Reference Point

Methods for analysis of cell-mediated immunity in domestic animal species

Matthew R. Sandbulte, PhD, and James A. Roth, DVM, PhD, DACVM

Past efforts to evaluate immune responses in domestic animals typically focused on humoral immunity. One reason for this is the relative ease with which serum antibody responses can be monitored by use of ELISAs and virus neutralization and hemagglutination assays. Immunological studies in recent years have highlighted the substantial roles of **cell-mediated immunity (CMI)** in defense against human and animal infections. This has accentuated the importance of examining CMI as part of any thorough effort to characterize an immune response to infection or vaccination.

When evaluating vaccines used in veterinary medicine, serum antibody titer is often cited as an important measure of vaccine efficacy. Yet, in a variety of situations, these data are insufficient to predict a positive or negative outcome for the vaccinated animal regarding development of disease:

- Specific antibodies against some infectious agents do not adequately protect the host from infection and disease.[1]
- Antibodies against certain infectious agents enhance disease. One mechanism for this is antibody-dependent enhancement[2] in which immunoglobulin-bound pathogens rely on Fc receptor-mediated phagocytosis for entry to host cells. Immune complexes can also contribute to the pathogenesis of infectious diseases by triggering complement activation and inducing a type III hypersensitivity response.[3]
- Antibodies may not always be detectable following an adaptive immune response. With regard to several viral agents, adaptive CMI responses can be mounted in the presence of passively transferred maternal antibody, even while humoral responses are suppressed.[4,5] Thus, animals with no detectable antibody titers can be fully resistant to challenge.

Cell-mediated immunity is a rather simple label for a broad and complex set of mechanisms that require T-cell activity. Effector T cells are induced against pathogens in response to primary infection or vaccination; some activated cells eventually differentiate into memory T cells that can expand and exert antimicrobial activity very rapidly in the event of secondary exposure.[6] **Cytotoxic T lymphocytes (CTLs)**, which express CD8 protein on their surface, have an important role against a wide range of intracellular pathogens.[7,8] These lymphocytes are programmed to kill infected cells when their **T-cell receptors (TCRs)** recognize pathogen-derived peptides that have been processed and displayed on **major histocompatability complex (MHC)** class I molecules via the endogenous pathway.[9,10] Peptides presented on MHC class II molecules by the exogenous pathway are recognized by $CD4^+$ helper T cells.[10] Helper T cells coordinate the overall adaptive immune response by modulating the activities of many other immune cells, both through direct cell-cell contact (eg, CD40-CD40 ligand signaling) and secretion of cytokines.[11,12] Cytokines can be categorized by the many different functional activities they mediate, and any cytokine may have multiple effects.[12,13] Some cytokines activate innate immune cells, such as phagocytes and natural killer cells, and stimulate their participation in inflammatory processes, whereas others suppress or resolve inflammation. Several cytokines promote the activation of and antibody production by B cells, as well as antibody class switching of B cells. Certain cytokines have effects further upstream in the ontogeny of cells, in that they direct the maturation and mobilization of distinct classes of cells. Chemokines control immune cell traffic in at least 2 ways: they attract the specific sets of cells that bear corresponding receptors and activate the integrin molecules that mediate leukocyte adhesion to vascular endothelium around the site of an infection.[14]

In recent years, the balance of immune regulation has been one of the foremost topics in immunology.[13] Successful defense against the majority of infections requires an early **T helper (Th)** 1 cell-mediated inflammatory response (type 1 response). Interferon-γ, **interleukin-2 (IL-2)**, and lymphotoxin-α are particularly important type 1 cytokines. Type 2 responses (ie, those mediated by Th2 cells) are generally characterized by high antibody production and often by recruitment of eosinophil and mast cell activity. Of the type 2 cytokines, IL-4, IL-10, and IL-13 are among the most dominant and best characterized. One purpose for type 2 responses appears to be mediation of immunity against helminth infections. Also, a transition to predominantly type 2 responses appears to be important for resolving inflammation after other kinds of infection. However, when

From the Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011. Dr. Sandbulte's present address is the Department of Infectious Diseases, St Jude Children's Research Hospital, 332 N Lauderdale St, Memphis, TN 38105.
Address correspondence to Dr. Roth.

