beans called *Phaseolus vulgaris* (Kennedy and Nager, 2006; Grasman, 2002; Calder, 2007; Abdukalykova and Ruiz-Feria, 2006).

The PHA-skin swelling technique is a widely used quantitative technique to measure the acquired immunity in terms of T-cell activity in birds. In this technique, birds are injected in their wing web, wattle, dewlap, or interdigitary skin with PHA. A control saline solution such as phosphate buffered saline (PBS) is injected into an alternate site. PHA response is then calculated 12-24 or 48 hours after injection as the difference in skin thickness increase between the two cases of PHA and PBS injections using a micrometer (Smits *et al.*, 1999; Grasman, 2002; Verhulst *et al.*, 2005; Martinez-Padilla, 2006; Moore and Siopes, 2002; Kliger *et al.*, 2000; Cheema *et al.*, 2003; Sijben *et al.*, 2001a;

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Bedecarrats and Leeson, 2006; Babu *et al.*, 2005; Korver and Klasing, 1997; Hudson *et al.*, 2004; Fritts *et al.*, 2004).

It was concluded by Martin *et al.* (2006) that PHA as a foreign protein induces both innate and acquired branches of the avian immune system. Directly after PHA injection, an influx of heterophils and thrombocytes occurs, followed by basophils and macrophages. This innate response is associated with inflammation response and skin trauma. After 24 hours post-injection, infiltration of lymphocytes, especially T-lymphocytes peaks at the site of injection. The pivotal involvement of T-lymphocytes in the PHA response assay was further confirmed by Edelman *et al.* (1986), and Schrank *et al.*(1990). These authors concluded that immuno-suppression of avian T-lymphocytes by irradiation or chemical drugs significantly decreased PHA skin response up to 50-60%. Similarly, the PHA response was suppressed by 30-45% as a result of organochlorines exposure, these chemicals are known to suppress T-lymphocytes function in birds (Grasman and Fox, 2001). Interestingly, some authors studied the primary and secondary PHA injection in birds and have shown that the secondary response induced greater swelling than the primary response, indicating that the immunity involved is characterised by a memory response (Johnsen *et al.*, 2000; Kondo *et al.*, 2004). On the contrary, Reid *et al.* (2003) showed no significant difference between the primary and secondary PHA responses.

ANTIGEN-SPECIFIC DELAYED TYPE HYPERSENSITIVITY TEST (DTH)

Unlike the PHA-skin test that induces large numbers of T-lymphocytes that are not specific to PHA, antigen-specific DTH such as the tuberculin skin test induces specific lymphocytes for a particular antigen. Primary injection induces memory and antigen presenting cells that are activated in a secondary response. Interestingly, both PHA and antigen-specific DTH skin tests give the same results (Fairbrother and Fowles, 1990; Grasman, 2002; Wang *et al.*, 2002).

Another modification of DTH skin tests that does not cause skin trauma is the use of skin patches of contact-sensitising agents such as oxazolone or dinitrofluorobenzene. These agents stimulate T-lymphocytes and natural killer cells upon primary and secondary applications. The resulting response can be measured in the same way as PHA and DTH skin tests (O'Leary *et al.*, 2006).

PHAGOCYTOSIS ASSAY

In vitro assays require the isolation of white blood cells of interest in order to use them in functional immunological assays. One of the most common methods of isolation of avian mononuclear cells (*i.e.* lymphocytes and monocytes) is by centrifugation of blood samples to create a density gradient. This separates more than 70% of the lymphocytes and monocytes at the top of the gradient (Fowles *et al.*, 1997; Trust *et al.*, 1994). Nevertheless, contamination of monocytes with quite large numbers of thrombocytes characterises this method. Gogal *et al.* (1997) have reported that thrombocyte contamination can be reduced by 5-20% by a slow-spin technique without a density gradient. Moreover, it was shown that diluting the blood samples with saline or culture medium with application of the density gradient may significantly reduce thrombocyte contamination (Grasman, 2002).

In addition the purified cell preparations, whole blood samples can be used to assess *in vitro* or *ex vivo* functional assays of the immune system. The latter has the advantage of retaining all the components of the *in vivo* situation in the same ratios. Nevertheless, purified cell cultures give information about the number of cells in the assay and the cells can be stored in a liquid nitrogen compartment for future use (Calder, 2007; Fairbrother and Fowles, 1990; Fowles *et al.*, 1997).

