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In vitro detection of antigen specific T lymphocyte subsets in cattle

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In vitro detection of antigen specific T lymphocyte subsets in cattle

by

Mark John Quade

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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ABSTRACT

Mitogen and specific antigen activation of bovine CD3⁺, CD4⁺, CD8⁺, and gamma delta T cell receptor⁺ T cells and B lymphocytes was examined. Lymphocyte activation was detected by measuring increased surface expression of activation markers (Interleukin-2 receptor (IL2r) and major histocompatibility complex class II molecules (MHC II)). Two color flow cytometry was used to simultaneously identify lymphocyte subsets and detect activation marker expression. For mitogen stimulated lymphocyte subsets, IL2r and MHC II expression was compared to subset proliferation as measured directly by flow cytometric analysis of the fluorescent cell membrane stain PKH2. Stimulation by the mitogens Concanavalin A (Con A), phytohemagglutinin (PHA), pokeweed mitogen (PWM), and anti-CD3 monoclonal antibody (MAb) caused increased expression of IL2r and MHC II as well as proliferation of CD4⁺ and CD8⁺ T cells. CD3⁺ T cells increased IL2r and MHC II expression in response to all four mitogens, and proliferated in response to Con A, PHA, and anti-CD3 MAb. Gamma delta T cells responded to all four mitogens with increased IL2r and MHC II expression, but only Con A and PHA caused detectable proliferation of gamma delta T cells. B lymphocytes generally responded to all four mitogens with increased IL2r and MHC II expression, but only Con A induced measurable B cell proliferation. Peripheral blood mononuclear cells (PBMC) of cattle hyperimmunized with bovine viral diarrhea virus (BVDV) were incubated in vitro for six days with or without live BVDV. CD3⁺, CD4⁺, CD8⁺, and gamma delta
T cells from hyperimmunized cattle became activated and increased IL2r expression when incubated with BVDV as compared to cells incubated without virus. Lymphocytes from BVDV negative control cattle did not increase IL2r expression when incubated in vitro with BVDV. PBMC of cattle immunized with a vaccine containing modified-live bovine herpesvirus 1 (BHV1) were incubated in vitro for six days with or without heat-inactivated BHV1. CD3^+, CD4^+, and gamma delta T cells were activated and increased expression of IL2r when incubated with BHV1 antigen as compared to cells incubated without virus. CD8^+ T cells of vaccinated animals did not increase IL2r expression when incubated with inactivated BHV1. Lymphocytes from non-vaccinated BHV1-negative animals did not increase IL2r expression when incubated with inactivated BHV1.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation organization

This dissertation is composed of a general introduction, a literature review entitled "In vitro detection of antigen specific memory T lymphocyte subsets," three manuscripts, and a general summary. The manuscript entitled "Dual-color flow cytometric analysis of phenotype, activation marker expression, and proliferation of mitogen stimulated bovine lymphocyte subsets" (Chapter 3) has been submitted to Veterinary Immunology and Immunopathology for publication. The manuscripts entitled "Antigen specific in vitro activation of T lymphocyte subsets of cattle immunized with a modified-live bovine herpesvirus 1 vaccine" and "Antigen specific up-regulation of Interleukin-2 receptor on T lymphocyte subsets of cattle hyperimmunized with bovine viral diarrhea virus" (Chapters 4 and 5) will be submitted for publication. References cited in each chapter appear at the end of the respective chapter. The last chapter presents a summary and general conclusions of the research, and recommendations for future research.
Research summary

For many important diseases, host antibody responses are relatively well understood, while T lymphocyte-mediated immune responses are less well characterized. T lymphocytes may be identified by the surface expression of CD3 molecules. CD3+ T cells may be divided into three subpopulations based on surface expression of CD4 and CD8 molecules, and gamma delta T cell receptor (TCR). The three T cell subsets recognize antigen in different ways and are functionally distinct, although some overlap of function may occur. The purpose of the research presented in this dissertation was to develop a method to monitor the response of all three T cell subsets to specific viral antigens.

Activated lymphocytes express a molecule known as Interleukin-2 receptor alpha (IL2r). Expression of IL2r is necessary for lymphocytes to undergo cell division and proliferation, which is an important aspect of the immune response to specific antigen. Monoclonal antibodies (MAb) have been developed that bind to bovine IL2r, as well as to CD3, CD4, CD8, and gamma delta TCR. It was hypothesized that MAb could be used to measure increased expression of IL2r on activated bovine T cell subsets using two-color flow cytometry.

To validate the ability of two-color flow cytometry to measure T cell subset IL2r expression, isolated bovine peripheral blood mononuclear cells (PBMC) were incubated with mitogens. The technique was shown to be able to measure differences of IL2r up-regulation between lymphocyte subsets stimulated with a given
mitogen, and differences in lymphocyte subset IL2r expression between various mitogens. Lymphocyte subset expression of IL2r was compared to mitogen induced proliferation, as measured by an assay that detects MAb binding to subset markers and the intensity of fluorescence of PKH2, a dye that stains cell membranes.

In order to determine whether antigen specific activation of T cell subsets could be measured, the two-color flow cytometric technique was used to monitor T cell subset responses of cattle to bovine viral diarrhea virus (BVDV) infection. It was shown that cattle hyperimmunized with BVDV had CD3⁺, CD4⁺, CD8⁺, and gamma delta T cells that responded to in vitro stimulation with live BVDV by up-regulation of IL2r. T cells from BVDV negative control animals did not respond to BVDV. The technique was then used to monitor T cell subset responses of cattle to bovine herpesvirus 1 (BHV1) following vaccination. It was shown that CD3⁺, CD4⁺, and gamma delta T cells, but not CD8⁺ T cells, of cattle immunized with a vaccine containing modified-live BHV1 responded to in vitro stimulation with inactivated BHV1 by up-regulation of IL2r. T lymphocytes from unvaccinated BHV1 negative control animals did not become activated by BHV1 stimulation. The significance of the experimental results and considerations for possible future research are discussed in the general summary chapter.
CHAPTER 2. LITERATURE REVIEW: IN VITRO DETECTION OF ANTIGEN SPECIFIC MEMORY T LYMPHOCYTE SUBSETS

Introduction: the importance of memory lymphocytes

Memory T lymphocytes are essential to animal health. When an animal is first exposed to a pathogen by vaccination or infection, there are relatively few lymphocytes that recognize specific antigens presented by the pathogen. The lymphocytes that do recognize their specific antigen become activated and proliferate, generating large numbers of effector cells to deal with the current infection, and memory cells for future use. Second exposure to the same antigen results in a faster, stronger, longer lasting response, the "memory response."

Detection of antigen specific memory T cells can provide health researchers and clinicians with valuable information. Since different T cell subsets can have distinct functions (Swain, 1995), identification of the specific lymphocyte subsets that respond to antigen and become memory cells may be important to the basic understanding of disease pathogenesis and host immune responses. The ability to detect antigen specific memory T cell subsets may improve evaluation of existing vaccines, and spur the development of safer and more effective vaccines. Additionally, detection of antigen specific memory lymphocytes may prove valuable in diagnostics and epidemiology.
What are memory lymphocytes?