Th2 cell-driven mechanisms dominate early in infection, microbial replication may proceed unchecked. This, as well as antibody-mediated hypersensitivity, can enhance the severity of disease. Importantly, cytokines from type 1 responses tend to suppress type 2 responses and vice versa, so that one or the other response predominates. Given the divergent properties of helper T cell subsets, the type 1 versus type 2 theory has been used to explain striking divergences between protection and disease development in response to certain infections in humans and rodent species, such as leprosy and leishmaniasis.

Measurement of cytokine activity and T helper subsets has been and promises to be a useful measure of vaccine efficacy against infectious agents of animals. Much of the research that has shaped the type 1 versus type 2 theory has involved studies of mice. Human helper T cell responses also polarize toward type 1 or type 2 profiles, although the definitions seem to be less rigid.[13] There is considerable evidence to support this paradigm in numerous domestic animal species as well.[15-18] However, it is important to be aware that the effects exerted by a cytokine in 1 species may not be identical to those exerted by its homologue in another species.[15]

Gamma delta (γδ) T cells are the other major T cell subset.[19] The TCR for this subset of T cells is a heterodimer comprised of a γ chain and δ chain, which are structurally distinct from the α and β chains that form the TCRs of $CD4^+$ and $CD8^+$ T cells. Antigen recognition by γδ T cells is not usually MHC-restricted. Certain host cell markers, such as stress-induced heat-shock proteins, can activate γδ T cells. The functions of γδ T cells in response to pathogens are not well understood, but their prominent distribution in mucosal surfaces is consistent with a role in surveillance and rapid response to invaders. There is also evidence that γδ T cells modulate inflammatory responses and promote tissue repair.[20]

Several important in vitro assays for antigen-specific cellular immune activity were developed approximately 30 years ago.[21,22] Most of these early in vitro CMI assays were used by veterinary researchers to some extent. Several review articles[23-26] describing those applications in domestic animals were published in past decades. Some of those classical methods for monitoring CMI continue to be used for immunologic studies of animals. However, technological advances in immunology have supplied researchers with a much wider array of CMI-related assays. Notable improvements have been made in the efficiency of experiments and the insight they can provide. In this article, the prevalent assays and technologies of the present day will be reviewed, with emphasis on contemporary approaches that have proven useful in studies of outbred, domestic animal species. The assays described here have been designed to monitor 4 categories of immune parameters: antigen-specific proliferation and activation of distinct T-cell subsets, antigen-specific cytokine production, cytotoxicity and associated effector molecules, and direct detection of peptide-specific receptors.

To set the context for this discussion, it is important to point out that there are 2 reasons to assay for T-cell activity. One reason is to examine basic immunologic mechanisms involved in an immune response and the roles of those mechanisms in protection. This can be done with in-depth, molecular analysis on small numbers of specialized animals (such as gene-knockout mice or National Institutes of Health mini pigs). The second reason is to monitor T-cell responses to vaccination in populations of animals over time. This requires assays that can be performed on large numbers of outbred animals. Some assays are very cumbersome to use for this purpose (eg, CTL assays). Other CMI assays are inherently more practical for this research aim (eg, in vitro tests for antigen-specific lymphocyte activation and proliferation or cytokine production). Ideally, some of these CMI methods will be enhanced to match the efficiency of serologic assays for immunologic monitoring of domestic animals. It is important to bear in mind that the results of any given assay must be interpreted cautiously, whether the assay evaluates antibody production or cellular activity; there is artificiality and variability in any in vitro test. Some careful study may be required to determine how well the readout from an assay correlates with immunity against the pathogen in question.

Antigen-Specific Activation and Proliferation of T Cells

Delayed-type hypersensitivity—The oldest technique for monitoring the cell-mediated response to an antigen is the **delayed-type hypersensitivity (DTH)** assay.[27] This method involves observation of the swelling that results from leukocyte recruitment to a site of ID injection of an antigen in an immune animal. Therefore, it permits in vivo detection of cell-mediated responses to a wide variety of infectious agents. Another advantage is that the test can be performed relatively easily without expensive equipment. Because of these features, the DTH assay is still used frequently.[28-30] There are several drawbacks to the assay, however. It is difficult to produce a quantitative readout of the results, and specifying which immune cells infiltrate the site of injection is often impractical. Also, the DTH test may only be valid the first time it is performed in an individual, if the test itself induces T-cell responsiveness.