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The phagocytic capability of avian heterophils and mononuclear cells can be measured by the phagocytosis assay that quantitatively measures the percentage of phagocytes and their enzymatic activity (*i.e.* the number of ingested antigens per phagocytic cell). Bacteria such as *Escherichia coli*, SRBC, and yeast cells are commonly used as substrates for phagocytosis. Opsonisation of these substrates by complements and/or antibodies is usually applied to facilitate phagocytosis, although unopsonised substrates may be used (Thies *et al.*, 2001; Arunachalam *et al.*, 2000). Opsonisation degree, temperature, incubation time and the ratio of substrates to phagocytes are the main factors to be considered when conducting phagocytosis assays. Labelling the substrates for phagocytosis with fluorescent stains enables detection and counting of these substrates by techniques such as flow cytometry or fluorescence microscopy (Janaczyk *et al.*, 2006).

OXIDATIVE BURST

Measuring the phagocytic activity of leukocytes is commonly associated with the oxidative burst assay that involves production of anti-microbial and cytotoxic substances in the form of reactive oxygen and nitrogen intermediates such as hydroxyl radicals, hydrogen peroxide and nitric oxide (Farnell *et al.*, 2003a;Farnell *et al.*, 2003b;Kogut *et al.*, 2001;Salgar *et al.*, 1991). The oxidative burst assay, commonly referred to as phago-burst or burst test, involves the use of unlabelled opsonised bacteria or any other antigen and a stimulus for the reaction that is usually a protein kinase C ligand phorbol, as well as a chemotactic factor such as N-formyl-MetLeuPhe. A flourogenic substrate is used to enable quantitative determination of the reaction by the use of flow cytometry or fluorescence microscopy techniques (Farnell *et al.*, 2003b; Chen *et al.*, 2003a; 2003b; Fritts *et al.*, 2004; Kew *et al.*, 2003a).

BACTERICIDAL KILLING BY WHOLE BLOOD AND PLASMA

In addition to phagocytosis by cellular components, bacteria can be killed by soluble immune elements in whole blood and plasma. For example, the complement system can initiate an enzymatic cascade capable of lysing targeted bacterial cells (Nordahl *et al.*, 2004). In addition, plasma lysozymes and acute phase proteins such as mannose-binding proteins exhibit bactericidal competence by attacking bacterial cell walls (Zheng *et al.*, 2005; Fernie-King *et al.*, 2002). This bacteria-killing ability contributes to the innate immune response, and can be easily measured *in vitro* by using whole blood or isolated plasma. One advantage of this assay is that it does not require species-specific reagents (Matson *et al.*, 2006).

CHEMOTACTIC RESPONSE

Chemotaxis is the movement of immune cells toward the site of infection under the influence of certain stimuli (Harmon, 1998; Qureshi, 2003; Qureshi *et al.*, 1993). Chemotactic response of heterophils and monocytes can be studied by using different stimuli such as leukotriene B4, antigenic peptides of bacterial cell walls, some interleukins, and autologous serum (Walrand *et al.*, 2001; Calder, 2007).

NATURAL KILLER CELL (NK) AND CYTOTOXIC T-LYMPHOCYTES ACTIVITY

These cytotoxic activities are usually combined with each other in *in vitro* assays. The target cells are incubated with NK cells and cytotoxic T-cells for specific periods of time and the assay is described by measuring the lytic ratio between the cytotoxic and the target cells (Calder, 2007; Calder *et al.*, 2002; Fowles *et al.*, 1997). Classical responses mediated by NK cells, monocytes and eosinophils can also be measured (Fritsche and Cassity, 1992; Fritts *et al.*, 2004). Antigen-coated chicken red blood cells are widely used

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to conduct the cytotoxicity assay (Boyd and Wick, 1980; Maccubbin and Schierman, 1986).