While the immunological memory response to antigen has been recognized for many years, the identity or existence of discrete "memory" lymphocytes has been debated. It is still unclear how memory T cells are generated, and whether antigen-specific memory cells live indefinitely, or perhaps require periodic restimulation for continued survival (Sprent, 1995; Bell et al., 1998). Memory and naive lymphocytes may be distinguished by the expression of different forms of a surface molecule called CD45R. The terminology used to describe the variations of CD45R differs somewhat depending on the species, but the idea remains the same. Naive lymphocytes are reported to express a high molecular weight form of this molecule called CD45RA (in mice), while memory lymphocytes express a lower molecular weight form called CD45RO (Plebanski et al., 1992). However, there is evidence that the situation may not be that simple.

Recent research supports two divergent models of T memory cell generation and maintenance. One model of CD4⁺ T memory cell generation postulates that antigen recognition by naive CD4⁺ T cells results in clonal expansion and differentiation of antigen specific lymphocytes, leading to the generation of short lived effector cells and two types of "memory" cells. One type of CD4⁺ memory cell, identified by the CD45RA surface antigen, exists in an activated state. CD45RA⁺ memory cells respond rapidly to antigen, but are relatively short-lived and will disappear soon after antigen is gone. Many of these CD45RA⁺ cells will revert to an
inactive state, identifiable by a change of the memory marker to CD45RO.

CD45RO⁺ T cells respond to antigen slowly, like a naive lymphocyte, but may live a long time in the absence of antigen. When subsequent exposure to antigen occurs, the memory response is due to the expanded numbers of antigen specific CD45RO⁺ T cells, rather than to any enhanced ability of individual cells to respond (Richards et al., 1997; Bunce et al., 1997; Bell et al., 1998).

Other research supports a second model of CD4⁺ T memory cell generation. This model states that exposure of a clone of T lymphocytes to antigen generates two separate lineages of antigen specific T cells, similar to the situation seen in B lymphocytes. One lineage proliferates and differentiates into activated effector cells that die when specific antigen is no longer present. A separate lineage of antigen stimulated T cells seems not to proliferate and differentiates into long-lived, partially activated memory cells that respond to antigen more rapidly and aggressively than naive T cells. Memory cells may be distinguished from naive or effector cells by biochemical properties, such as differences in phosphorylation patterns of signal transduction molecules (Bemer et al., 1995; Gupta et al., 1997; Farber, 1998).

Most research has been done with laboratory rodent or human CD4⁺ lymphocytes. The situation is even less clear for CD8⁺ memory T cells, and for lymphocytes of other species. There is evidence that supports the theory that memory CD8⁺ T cells may persist in the absence of antigen or other immune cells, but may require periodic stimulation by cytokines or contact from some type of stromal cell. Other research indicates that recurring exposure to persistent antigen is
necessary for CD8\(^+\) memory cell maintenance (Arvin et al., 1991; Sprent, 1995; Di Rosa et al., 1996). It is reported that bovine CD4\(^+\) memory T cells maintain expression of CD45R0, while CD8\(^+\) memory T cells initially express CD45RO, but soon down-regulate CD45RO expression (Bembridge et al., 1995).

**Applications for detection of antigen specific memory lymphocytes**

In basic research, detection of expanded clones of antigen specific memory lymphocytes may lead to a greater understanding of pathogenesis and immune responses to many diseases. Techniques to study generalized T lymphocyte responses to antigen have been available for many years. For example, lymphocyte blastogenesis tests measure isolated lymphocyte DNA synthesis (proliferation) when exposed to antigen or mitogens *in vitro*. However, this assay yields no information regarding which of the functionally distinct lymphocyte subsets recognizes and responds to specific antigen. Several newer techniques are able to identify lymphocyte subsets and detect cell activation or functional activities. For example, flow cytometry can be used to identify lymphocyte subsets and simultaneously detect various measures of activation. Antigen-induced cytokine production can give clues regarding the functional activities of the lymphocyte subsets that are activated by antigen. Such techniques have been used to detect antigen specific lymphocytes and can yield much useful information about disease pathogenesis and immune responses (Clough et al., 1995).
Detection of antigen specific T cells should be very useful in the field of vaccinology as well. Antibody responses to vaccines are well studied. However, T cell mediated immune responses (CMI) to many vaccines are poorly characterized, despite the fact that CMI is an important protective response for many diseases, especially intracellular bacteria, viruses, fungi, and protozoal diseases. The ability to detect and quantify CMI responses and activation of specific lymphocyte subsets would provide ways to identify protective T cell epitopes, evaluate vaccine efficacy, and aid in the development of novel vaccine technology (Herr et al., 1996; Hackett, 1997). Many new vaccine techniques are being developed to specifically stimulate cell mediated immune responses (Bona et al., 1998). To fully exploit such new developments, T lymphocyte subset responses to diseases must be well characterized, and specific T cell subset responses to vaccination must be measurable.

Vaccine technology has advanced to the point of being able to stimulate specific types of immune cells to achieve the optimal protective response. Use of the appropriate adjuvant can determine which lymphocyte subsets are activated (Neuzil et al., 1997; Baldrige et al., 1997; Benne et al., 1997; Krivorutchenko et al., 1997). Antigens may be bound to synthetic particulate carriers or immune-stimulating complexes (ISCOMS) to enhance presentation to T cells (Prieur et al., 1996; Challacombe et al., 1997; Sjolander et al., 1997; Men et al., 1997). Vaccines can be designed using native or synthetic antigens, or combinations, so that antigen is presented to a given type of lymphocyte or antigen presenting cell (APC) or both (Condon et al., 1996; Zhang et al., 1997; Nakamura et al., 1997; Yang et al., 1997; del
Guercio et al., 1997). When DNA vaccines are administered by different routes, antigen may be presented and processed by different immune cells to induce different immune responses (Yokoyama et al., 1997; Prayaga et al., 1997; Barry et al., 1997). Other genetic immunization techniques include fusing an antigenic protein to a molecular adjuvant to increase stimulation of APCs (Kowanko et al., 1991), and using DNA vaccines that code for production of antigenic peptides together with genes for the production of cytokines to guide the immune response (Maecker et al., 1997; Barry et al., 1997; Lewis et al., 1997; Prayaga et al., 1997). Immune responses could be modulated by changing the intracellular location of an antigen produced by DNA vaccine by adding signal sequences or trans-membrane domains (Ertl et al., 1996). Such techniques may lead to vaccines that stimulate the most appropriate cell types to maximize protective immune responses while eliminating unnecessary or harmful antigens (Bona et al., 1998).