Proliferation—Antigen-specific lymphocyte proliferation (blastogenesis) has been widely used to assess antigen recall responses in vitro. A high level of proliferation in response to an antigen correlates with the expansion of antigen-specific lymphocytes in response to vaccination or infection and indicates the superior recall responses of memory cells in immune individuals. Lymphocyte proliferation has typically been analyzed by measuring the cellular uptake of tritiated thymidine.[23,31] Tritiated thymidine is added to lymphocyte culture during the final 8 to 24 hours of incubation with antigen. This technique offers a quantitative readout via a scintillation counter, and it can be repeated routinely with **peripheral blood mononuclear cells (PBMCs)** from an individual animal. However, data from the assay do not indicate which lymphocyte populations are responding to a given antigen; the assay only provides information about proliferation during the period when tritiated thymidine is present. The hazards and restrictions of handling radioactive material are important disadvantages.

Many of the newer methods for CMI assessment are akin to the conventional lymphocyte proliferation assay, in that they are based on in vitro culture of PBMCs with antigen. Lymphocyte proliferation is still a very widely used indicator of antigen-driven cellular activity in these systems, but considerable efforts have been made to develop alternative proliferation assays that use nonradioactive signals and provide more information about the responding cell populations. Colorimetric assays can serve as effective quantitative tests for lymphocyte proliferation.[32,33] Two indicator reagents that are effective in domestic animal studies are alamar blue and the tetrazolium salt MTT (**Appendix 1**). Both of these reagents undergo color changes under the metabolic conditions of proliferating lymphocytes, and they can be quantified via standard spectrophotometry. An advantage of colorimetric readouts over measurements of tritiated thymidine incorporation is that radioactive materials are not required.

Flow cytometry-based proliferation assays have gained widespread acceptance in recent years.[51-53] With these techniques, markers are incorporated into cells during culture and precise measurements of antigen-driven proliferation can be derived from changes in marker intensity. Reagents that have been used for this purpose include **5-bromo-deoxyuridine (BrdU)**, the PKH series of dyes, and carboxyfluorescein diacetate succinimidyl ester (generally referred to as CFSE, although CFSE is actually the fluorescent molecule derived after intracellular esterases remove 2 acetate side chains from the original molecule). All 3 of these have been used successfully in immunologic studies of domestic animal species (Appendix 1). The nucleotide analog BrdU is added to cells late in culture for incorporation into replicating DNA. Before flow cytometric analysis, BrdU must be bound with a fluorescent antibody.[42] Both PKH dyes and CFSE are typically used to stain cells before culture with antigen, and no further reagents are required to achieve fluorescence. The PKH dyes stain cells via integration into the membrane, whereas CFSE is a membrane-permeant dye that binds covalently to cytoplasmic proteins. With either type of dye, each cell division results in 2 daughter cells with half the fluorescence intensity of the parent cell. This allows one to determine the proportion of cells that have undergone division and the number of generations that have occurred.[54] Cells undergoing as many as 8 rounds of division are routinely differentiated with these protocols. Another advantage of flow cytometric proliferation techniques is the capacity to phenotype the proliferating and nonproliferating cells. Fluorescent antibody conjugates are used directly or indirectly to label T lymphocyte subset markers, such as CD4, CD8, and the γδ TCR.