MITOGEN-STIMULATED PROLIFERATION OF T- AND B- LYMPHOCYTES

T- and B- lymphocytes are well known to proliferate *in vitro* by increasing in number and by synthesizing DNA during blastogenic transformation in response to certain stimuli such as mitogens, antigens, antibodies, or even vaccines. For example, concanavalin A (Con A) and PHA mitogens induce proliferation of T-lymphocytes, while bacterial lipopolysaccharides (LPS) and *Staphylococcus aureus* stimulate proliferation of B-lymphocytes. Pokeweed mitogens stimulate proliferation of both T- and B-lymphocytes (Meydani *et al.*, 1991; Fritsche *et al.*, 1991; Calder, 2007; Sijben *et al.*, 2000a; Barber *et al.*, 2005; Lee, 1978).

Lymphocyte proliferation is traditionally measured by radioactivity of incorporated thymidine into the DNA of mitotic lymphocytes. Incorporation of bromodeoxyuridine into the deoxyribonucleic acid (DNA) of lymphocytes may also be used as a measure of lymphocyte proliferation (Wang *et al.*, 2000; Calder, 2007; Takahashi *et al.*, 1999; Hangalapura *et al.*, 2003). A flow cytometry technique was recently used to measure cell proliferation by incorporation of carboxyfluoroscein succinimidyl ester (CFSE) stain (Lyons, 2000; Lyons and Parish, 1994; Angulo and Fulcher, 1998; Bird *et al.*, 1998; Hodgkin *et al.*, 1997).

PRODUCTION OF CYTOKINES

Cytokines are known to be produced upon stimulation of lymphocytes and monocytes as regulators of the immune response. Cytokines can be measured in cultures using ELISA techniques in which monoclonal antibodies are directed to them. Flow cytometry techniques are used to measure cytokines and to phenotype the cells producing them. In addition, intracellular cytokines can be measured by flow cytometry by using specific stains after treatment of cell membranes (Meydani *et al.*, 1991; Thies *et al.*, 2001; Tricon *et al.*, 2004; Yang and Yuming, 2006; Kromer *et al.*, 1984). Up-regulation or down-regulation of different types of cytokines can be used as an indication of the immune status. For example, immuno-suppression in chickens is commonly evaluated as a decrease in IFN- α and IFN-γ m-RNA that can be detected by using nucleic acid hybridisation micro-titre assays (Kew *et al.*, 2003b; Novak and Scheideler, 2001; Chen *et al.*, 2003a; 2003b; Sijben *et al.*, 2001b; Meydani *et al.*, 1991).

EXPRESSION OF CELL MEMBRANE MOLECULES

Stimulation of different types of immune cells induces the expression of specific molecules on their surfaces. Consequently, determination of different immune cells can be achieved by identifying molecules expressed on their cell membranes. This requires the use of specific stains or markers for these surface molecules. Examples of cell surface molecules include toll-like receptors, major histocompatibility complex (MHC) subtypes, clusters of differentiation (CD) subtypes and cytokine receptors. Determination of the profiles of different cell types is then achieved by the use of the flow cytometry technique (Farnell *et al.*, 2003a; Calder, 2001; 2007). In addition, other immuno-fluorescence techniques such as fluorescence microscopy can be used to detect reactions with surface antigens (Swamy *et al.*, 2004). Some antibodies have been developed to chicken antigens such as to chicken CD3, CD4, CD8, and to cytokines (Bacon, 1992; Chan *et al.*, 1988). This approach enables identification of the morphological characteristics of lymphocytes during their differentiation and developmental stages. For example, Chen *et al.* (1988) showed that two receptors TcR1 and TcR2 are expressed on separate T cell sub-lineages during their early

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development in the thymus and then in the periphery. This technique of labelling immune cells with monoclonal antibodies directed against surface antigens is called immunophenotyping.

Conclusions

Taken together, there are a wide range of immunological techniques to be used to investigate the health status of avian species. The decision of using certain an immune technique depends on the purpose of the study and the immune function to be investigated. There are various vital applications of these techniques field of chemical, clinical and immunological studies of both human and animal species, including avian species. They can be applied in immunological avian studies to investigate the effect of immunomodulators on the immune system and general health status. Monitoring the health status of poultry flocks helps in surveillance programs to maintain the general health of poultry flocks and to discover any outbreaks of diseases related to abnormal range of immune functions.

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