For diseases under eradication programs, marker vaccines have been developed that allow serological differentiation of infected animals from vaccinates. Such vaccines have been developed for swine pseudorabies and bovine herpesvirus 1 (Stegeman et al., 1994; Bosch et al., 1998). These vaccines are engineered to contain protective epitopes, but lack some antibody producing antigens found in wild type viruses. For diseases in which CMI is more protective than humoral immunity, it may be possible to design a vaccine that induces memory T cells to a very restricted range of epitopes, or perhaps produces CMI without antibody. *In vitro* detection of antigen specific T cells would allow differentiation of vaccinates and infected animals.
In the field of diagnostics, \textit{in vitro} detection of antigen-specific memory cells could prove a valuable addition to serology and delayed-type hypersensitivity (DTH) skin testing. Diagnosis of some diseases, bovine paratuberculosis for example, has been a difficult or lengthy process prone to false negative results (Onet, 1997). An \textit{in vitro} assay to detect memory T cells specific for paratuberculosis has recently been described (Stabel, 1996), which should improve the speed and accuracy of diagnosis of this disease. For some diseases, antibody titers decline below detectable levels over time, or little antibody is produced. Expanded clones of memory T cells may be detectable for longer times than antibody, or in situations where little or no antibody is produced (Di Rosa et al., 1996), improving disease surveillance. Antibody in serum of young animals may be due to passively acquired maternal antibody or active response to disease. Detection of antigen specific memory cells could differentiate between the two options. The presence of maternal antibody may prevent neonates from seroconverting when infected or vaccinated (Bradshaw et al., 1996; Ellis et al., 1996); detection of antigen specific memory cells may be a more reliable way to detect exposure of neonates to some agents.

\textbf{T lymphocyte subsets}

Two functionally distinct subsets of T lymphocytes are described on the basis of surface expression of accessory molecules CD4 or CD8. CD4$^+$ T cells are generally regarded as cytokine producing or "helper" cells, while CD8$^+$ T cells are
cytotoxic cells (McKeever, 1994), but both types of cells may be capable of either activity under some conditions (Arvin et al., 1991; Choi et al., 1994; Herr et al., 1996). Most CD4^+ and CD8^+ T lymphocytes possess the alpha beta T cell antigen receptor (TCR). Alpha beta T lymphocytes cannot recognize native antigen; they respond only to peptide antigens that have been processed by antigen presenting cells (APC) and presented on the surface of APCs bound by major histocompatibility molecules (MHC).

Gamma delta T lymphocytes possess a T cell receptor that consists of two chains analogous to, but distinct from, that of alpha beta T cells. Gamma delta T cells are commonly found at dermal and mucosal surfaces. Gamma delta T cells are a minor population in peripheral blood of most species, but are prominent in the blood of ruminants (Parker et al., 1990; Hein et al., 1991). Gamma delta T cells can secrete cytokines that modulate immune responses and attract inflammatory cells to sites of infection, and may possess cytolytic activity (Lang et al., 1995; Boismenu et al., 1996). The antigens they recognize and how they recognize them is poorly understood. At least some gamma delta T cells recognize phosphorylated non-peptide antigens that are not processed by antigen presenting cells or presented by classical MHC molecules (Morita et al., 1995). The phosphoantigens recognized by such gamma delta T cells are components of the cell wall of many bacteria. Gamma delta T cells seem to be particularly important in responses to mycobacterial infections, but have been reported to respond to neoplastic and virus-infected cells as well (Fisch et al., 1990; Bukowski et al., 1994).
It has been suggested that gamma delta T lymphocytes are intermediate between non-specific defense mechanisms, such as neutrophils and macrophages, and specific immunity mediated by alpha beta T cells. They recognize antigen more specifically and respond more slowly than neutrophils and macrophages; they recognize antigen less specifically than alpha beta T lymphocytes and respond more rapidly (Kaufmann, 1996). Gamma delta T cells may be of particular importance during first exposure to an organism, when only 0.1 - 0.0001% of alpha beta T cells respond to a specific peptide-MHC complex, while 1-8% of gamma delta cells respond to phosphoantigens. They may be of less importance in secondary exposure when antigen specific alpha beta T cell clones have expanded (Poccia et al., 1997).

Methods of in vitro detection of T cell activation

Humoral responses to many diseases have been extensively studied. However, the ability to study CMI has developed more slowly. CMI may be demonstrated by detecting the presence of expanded clones of T lymphocytes, i.e. memory cells, that recognize pathogen-specific antigens (Roth, 1993). Several parameters have been used in vitro to differentiate mitogen- and antigen-stimulated lymphocytes from unstimulated resting lymphocytes. Activated cells may be identified by changes in metabolism or expression of surface molecules. Cytokine production, cytotoxicity, or other functional assays may be used to identify stimulated lymphocytes. Proliferation
of lymphocytes is often used as a way to demonstrate activation of lymphocytes by mitogens and antigens (Clough et al., 1995).

Some assays, such as lymphocyte blastogenesis, can detect T cell responses, but give no information regarding which of the functionally different T cell subsets are responding to antigen. In an attempt to determine the roles of different subsets, isolated lymphocytes may be depleted or enriched of T cell subsets for these assays (Chiodini et al., 1992; Lutje et al., 1992). However, the interactions of different subsets cannot be studied in separated populations of lymphocytes.

Detecting activation of memory T lymphocytes

Metabolic changes may be detected in lymphocytes within minutes following stimulation. Fluorescent probes allow rapid measurement of several different types of metabolic changes that occur early in activation. Intracellular calcium flux (Rabinovitch et al., 1986; June et al., 1986; Gelfand et al., 1988b), cytoplasmic pH changes (Rogers et al., 1983; Gelfand et al., 1988a), changes in oxidative metabolism (Shapiro, 1995b), and altered membrane potential (Shapiro et al., 1979) have been used to observe early lymphocyte activation.

Lymphocyte activation results in the expression of cell surface molecules not present on resting cells. Some surface markers of activation are expressed within a few hours of stimulation, others do not appear for days. Some surface molecules that have been used to detect lymphocyte activation, in order of their appearance
following stimulation, include CD69 (Lopez-Cabrera et al., 1993), CD98 (Friedman et al., 1994), CD25 (interleukin-2 receptor), insulin receptor (Cebrian et al., 1988; Biselli et al., 1992), CD21 (transferrin receptor) (Suomalainen, 1986), and MHC class II molecules (Uchiyama et al., 1981; Haynes et al., 1981; Shapiro, 1995b).

Of these activation markers, interleukin-2 receptor (IL2r) may be the most relevant physiologically. Soon after antigen recognition, lymphocytes begin to express high affinity IL2r on their surface. Activated T cells also secrete interleukin-2. Binding of IL-2 to its receptor is a powerful stimulus to induce cell division. In an immune response, only lymphocytes that have recognized specific antigen express high levels of IL2r and bind enough IL-2 to undergo proliferation (Miyawaki et al., 1982; Smith, 1988; Minami et al., 1993; Abbas et al., 1994). IL2r expression requires new protein synthesis, but occurs before measurable DNA synthesis or cell division (Uchiyama et al., 1981; Rubin et al., 1985; Redelman et al., 1986). Cells expressing IL2r at 12-24 hours post-stimulation correlate well with cells synthesizing DNA 120 hours after stimulation; it appears that cells bearing IL2r early during activation are the T cells which proliferate later (Shapiro, 1995b). Expression of IL2r may be induced by specific antigen, mitogens, or by some T cell specific MAb (Cantrell et al., 1984; Smith, 1988).