Activation marker induction—Along similar lines, lymphocyte responses to antigens are detectable in vitro by flow cytometric analysis of activation-induced cell surface markers. Fluorescent antibodies can be used to label these molecules. Activation markers that have been used for the purpose of detecting recall responses include CD25 (IL-2 receptor α subunit), CD69, and MHC class II[48,55-57] (Appendix 1). The expression of such markers can be analyzed in parallel with T lymphocyte subset markers to assess the level of responses by distinct cell populations, such as CD4, CD8, and γδ T cells. Levels of activation marker expression in a cell population can be measured in terms of 2 values: the percentage of cells that express the marker and the mean fluorescence intensity of staining for the marker. Monoclonal antibodies against subset markers and activation markers are commercially available for a number of animal species, which makes this approach a practical tool for veterinary researchers. Under some circumstances, antigen-stimulated cells express activation markers but do not proliferate, suggesting that activation marker-based assays should detect some T-cell responses that remain undetected via proliferation assays.[55,58]

There are some important considerations regarding the interpretation of data from assays of lymphocyte proliferation or activation. The demonstration of either type of response following exposure to antigen does not prove any particular effector function. If 1 subset of lymphocytes is better adapted to survive and proliferate in vitro, then the relative levels of antigen-driven proliferation or activation across subsets may be skewed in favor of that subset. The dyes used to monitor proliferation are often somewhat toxic to cells, and this complicates the interpretation of data, particularly with negative results. In addition, it is possible that antigen-specific cytokine production by 1 T-cell population in an in vitro system might promote nonspecific bystander responses by other T cells. Ordinarily, the activation of a T cell depends on antigen recognition by the TCR, and further stimulation by cytokines amplifies that antigen-specific response. However, memory T cells can be sensitive to stimulation by certain cytokines in the absence of antigen.[59] Thus, it is plausible that an antigen-specific T-cell response in vitro might lead to the activation of accompanying memory T cells that do not share the same specificity. It has not been proven conclusively whether this causes measurements of antigen-specific T-cell activity to be overestimated. Of course, even bystander T-cell activation presumably depends on antigen-specific cells having made a recall response in the first place.

Antigen-Specific Cytokine Production

A variety of methods are used to analyze the capacity of T cells for antigen-specific cytokine expression, and similar to the aforementioned methods, most of these involve the incubation of cells with antigens. Two of the most frequently assayed cytokines are interferon-γ and IL-4 (the signature products of Th1 and Th2 cells), but many experimental situations call for the analysis of other cytokines.

Bioassays—One approach to the analysis of antigen-specific cytokine production is to perform bioassays.[60,61] In these assays, supernatants from antigen-stimulated lymphocytes are tested for biological effects on a specific type of cell in culture. For instance, B-cell proliferation can be associated with the presence of IL-4. Numerous bioassays have been used for studies of domestic animals (**Appendix 2**), especially in instances

of limited reagent availability. One distinct disadvantage to these methods is that a particular cytokine is typically not the only factor capable of causing a particular biological effect. The presence of a cytokine can be assumed more confidently if the activity is blocked by a cytokine-specific antibody. Because of the subjectivity inherent in bioassays, direct detection of cytokines or the transcripts that encode them is generally considered to be more accurate and reliable.

Cytokine mRNA detection—Starting at the level of gene transcription, the first approach is to detect shifts in the amounts of cytokine mRNA after exposure of cells to antigen. Methods of analyzing cytokine gene transcription include northern hybridization, ribonuclease protection assays, and several **reverse transcription-polymerase chain reaction (RT-PCR)** assays.[84,85] Total RNA can be isolated for any of these methods with a number of commercially available kits. Because there are published genetic sequences for many of the key cytokines in domestic animal species, the development of probes or primers suited for these techniques is generally very feasible. In their basic forms, the mRNA-based assays yield data that are qualitative or semiquantitative, but adaptations with better quantitative readouts have been developed.

A ribonuclease protection assay for cytokine transcription relies on the construction of a radioactively labeled antisense RNA probe derived from the gene sequence.[86] The probe is incubated with total RNA so that it may hybridize with target mRNA. Next, the RNA mixture is treated with ribonuclease so that single-stranded RNA is digested, whereas the double-stranded RNA complexes are protected. The sample is separated in a denaturing polyacrylamide gel. Radioactive signals from the dried gel are measured, and the intensity of the signal is proportional to the amount of mRNA. The RNA messages for multiple cytokines can be analyzed in a single assay, provided that the probe sizes can be differentiated on the gel.[66,86]

Advantages of the RT-PCR assay are that it is partially automated and requires no radioactive isotopes.[85] Total RNA is reverse transcribed into DNA by standard procedures that involve heat-stable reverse transcriptase. A pair of PCR primers derived from the cytokine sequence is used to amplify the specific signal of interest. In the conventional technique, the product is electrophoresed on an agarose gel so that the target band can be visualized with ethidium bromide under UV light. Densitometry techniques can be used to compare band intensities.

A competitive RT-PCR assay is a modified version of the RT-PCR assay designed to make results somewhat more quantifiable[87] and has been used often for the measurement of cytokines from domestic animals (Appendix 2). This technique requires the construction of a competitor template that is recognized by the same 2 primers that target the cytokine mRNA. Amplification of the cDNA template and competitor template yields products of distinct sizes. In a set of parallel reactions, the quantity of competitor template is reduced by serial dilutions. The key concept is that greater initial quantities of mRNA-derived template will outnumber the competitor template; thus, the signal will be delivered earlier in the series of competitor dilutions. Polymerase chain reaction products are separated and visualized on agarose gels. The concentration of cDNA can be estimated by assessing the concentration of competitor template DNA in the particular lane where the 2 bands have equal intensity.

A major advance in this area is the real-time RT-PCR assay, which enables a researcher to quantify cytokine transcripts with high sensitivity and accuracy.[88] The readout from this assay is derived from fluorescence signals that correspond with the synthesis of the PCR product. The accumulation of signals is monitored in real time from 1 PCR cycle to the next. One strategy is to use a dye in the reaction mixture that specifically labels double-stranded DNA. Greater specificity is attainable with fluorescently labeled DNA probes. These oligonucleotide probes have a reporter dye at 1 end and a quencher at the other. Fluorescence is quenched unless the probe is cleaved. The probe is specific to the DNA sequence of the target gene, and enough of it must be present to tag all amplified DNA strands. During the next primer elongation step, the 5' exonuclease activity of *Taq* polymerase cleaves the probe and unquenches the reporter fluorochrome. A fluorimeter built into the thermocycler measures this signal. The number of cycles required to achieve a detectable signal and the kinetics of signal amplification are used to quantify the mRNA template, typically with the help of computer software. The real-time RT-PCR technique has been used in recent years to monitor cytokine expression in animal studies (Appendix 2).

Generally speaking, the key advantage of mRNA sequence-based tools for studying cytokine expression is their versatility in settings where monoclonal antibody reagents are not available, which is common in domestic animal research. However, several disadvantages must be taken into account. First, the transcription of genes does not always correlate with the synthesis of respective proteins. Second, the expense and labor required to perform RNA isolation, reverse transcription, and PCR procedures, in addition to the initial cell culture, may be disadvantageous in experiments in which a large number of samples must be tested. Real-time PCR assays require specialized equipment, which represents another considerable expense.

Cytokine detection in lymphocyte culture supernatant—Cytokines are routinely measured in supernatants from antigen-stimulated cells by use of ELISAs.[60] The sandwich ELISA is preferred for this purpose because of its high specificity and sensitivity. This technique requires a pair of antibodies to a specific cytokine, each of which ideally should bind to different epitopes. The detection antibody is frequently conjugated to biotin so that an enzyme-streptavidin conjugate can be added in the next step, followed by the addition of the enzyme substrate. Alternatively, an unlabeled detection antibody can be used, which subsequently requires indirect labeling with an isotype-specific antibody-enzyme conjugate. For many human and murine cytokines, ELISA kits are available commercially, but there are only a few kits marketed for

domestic animal species. In some instances, in the absence of available kits, pairs of monoclonal antibodies can be purchased or developed to produce ELISAs in-house.[89] For the detection of cytokines, one of the advantages of an ELISA is that many samples can be collected, frozen for storage, and then tested efficiently at 1 time. Equipment suitable for this technique is available in most research laboratories. Quantitative results can be obtained by use of a standard curve that is derived via assessment of purified or recombinant cytokine. Drawbacks to conventional ELISAs are that they do not identify the cell populations that produce a cytokine or indicate the frequency of cells that express it. Also, detection of a cytokine protein does not ensure that the protein is in its biologically active form. Cytokines that are characteristically produced at low protein concentrations, such as IL-4, can be difficult to detect via sandwich ELISA.[90]