Expression of IL2r is an early indication of lymphocyte activation and represents the potential to proliferate. However, lymphocytes may express IL2r without dividing (Rubin et al., 1985). There is evidence that some antigens may activate lymphocytes enough to increase IL2r expression, but the cells become anergic
or unresponsive; these anergic lymphocytes expressing IL2r may even act as suppressor cells by consuming locally produced IL-2 and preventing potentially responsive lymphocytes from receiving adequate stimulation to proliferate (Lombardi et al., 1994).

Expression of activation molecules is commonly studied using fluorescent immunohistochemistry and flow cytometry. A wide selection of monoclonal antibodies and other reagents is available for use with human and rodent lymphocytes. There are relatively fewer antibodies available for studying leukocytes from species of veterinary importance, but the selection of such products is expanding, especially for dogs (Cobbold et al., 1994), ruminants (Naessens et al., 1996), swine (Saalmuller et al., 1996), and horses (Lunn et al., 1996). For example, MAb are available to differentiate bovine T lymphocyte subsets CD3⁺ (Davis et al., 1993), CD4⁺ and CD8⁺ (Larsen et al., 1990), gamma delta T cells (Davis et al., 1996a), and B cells (Sopp, 1996), as well as activation markers such as IL2r (CD25) (Naessens et al., 1992; Davis et al., 1996b) and MHC II (Davis et al., 1987).

Monoclonal antibodies have been used in dual color flow cytometry to determine which specific lymphocyte subsets upregulate expression of activation molecules in response to in vitro culture with antigen or mitogen in several animal species including swine (Dillender et al., 1993), sheep (Bujdoso et al., 1993; Begara et al., 1995), cattle (Stone et al., 1995; Ferens et al., 1998), and deer (Hesketh et al., 1993), as well as humans (Rubin et al., 1985; Prince et al., 1986; Ito et al., 1992; Gaines et al., 1996).
Another method of determining lymphocyte activation is by measuring synthesis of DNA. DNA synthesis begins about 30-36 hours following stimulation (Shapiro, 1995b). Since the 1960's, DNA synthesis has been measured by the incorporation of tritiated thymidine into newly synthesized DNA, called lymphocyte blastogenesis or lymphocyte transformation. This technique has been used to detect mitogen and antigen stimulated lymphocytes of many species (Chiodini et al., 1992; Lutje et al., 1992; Brown et al., 1993; Baca-Estrada et al., 1996; DeBey et al., 1996). While blastogenesis provides valuable information, it reveals nothing concerning which lymphocyte subsets are responding or their effector function (Clough et al., 1995). Specific subsets may be enriched or depleted and the results compared to those obtained using the total lymphocyte population, thereby giving clues as to which subsets are proliferating.

Synthesis of RNA and DNA can be measured using a number of different substances that bind to or incorporate into nucleic acids. Increased amounts of cellular RNA or DNA indicate that lymphocytes have been activated by mitogen or antigen. Many of these nucleic acid markers are fluorescent and may be detected by flow cytometry, which allows the simultaneous identification of lymphocyte subsets using MAb (Shapiro, 1995b). Acridine orange (Braunstein et al., 1975; Darzynkiewicz et al., 1976; Traganos et al., 1977) and pyronin Y (Redelman et al., 1986) have been used to quantitate RNA, while prodidium iodide (Rosat et al.,
1994), bromodeoxyuridine (Gaines et al., 1996), Hoechst DNA-binding dyes (Blaheta et al., 1991), and DNA-binding antibiotics (Costa et al., 1975; Shenker et al., 1995) have been used to quantitate DNA. Monoclonal antibody against proliferating cell nuclear antigen (PCNA) has also been used to measure increased lymphocyte DNA content following stimulation (Hesketh et al., 1993). Fluorescent markers of nucleic acid synthesis can detect smaller numbers of activated cells at an earlier time than tritiated thymidine incorporation (Shapiro, 1995b). Many of these nucleic acid markers have been used to detect antigen specific and mitogen activation of lymphocytes (Le Moal et al., 1992; Hesketh et al., 1993; Gaines et al., 1996).

Proliferation of lymphocytes has been measured using fluorescent dyes that incorporate into cell membranes. Derivatives of cyanine dyes (e.g. dioctadecylindocarbocyanine) have been used, and the recently introduced series of PKH cell tracking dyes (Sigma) have been used for this purpose (Weston et al., 1990; Ragnarson et al., 1992; Shapiro, 1995a). Lymphocyte membranes may be stained with dyes prior to incubation with antigen or other stimulants. Following incubation with stimulants, lymphocytes may be labeled with MAb against subset markers to identify which subsets are responding to stimulation by proliferating. When analyzed by flow cytometry, proliferating cells contain progressively less dye with each new generation, giving information regarding what percentage of cells of a given lymphocyte subset are proliferating and to what extent they have divided (Horan et al., 1990; Shapiro, 1995a).
Cytokine production as a method of detecting memory T lymphocytes

Production of cytokines may be used to detect activated memory lymphocytes. There are several methods available to measure the production of cytokines, such as bioassays using cytokine-dependent cell lines, and direct measurement of cytokines using ELISA techniques. Some bioassays and ELISA reagents are species specific and the availability of reagents specific for species of veterinary interest has been limited, but is improving (Clough et al., 1995). A number of cytokine-dependent cell lines are available to perform bioassays to measure cytokine production from stimulated lymphocytes, especially for human and rodent cytokines. Bioassays have been used to detect antigen specific production of IL-2, IL-4, IL-5, and interferon-gamma (INF-gamma) (Kelso et al., 1995; Prigione et al., 1995). Bioassays of lymphocyte production of INF-gamma, tumor necrosis factor (TNF), and IL-2 have been used to detect antigen specific memory T cells in cattle (Brown et al., 1993).

ELISA technology is available to measure several cytokines. Antigen specific memory T cell production of IFN-gamma, IL-2, IL-4, IL-5, and IL-12 have been demonstrated in human (Prigione et al., 1995) and mouse lymphocytes (Falchetti et al., 1995; Cooper et al., 1995). ELISA detection of IFN-gamma production has been used to demonstrate antigen specific memory T cells in cattle infected with Mycobacterium bovis (Kee et al., 1997), Mycobacterium paratuberculosis (Stabel, 1996), and Pasteurella haemolytica (DeBey et al., 1996).
ELISPOT is a type of ELISA that can quantitate the number of cells that are producing a certain cytokine. Antigen specific cytokine secretion of INF-gamma, IL-2, IL-4, IL-5, TNF has been detected by ELISPOT primarily in laboratory rodents (ElGhazali et al., 1993; Xu-Amano et al., 1994; Murphy et al., 1997; Culshaw et al., 1997), but the technique has been used to detect antigen specific IFN-gamma production by lymphocytes of swine (Husmann et al., 1996).