Cytokine detection within single cells—The frequency of cytokine-secreting cells can be determined with an **enzyme-linked immunospot (ELISPOT)** assay, which is a variation on the sandwich ELISA.[53,91] In this method, capture antibody is coated on the bottom of plastic wells before medium, cells, and antigen are incubated in them. At each locus in the well where a cytokine-secreting cell lies, that cytokine will bind to capture antibodies within a small radius. At the completion of the culture period, approximately the same detection steps are carried out that would be involved in a conventional sandwich ELISA. The result is a pattern of spots that corresponds with the distribution of cytokine-secreting cells. These spots are examined microscopically and quantified either manually or via a computer; by use of a straightforward calculation, the frequency of cytokine-secreting cells can be ascertained. This approach is often used to count effector cells after only 1 or 2 days of ex vivo incubation, although the detection of memory cells can require more time.[53] Limitations to the ELISPOT method include the lack of information about cytokine quantities and the identity of secretor cells. The latter can be addressed if cells are sorted by phenotype before beginning the assay. For studies involving domestic animal species, reagent availability for ELISPOT assays is likely to be similar to that associated with conventional sandwich ELISAs.

Cytokine-producing activity can be measured in distinct T-cell subsets by use of intracellular cytokine staining techniques and multiparameter flow cytometry.[92,93] Two key requirements for the success of this approach are that cells retain the cytokine molecules they produce and that cells are permeable to antibody during the staining steps. The first requirement is met by treating cells with an inhibitor of the Golgi complex, such as brefeldin A, during culture. The second requirement is met by fixing cells and making their membranes permeable with a detergent solution before labeling (permeabilization). All of the reagents necessary for blocking secretion, fixation, and permeabilization can be purchased together in a kit. Data are acquired and analyzed by almost the same approach as the aforementioned flow cytometric assays for T-cell activation. Antigen-specific cytokine-producing cells are often present at rather low frequencies, making assay sensitivity a potential problem. One approach to overcoming this problem is to amplify the responding cells by adding phorbol myristate acetate-ionomycin to the cell culture near the end of incubation. Clearly, with this approach, it is essential to run controls for nonspecific T-cell activation. The main advantage of intracellular staining is its powerful capacity to trace cytokine production to specific populations of effector lymphocytes. However, the assay is complicated by the special requirements for inhibition of protein secretion and treatment of cells to induce membrane permeability. Altered cellular activity may distort normal antigen-driven processes, and cell-labeling procedures inevitably allow some leakage of cytokines. In addition, the technique is more time-consuming than flow cytometric assays for T-cell activation markers.

Cytotoxicity and Associated Effector Molecules

Conventional CTL assay—Cytotoxic T lymphocytes are a crucial component of many immune responses and have long been assayed by use of an in vitro method that measures the lysis of antigen-loaded autologous target cells. In the conventional chromium-release format for this technique, **chromium Cr 51 (^{51}Cr)** is loaded in the target cells, and its release is an indicator of target cell lysis.[23,94] Several controls are necessary to demonstrate antigen specificity and MHC restriction. One of the difficulties with this technique is handling radioactive material. Several related techniques that use nonradioactive labeling for target cells have been described, but none of them seem to have been widely adopted. In investigations involving outbred animals, the major disadvantage of this method is that autologous target cell lines that are susceptible to infection with the agent being studied must be maintained for each individual tested.

Effector molecules associated with cytoxicity—Cytotoxic T lymphocytes express some distinctive molecules that function in target cell killing. Some of these have been used as markers for immunity, and although these methods are potentially very useful, they have not been widely used in veterinary research to date. Effector molecules that originate in the granules of CTLs (most notably perforin, granzyme A, and granzyme B) are of particular interest. The synthesis of these proteins is induced upon activation of the naïve CTL precursor.[95] Then if the primed effector CTL recognizes an infected cell, it releases perforin in a polarized fashion. Perforin facilitates the delivery of other granule contents into the infected cell. Among these contents are granzymes, which are serine proteases that trigger apoptosis pathways. An ELISPOT assay designed to directly measure granzyme B secretion was used to detect peptide-specific CTLs among PBMCs from humans.[96] An alternative approach, which probably requires less investment in the development of reagents, is to measure granzyme activity with serine protease substrates as indicators. Correlations have been detected between enzymatic activity and target cell killing.[97,98] For instance, a strong correlation has been reported between the results of parallel CTL assays that

analyze chromium release and granzyme activity in human cells.[99] In the latter method, granzyme protease activity is detected in PBMCs by measuring digestion of a substrate that is specific to granzyme B. Techniques for monitoring granzyme production or activity would appear to offer better efficiency and fewer difficulties with radioactive materials and target cell preparation, compared wth conventional ^{51}Cr release assays.

Direct Detection of Peptide-Specific Receptors

A major breakthrough in immunology in the past several years has been the development of reagents and techniques that allow the direct identification of peptide-specific CD8 T cells.[91,100] The key concept is that peptide-specific CD8 T cells can be identified from within a polyclonal T-cell population by staining the cells with a fluorescently labeled complex of peptide and autologous MHC class I. This is a powerful tool for identifying critical epitopes and measuring the dynamics of a complex immune response. To achieve sufficient avidity to label T cells, multimers of the MHC peptide must be constructed. Typically, 4 MHC-peptide units are combined in a tetramer. Tetramer is a term that has become shorthand for this class of labeling reagents. More recently, MHC class II tetramers have been developed for labeling peptide-specific CD4 T cells.[101] Because there are typically many peptide targets in a given protein, a large set of tetramers may be necessary to broadly characterize a CD4 or CD8 T-cell response to an antigen. It is much easier to prepare the necessary tetramers when working with a strain of inbred mice than with outbred groups of individuals because the MHC genotype is uniform, well-defined, and shared amongst mice in many different laboratories. This technology has been used to analyze murine immune responses to many pathogens and tumor antigens. There is a growing number of studies in humans involving MHC class I tetramers that are matched to the **human leukocyte antigen (HLA)** genotypes of study participants. A helpful factor for some of this work is the high prevalence of HLA-A*0201 in humans (> 95% of the US population).[102] It is difficult to predict if or when sufficient resources might become available for the practical use of tetramer technology in veterinary research. Even if it eventually proves feasible, there are limitations to the technique. It would be rather cumbersome to use in studies to assess the overall T-cell response to a pathogen because of the many peptides that would have to be tested in each of the 6 MHC I or II types in each individual. Also, tetramer labeling by itself does not characterize any functions of the cells that it identifies, so other assays would often need to be performed with tetramer-positive cells.

Considerations for Experimental Design

Antigen-lymphocyte incubation strategies— There are important choices to be made in terms of the population of leukocytes to test for antigen-specific priming. The most convenient source of cells for repeated assays of a systemic immune response is blood obtained from the peripheral vasculature, and PBMCs are therefore used very frequently. Arguably, cells from lymphoid organs can provide a more direct indication of immune induction, especially in the early stages. Responses to systemic infections or parenteral vaccination are typically initiated in draining lymph nodes or the spleen. Infections of the respiratory or intestinal tracts (or vaccination via the intranasal route) will likely result in immune responses in mucosal lymphoid tissues, such as tonsils or Peyer patches. In some animal studies, biopsy specimens of draining lymph nodes can be obtained, and in other instances, cells are collected from lymphoid tissues at necropsy. One technically demanding but insightful approach is to obtain samples of pulmonary lymph from cannulated lymphatics during the course of an immune response.[103]

It is sometimes desirable to examine fractionated lymphocyte populations for antigen-specific responses. For instance, if CD8 T-cell proliferation or activation in response to antigen is detected via flow cytometry, it can be useful to test a purified CD8 T-cell population for dependence on CD4 T-cell help. Separation of subsets before antigen stimulation also enables one to measure the contribution of each subset to cytokine production via techniques such as RT-PCR assay, ELISA, or ELISPOT assay. Several antibody-mediated techniques are available to either isolate 1 subset of T cells or deplete a given subset from the mixed population. These techniques include fluorescence-activated cell sorting, magnetically activated cell sorting, and complement-mediated subset depletion. Homogenous lymphocyte subset populations may be inferior to mixed PBMCs with regard to the creation of an in vitro environment where antigen presentation, cognate T-cell help, and the cytokine milieu can support T-cell activation.