Activation of T cells may be analyzed by demonstrating intracellular cytokine mRNA. Northern blotting (Brown et al., 1993) and reverse transcription-polymerase chain reaction (RT-PCR) can be used to identify and quantitate cytokine mRNA in lymphocytes (Kelso et al., 1995; Prigione et al., 1995). Reagents and techniques for RT-PCR detection of mRNA for IL-2, IL-4, IL-6, IL-10, IL-12, interferon gamma, and tumor necrosis factor have recently been described for use in cattle, cats, dogs, horses (Rottman et al., 1996), swine (Rottman et al., 1996; Dozois et al., 1997), and sheep (Egan et al., 1996). Antigen specific memory cell cytokine mRNA has been detected in lymphocytes from cattle infected with *Mycobacterium bovis* (Kee et al., 1997) and *Babesia bovis* (Brown et al., 1993). Monoclonal antibody against IL-2 and IL-4 may be used to detect intracellular cytokine molecules. These antibodies have been used in conjunction with surface subset markers to identify lymphocyte subsets and production of specific cytokines in mice (Falchetti et al., 1995).
Cytotoxicity assays for detection of memory T cells

Cytotoxicity assays measure the ability of cytotoxic T lymphocytes (CTL) to destroy target cells. Cytotoxic T cells are typically of the CD8^+ subset, but CD4^+ and gamma delta T lymphocytes may also possess cytotoxic properties (Arvin et al., 1991; Choi et al., 1994; Lang et al., 1995). Target cells are labelled with a substance that is taken up by the cells and maintained intracellularly. When labelled target cells are incubated with potential CTLs, release of marker substance into the medium indicates target cell lysis. The most commonly used marker for CTL assays is radioactive chromium, but tritium (Matzinger, 1991) and non-radioactive Europium have also been used (Blomberg et al., 1986). Alternatively, the release of lymphocyte-specific substances, such as granzyme, may be used to demonstrate that CTLs have recognized and responded to target cells (Baetz et al., 1995).

Since CD8^+ T cells recognize antigen only when it is presented on "self" MHC class I molecules, target cells for CTL assays must be histocompatibility matched with the CTLs to be tested. Histocompatibility matching means that cytotoxicity assays are relatively simple with the genetically identical strains of laboratory rodents that are available (Pemberton et al., 1987), but presents the challenge of haplotype matching or establishing and culturing autologous target cells when testing outbred species for CTLs. Nevertheless, antigen specific CTL activity has been demonstrated in several species of veterinary importance, including cattle (Denis et al., 1993; Gaddum et al.,
1996), sheep (Woldehiwet et al., 1994), swine (Pauly et al., 1995), poultry (Thacker et al., 1995), horses (McGuire et al., 1997), and cats (Tellier et al., 1997).

Summary

*In vitro* detection of antigen specific memory T lymphocytes could provide information valuable to basic understanding of host-pathogen interactions, vaccine development and evaluation, and disease diagnosis. Memory T cells may be detected by increased expression of activation markers, proliferation, cytokine production, and lysis of target cells. Some measures of lymphocyte activation may represent incomplete activation that does not result in a functional response to antigen. It may be that proliferation assays most accurately determine which immune cells are responding to antigen in a protective manner. Perhaps some combination of surface molecule expression, proliferation, and cytokine secretion will provide the most accurate information regarding what cells are activated and how those cells are responding functionally to specific antigen.
References


Bunce, C. and Bell, E.C., 1997. CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. The Journal of Experimental Medicine, 185:767-776.


Suomalainen, H.A., 1986. The monoclonal antibodies trop-4 and 4F2 detect the same membrane antigen that is expressed at an early stage of lymphocyte activation and is retained on secondary T lymphocytes. The Journal of Immunology, 137:422-427.


CHAPTER 3. DUAL-COLOR FLOW CYTOMETRIC ANALYSIS OF PHENOTYPE, ACTIVATION MARKER EXPRESSION, AND PROLIFERATION OF MITOGEN STIMULATED BOVINE LYMPHOCYTE SUBSETS

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Abstract

Bovine peripheral blood mononuclear cells were cultured in vitro for three days with the mitogens Concanavalin A (Con A), pokeweed mitogen (PWM), phytohemagglutinin (PHA), and anti-CD3 monoclonal antibody (MAb). Activation of T lymphocyte subsets (CD3+, CD4+, CD8+, and gamma delta T cell receptor+ (TCR)) and of B lymphocytes was measured by blastogenesis and by two-color flow cytometric analysis of subset expression of IL-2 receptor (CD25) and MHC class II. Proliferation of lymphocyte subsets was directly measured by two-color flow cytometric analysis of fluorescence intensity of PKH2, a fluorescent dye that stably incorporates into cell membranes. CD3+, CD4+, and CD8+ T cell subsets were activated by all the stimulants to increase expression of IL2r and MHC II and to proliferate, except that PWM did not cause measurable CD3+ cell proliferation. Gamma delta T cells responded to all four stimulants with increased IL2r and MHC II expression. Con A and PHA caused measurable proliferation of gamma delta T cells, but PWM and anti-CD3 MAb did not. B cells generally responded to the
stimulants with increased IL2r and MHC II expression. B cells proliferated when incubated with Con A, but did not measurably proliferate in response to PWM, PHA, or anti-CD3 MAb.

**Keywords** bovine, mitogens, T cell subsets, lymphocyte proliferation, activation

**Introduction**

Mitogens have been used to activate lymphocytes and to induce them to proliferate in many species, including cattle. To study the effects of mitogens on different lymphocyte subsets, lymphocyte blastogenesis has been measured following depletion or enrichment of subsets from the total population of lymphocytes. It was concluded that for bovine lymphocytes, concanavalin A (Con A) and phytohemagglutinin (PHA) stimulated T lymphocytes, while pokeweed mitogen (PWM) stimulated both T and B cells (Schultz, 1981; Usinger et al., 1981; Lutje et al., 1992). Early work on the effects of mitogens on lymphocytes did not examine the effects on gamma delta T cells, which have been shown to be a major subpopulation of circulating lymphocytes in cattle (Hein et al., 1991). *In vivo* depletion of bovine gamma delta T cells has been shown to increase blastogenesis due to PWM (Howard et al., 1989), and there is evidence that the *in vitro* proliferative responses of gamma delta T cells may be slower than other T lymphocyte subsets when stimulated by PWM (Franklin et al., 1994).
The availability of monoclonal antibodies (MAb) for cattle lymphocyte subset markers, along with the use of flow cytometry, has enabled researchers to more directly study the effects of mitogens on bovine lymphocyte subpopulations. Monoclonal antibodies have been produced that define bovine T lymphocyte antigens CD3 (Davis et al., 1993), CD4 (Bensaid et al., 1991), CD8 (Larsen et al., 1990), and gamma delta T cell receptor (TCR) (Davis et al., 1996a), as well as B cell markers such as CD21 (Mukwedeya et al., 1993; Sopp, 1996). Single color flow cytometry has been used to study mitogen induced changes in percentages of lymphocyte subsets (Hurley et al., 1994; Franklin et al., 1994) and surface expression of lymphocyte activation markers, such as Interleukin-2 receptorα (IL2r) (Miyawaki et al., 1982; Prince et al., 1986) and major histocompatibility complex class II (MHC II) (Davis et al., 1987). Using two-color flow cytometry, it is possible to identify the specific subsets of lymphocytes of humans (Uchiyama et al., 1981; Mach et al., 1996), cattle (Hurley et al., 1994; Davis et al., 1996b; Ferens et al., 1998; Isaacson et al., 1998) and other species (Le Moal et al., 1992; Dillender et al., 1993) that increase expression of IL2r and MHC II when activated by mitogens, antigens, and superantigens.

Lymphocyte activation may lead to proliferation. Proliferation may be measured using PKH2 fluorescent cell tracking dye (Sigma), which incorporates into the membrane of living cells. It has been shown that PKH2 dye does not interfere with lymphocyte proliferation (Samlowski et al., 1991). Labeled lymphocytes are cultured in vitro with stimulants, and proliferating cells may be identified by a reduction in fluorescence intensity relative to cells that have not divided (Horan et
The phenotype of PKH2-labeled proliferating lymphocytes can be identified using lymphocyte subset specific MAb and dual-color flow cytometry (Shapiro, 1995b).

In this study, bovine lymphocyte subset responses to four commonly used stimulants were compared using three different methods. Isolated bovine peripheral blood mononuclear cells (PBMC) were cultured with Con A, PHA, PWM, and anti-CD3 MAb for three days. Activation of lymphocytes was compared using lymphocyte blastogenesis, two-color flow cytometry to measure lymphocyte subset expression of IL2r and MHC II, and two-color flow cytometric analysis of lymphocyte subset proliferation.

Materials and methods

Reagents

Mitogens were used at final concentrations determined to be optimal for lymphocyte blastogenesis (incorporation of $^3$H-thymidine) in our laboratory (Con A 2.5μg/ml, PWM 1:500, and PHA 0.8μg/ml) (Sigma). Culture medium (RPMI+) consisted of RPMI 1640 (Celox) containing 10% heat inactivated fetal bovine serum negative for bovine viral diarrhea virus (BVDV) and BVDV antibody (Hy-clone) and 0.5% cell culture penicillin/streptomycin (Sigma). The PKH2 fluorescent membrane dye kit was purchased from Sigma.
Monoclonal antibodies

Mouse MAb that bind to bovine antigens were purchased from VMRD Inc. (Pullman, WA) and are shown in Table 1. Affinity purified goat anti-mouse isotype specific secondary antibodies were purchased from Caltag (Burlingame, CA): phycoerythrin (PE) labeled anti-IgG2a (M32204), fluorescein isothiocyanate (FITC) labeled anti-IgG1 (M32101), FITC-labeled anti-IgG2b (M32501), and FITC-labeled F(ab)2 anti-IgM (M31601). Mouse MAb of irrelevant specificity used as isotype controls were purchased from Caltag: IgG1 (MG100), IgG2a (MG2a00), IgG2b (MG2b00) and IgM (MGM00).

Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from healthy 3-6 month old Holstein steers (n=5-8) using a previously described method (DeBey et al., 1996). Briefly, citrated blood was centrifuged, the buffy coat was collected, and contaminating erythrocytes lysed by hypotonic flash lysis. PBMC were washed twice in Hanks balanced salt solution without Ca++ and Mg++ (HBSS), and resuspended at 2 x 10^6/ml in 5ml RPMI+ in 6-well tissue culture plates. Isolated cells were >95% viable by trypan blue exclusion.

Control wells contained medium and cells only, while wells with mitogen stimulated cells contained Con A, PHA, PWM, or anti-CD3 MAb. All treatments
were set up in duplicate. Con A and PWM were tested simultaneously, PHA and anti-CD3 were tested on separate occasions. For use as a stimulant, anti-CD3 MAb (MM1A, 0.5ml at 10μg/ml HBSS) was adhered to plates by incubating overnight at 4°C. Unbound anti-CD3 was removed by rinsing wells twice with HBSS. Plates containing medium, cells, and stimulants were incubated in humidified 5% CO₂ atmosphere at 39°C for three days.

For the proliferation assay, isolated PBMC were labeled according to the manufacturer's procedure with green-fluorescent PKH2 before being placed in culture. PBMC (2 x 10⁷) were suspended in 1 ml proprietary diluent (Sigma), and incubated with an equal volume of PKH2 dye (4 x 10⁻⁶M) in diluent for 6-7 minutes. Fetal bovine serum (2ml) was added to stop the reaction, and the cells were washed three times with RPMI 1640. One third of each sample was immediately labeled with MAb against CD3, CD4, CD8, CD21, or gamma delta T cell receptor (TCR) and analyzed flow cytometrically. The remaining PKH2 labeled PBMC from each animal were divided equally and 4 x 10⁶ cells were resuspended in 5ml RPMI+ in 6 well culture plates and incubated (39°C, 5% CO₂) with or without stimulants as described above.

Preparation of cultured PBMC for flow cytometric analysis

After three days of incubation, plates were chilled on ice. Media and non-adherent cells were collected by gentle pipetting, followed by a gentle rinse with cold
HBSS. Wells were then treated with 1 ml cold trypsin/EDTA for 3-4 minutes, followed by vigorous rinsing with cold HBSS. Cells recovered with media and rinses were pooled, and cells from duplicate wells were pooled for further processing. A small percentage of cells remained adhered and could not be recovered by this method. Recovered cells were washed twice in cold HBSS, then washed and resuspended in cold phosphate buffered saline containing 0.5% bovine serum albumin and 0.1% NaN₃ (PBS+ +). Cells (1 x 10⁶) were placed in replicate wells of 96-well u-bottom microtiter plates. All cells were incubated with two primary antibodies: a lymphocyte subset specific antibody and antibody against IL2r or MHC II. All primary MAb were diluted to 10μg/ml PBS+ +, except anti-CD4 was 20μg/ml. Duplicate wells received 25μl of primary MAb specific for either CD3 (MM1A), CD4 (CACT138A), CD8α (CACT80C), gamma delta TCR (GB21A), or B cells (BAQ15A). Half of the wells containing each subset specific MAb then received 25μl anti-IL2r MAb (CACT108A) and half received 25μl anti-MHC II MAb (TH14A). Isotype control wells containing MAb of irrelevant specificity were run for mitogen treated and untreated cells from each animal. All isotype control wells contained 25μl of IgG2a isotype (control for anti-IL2r and -MHC II), and either 25μl IgG1 isotype (control for anti-CD3, -CD4, -CD8, -CD21) or 25μl IgG2b isotype (control for anti-gamma delta TCR), each diluted 10μg/ml PBS+ +.