Researchers have used several approaches to culture lymphocytes for antigen stimulation. In some studies, antigens are simply added to suspensions of mononuclear cells in media. Alternatively, T-cell activation is often facilitated with differentiated **antigen-presenting cells (APCs)** prepared in advance. Mitomycin C-treated PBMCs and adherent cells from peripheral blood monocyte culture have both been used as renewable sources of syngeneic APCs.[104,105] Recall antigens used in published studies include whole viruses (live and inactivated), recombinant viruses, protein extracts from bacterial culture, individual proteins, and peptides. The more complex antigens may be better at provoking APC activity; they are also likely to contain more diverse epitopes for the stimulation of polyclonal T-cell responses. According to dogma, live intracellular pathogens have optimal access to the endogenous antigen presentation pathway. This suggests that viable microorganisms may be ideal antigens with which to assess recall responses by CD8 T cells. Synthetic peptide libraries that contain arrays of short sequences from within antigenic proteins are useful to screen for prominent T-cell epitopes.[106] Libraries tailored for the analysis of CTL epitopes usually consist of peptides that are approximately 8 to 11 residues long, whereas libraries for Th-cell epitopes often have peptide lengths of 13 to 25 residues. One example of a CD4+ T-cell epitope library used in veterinary research featured peptides (each composed of 16 amino acids) that represented the full sequence of 2 bovine respiratory syncytial virus proteins.[107]

Assays of CMI are inherently more complex than serologic assays because they measure the in vitro functions and activities of live cells. Therefore, considerable day-to-day variability is typical in experiments that incorporate these assays. Because of this, it is essential that statistical analyses of data are designed to test for differences between a control group and treatment groups that are vaccinated or infected, rather than testing for differences across time. Valid statistical comparisons are only possible if cells from control and subject animals are tested in parallel on the same day.

Vaccine Development and CMI Assays

Many of today's experimental vaccines against pathogens of importance in veterinary medicine are designed to target critical epitopes by use of recombinant gene technology.[108] In these sophisticated approaches, genes are more and more routinely being deleted from wild-type microorganisms and vectored in heterologous viruses or bacteria or inserted into plasmids for DNA vaccination. To thoroughly evaluate these cutting-edge vaccines and optimize them for practical use, it is important that researchers and regulatory professionals use effective and comprehensive tests of immunity. There is also a strong impetus for the application of methods for evaluating vaccines and therapeutics that minimize the use of challenge infections in animals. Modern assays for CMI will very likely be essential for accomplishing all of these goals.

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Appendix 1

In vitro assays for lymphocyte proliferation and activation used in domestic animal species.

Technique	Host species
Tritiated thymidine uptake	Cow[34] Pig[35] Chicken[36]
Tetrazolium salt MTT proliferation	Dog[37] Chicken[38]
Alamar blue proliferation	Cat[39] Chicken[40]
BrdU (5-bromo-deoxyuridine) flow cytometry	Cow[41] Dog[37] Chicken[42]
PKH flow cytometry	Pig[43] Cow[44]
CFSE (carboxyfluorescein diacetate succinimidyl ester) flow cytometry	Cow[45] Cow[46,47]
CD25 flow cytometry	Goat[48] Pig[49]
Major histocompatability complex II flow cytometry	Cow[46] Pig[49,50]

Appendix 2

Cytokine detection methods used in domestic animal species.

Technique	Host species
Bioassay	Cow[62] Chicken[63] Pig[64,65]
Ribonuclease protection	Cow[66]
Standard reverse transcription-polymerase chain reaction (RT-PCR) assay	Cow[67]
Competitive RT-PCR assay	Chicken[68] Cow[66,69] Pig[70] Cat[71] Sheep[72]
Real-time RT-PCR assay	Chicken[73,74] Cow[75] Horse[76]
Sandwich ELISA	Cow[75] Chicken[77] Pig[78] Sheep[79]
Enzyme-linked immunospot (ELISPOT) assay	Cow[75]
Intracellular staining	Pig[80,81] Cow[82,83]