Cells and primary antibodies were incubated at 4°C for 30 minutes. Cells were then washed four times in PBS+ + and resuspended in PBS+ + containing fluorescently labeled secondary antibody. All wells received 25μl of PE-labeled anti-
IgG2a to label IL2r and MHC II MAb, and 25μl FITC-labeled secondary antibody against the appropriate isotype of subset specific MAb. All secondary antibodies were diluted to 10μg/ml PBS+. Cells and secondary antibodies were incubated for 30 minutes at 4°C in the dark. Cells were washed five times with HBSS, then fixed in 1% paraformaldehyde in PBS for flow cytometric analysis.

When anti-CD3 MAb (MM1A) was used as a stimulant, the procedure was the same as above, except the following IgM isotype primary antibodies were used (VMRD): CD4 (GC50A1), CD8α (BAQ111A), gamma delta TCR (CACT61A), B cell (BAQ44A). Fluorescently labeled secondary antibody was FITC-labeled anti-IgM (M31601) and isotype control antibody was IgM (MGMO0) (Caltag).

Fluorescence of lymphocyte subsets and IL2r or MHC II was analyzed using a Coulter XL-MCL flow cytometer equipped with standard software. Lymphocytes were discriminated by forward and side light scatter parameters. Ten thousand cells from each sample were analyzed. Quad-stat gates were set using unstimulated control cells stained with isotype control primary MAb and isotype specific FITC- and PE-labeled secondary antibodies.

For PKH2 flow cytometric analysis, labeled cells were cultured and harvested as above. Cells were incubated for 15 minutes at 25°C with MAb against CD3 (MM1A), CD4 (GC50A1), CD8α (CACT80C), gamma delta TCR (CACT61A), and B cells (BAQ44A), all at 7μg/ml final concentration. Cells were washed and incubated with isotype-specific PE-labeled secondary antibody for 15 minutes at 25°C. Cells were washed to remove unbound antibody and analyzed without fixation.
Proliferation of each lymphocyte subset was analyzed on a FACScan (Becton Dickinson) with argon laser excitation at 488nm. PKH2 fluorescence was collected in the FL1 channel (515-545nm), secondary antibody PE fluorescence was collected in the FL2 channel (564-606nm). Five thousand cells from each sample were analyzed. Cells positive for a given phenotype marker were selectively gated, and PKH2 fluorescence was analyzed using Proliferation Wizard software (Verity Software House, Topsham, ME). The fluorescence intensity of PKH2 labeled cells that were analyzed on day 0 was used to establish the fluorescence intensity of the undivided or parent generation of cultured cells.

**Lymphocyte blastogenesis**

Lymphocyte blastogenesis was performed as previously described (Roth et al., 1982). Briefly, isolated PBMC were resuspended at 1 x 10⁶/ml culture medium. Two hundred µl of cell suspension was placed in wells of flat-bottom 96-well microtiter plates. Treatments consisted of no mitogen added (background), or 25µl of Con A, PWM, or PHA at the same concentrations used for the other assays. Anti-CD3 MAb, 100µl at 10µg/ml, was adhered to wells as described. All treatments were set up in triplicate. Plates were incubated in humidified 5% CO₂ atmosphere at 39°C. Cells were pulsed with 1µCi ³H-thymidine per well after 2 days of incubation, harvested 18 hours later, and counts per minute (cpm) of radioactivity measured.
Data Analysis

Blastogenesis results are reported as mean counts per minute (cpm) of radioactivity. Stimulation indexes were calculated using this equation:

\[
\frac{\text{cpm of mitogen stimulated cells}}{\text{cpm of same animals’ unstimulated control cells}}
\]

For lymphocyte subset expression of activation markers, the percentage of cells expressing each phenotype marker that were positive for either IL2r or MHC II was calculated. Mean fluorescence intensity of IL2r or MHC II expression (phycoerythrin), which correlates to the density of surface molecule expression (Shapiro, 1995a), of double positive cells was determined. IL2r or MHC II expression indexes were calculated for each lymphocyte subset using the following equation:

\[
\frac{\% \text{ stimulated cells in subset positive for IL2r}}{\% \text{ same animal control cells positive for IL2r}} \times \frac{\text{mean PE fluorescence intensity of stimulated cells}}{\text{mean PE fluorescence intensity of control cells}}
\]

For PKH2 stained lymphocytes, for each subset the percentage of cells in the parent generation and generations 2-6 was determined, and a proliferation index (PI) was calculated. The PI is the calculated expansion of a population of cells: for example, if 100 cells expand to 200 (regardless of distribution of generations) the PI is 2.0.
All data are presented as means. Error bars represent standard error of means. Differences were analyzed by analysis of variance using SAS software (SAS Inc., Cary, NC). Differences are considered significant at $P<0.05$.

Results

Lymphocyte blastogenesis

Concanavalin A, PWM, PHA, and anti-CD3 MAb all caused increased uptake of $^3$H-thymidine in the lymphocyte blastogenesis assay. Figure 1a shows mean counts per minute for unstimulated PBMC and for PBMC incubated with mitogens. Anti-CD3 was tested at a separate time, so cpm for its own controls are included. Mitogens were titrated to determine optimum concentrations (data not shown). The same concentration of mitogens was used in all subsequent assays. Blastogenesis stimulation indexes were calculated for each mitogen (figure 1b). All mitogens caused significant increases in $^3$H-thymidine uptake compared to unstimulated samples ($P<0.01$).

IL2r and MHC class II surface expression

The effects of mitogens on IL2r and MHC class II surface expression on lymphocyte subsets are presented in figures 2-6. Con A and PWM were tested in
replicate wells on one occasion, PHA and anti-CD3 were tested on separate occasions. Values for unstimulated control cells are presented together with the appropriate stimulant.

All T cell subsets (CD3\(^+\), CD4\(^+\), CD8\(^+\), and gamma delta TCR\(^+\)) responded to Con A, PWM, PHA, and anti-CD3 MAb with significantly increased (P<0.05) percentage of cells expressing IL2r and MHC II, increased IL2r and MHC II mean fluorescence intensity, and increased IL2r and MHC II expression indexes to varying extents (figures 2-5).

The percentage of B cells expressing IL2r increased significantly (P<0.05) in response to all mitogens. Mitogens did not increase the percentage of B cells expressing MHC II, since over 99% of unstimulated B cells expressed MHC II (figure 6a). Con A, PWM, and anti-CD3 significantly increased IL2r mean fluorescence intensity of B cells (P<0.05), while PHA did not. All four mitogens caused significant increases in B cell MHC II mean fluorescence intensity (P<0.05) (figure 6b). All the mitogens increased B cell IL2r expression index, but none caused changes in B cell MHC II expression index (figure 6c).

Lymphocyte proliferation

The distribution of generations of lymphocyte subsets from control, Con A, PWM, PHA, and anti-CD3 treated PBMC is shown in figure 7. For each series of bars, the first bar on the left depicts the average percentage of cells in the undivided
or parent generation. Bars to the right show the average percentage of cells containing progressively less PKH2 dye, which is interpreted as cells that have undergone division and are determined to be in generations 2-6. Treatments causing the greatest proliferation resulted in only small percentages of cells beyond the 6th generation, so further generations are not shown.

The distribution of CD3+ T lymphocyte generations is shown in figure 7a. In unstimulated cultures, about 75% of the CD3+ cells are in the undivided parent generation after three days of incubation, with smaller numbers of cells in generations 2-6. The distribution of fluorescence from Con A, PHA, and anti-CD3 treated cultures shows a shift to generations 2-6, indicating that a large percentage of cells proliferated. CD3+ cells from PWM treated cultures show only a small change in the distribution of cell generations compared to unstimulated control cells; 61% remained in the parent generation. CD4+ T cells (figure 7b) and CD8+ T cells (figure 7c) both proliferated noticeably to all four mitogens. The distribution of cell generations for both lymphocyte subsets shifted to generations 2-6 compared to unstimulated cells.

CD3+ T cell proliferation indexes (PI) are shown in figure 8a. Con A, PHA, and anti-CD3 stimulated cells show significantly (P<0.05) increased PI compared to unstimulated cells, while the PI for PWM is not significantly different (P>0.05). CD4+ cells (figure 8b) and CD8+ cells (figure 8c) responded to all four mitogens with significantly higher PI (P<0.05).
Gamma delta T cell distribution of generations is shown in figure 7d. Con A and PHA caused noticeable shifts to generations 2-6. The distribution of generations of PWM and anti-CD3 treated cells is little different than for untreated cells, indicating little proliferation of gamma delta T cells treated with these two stimulants. Gamma delta T lymphocytes treated with Con A or PHA had significantly increased PI (P<0.05), but PWM and anti-CD3 did not cause an increase in PI (figure 8d).

B cells proliferated less than T cell subsets when incubated with stimulants. Con A caused a small shift to generations 2-6, but PWM, PHA, and anti-CD3 induced little or no change compared to untreated cells (figure 7e). Fewer than 60% of B cells remained undivided when treated with Con A, while more than 75% of B cells treated with PWM, PHA, or anti-CD3 remained in the parent generation. B cell proliferation indexes were significantly increased only by Con A stimulation (P<0.05); neither PWM, PHA, nor anti-CD3 resulted in a significantly higher PI (P>0.05) (figure 8e).

Discussion

Lymphocyte blastogenesis has been used to demonstrate activation of lymphocytes by mitogens and antigens. However, measuring blastogenesis of lymphocytes that have not been separated into subsets does not identify which subpopulations of lymphocytes are activated. If lymphocytes are separated into
subsets, then tested in blastogenesis assays, the interaction of subsets cannot be studied. The blastogenic responsiveness of the various subsets to mitogens may be different when they are separated as compared to when they are incubated together in a mixed population. Cytokines from other subsets and cell-cell interactions may influence the blastogenic responsiveness of each subset. The methods employed here allow the quantitation of lymphocyte activation and proliferation for each subset of lymphocytes when they are incubated together and allowed to interact.

Increased surface expression of IL2r and MHC II has been used in several species to detect mitogen and antigen activated lymphocytes (Ito et al., 1992; Hesketh et al., 1993; Dillender et al., 1993; Begara et al., 1995). The percentage of lymphocytes expressing IL2r and the density of surface IL2r expression have been reported to be sensitive and specific for detecting activated human lymphocytes (Prince et al., 1986). Single color flow cytometry has been used to study changes in expression of IL2r and MHC II for mitogen activated lymphocytes from healthy cattle, as well as from animals treated with immunosuppressive doses of dexamethasone (Oldham et al., 1992) and cattle with specific infectious diseases (Begara et al., 1995; Stone et al., 1995). Single-color analysis also has been used to study mitogen-induced changes in the percentage of ruminant lymphocyte subsets or the percentage of cells expressing surface activation markers on lymphocytes isolated from peripheral blood (Franklin et al., 1994; Davis et al., 1996b; Schuberth et al., 1996), lymph nodes (Hurley et al., 1994), and bronchoalveolar lavage fluid (Begara et al., 1995). Two-color flow cytometry has been used to study bovine lymphocyte
subset expression of IL2r and MHC II in response to Con A and superantigen (Ferens et al., 1998), and the effect of viral infection on bovine lymphocyte subset expression of IL2r and MHC II in response to mitogens (Stone et al., 1995; Isaacson et al., 1998).

In this study, the response of bovine CD3⁺, CD4⁺, CD8⁺, gamma delta T lymphocytes, and B cells to four commonly used mitogens was examined using three different methods to measure cell activation. Activation of unseparated lymphocytes was monitored using blastogenesis and by two-color flow cytometric detection of increased expression of IL2r and MHC II by lymphocyte subsets. Two-color flow cytometry was also used to directly measure proliferation of lymphocyte subsets of mitogen stimulated unfractionated PBMC by analyzing fluorescence intensity of PKH2, a fluorescent dye that incorporates into cell membranes. With each new generation of cells, proliferating cell populations contain progressively less PKH2 dye in their membranes.

CD3⁺, CD4⁺, and CD8⁺ T lymphocyte subsets responded similarly to Con A, PWM, PHA, and anti-CD3 stimulation. All three subsets had significant increases in the percentage of cells expressing IL2r and MHC II, fluorescence intensity of IL2r and MHC II, and significant proliferative responses, except that CD3⁺ cells did not proliferate significantly to PWM.

Gamma delta T lymphocytes responded somewhat differently from the other T cell subsets to mitogenic stimulation. All the mitogens caused gamma delta T cells to increase surface expression of both IL2r and MHC II. Con A and PHA induced
significant proliferation of gamma delta T cells. However, even though PWM and anti-CD3 stimulated gamma delta T cells to upregulate surface markers of activation, neither mitogen caused a noticeable shift in the distribution of generations or significantly increased PI. CD3 is a pan-T cell marker found on CD4^+, CD8^+, and gamma delta T cells (Howard et al., 1994). Gamma delta T cells comprise a large percentage of circulating T lymphocytes in cattle. The lack of gamma delta T cell proliferation in response to PWM is apparently responsible for the lack of significant proliferation of CD3^+ T cells to PWM, even though CD4^+ and CD8^+ cells did have detectable proliferation in response to PWM.

The lack of proliferation of gamma delta T cells to PWM may be due to suboptimal incubation time. In this study, PBMC were incubated with mitogens for 3 days. A previous study found that the number of PWM stimulated bovine "null cells" (probably mostly gamma delta T cells) that could be recovered from culture did not increase until day six of incubation (Franklin et al., 1994). In the case of anti-CD3 MAb stimulation, cross-linking of gamma delta T cell anti-CD3 molecules by MAb may provide sufficient stimulation to cause upregulation of IL2r and MHC II, but not enough stimulation to cause proliferation. It has been shown that blastogenesis of purified bovine gamma delta T cells stimulated with anti-CD3 was significantly increased by co-stimulation with MAb against WC-1, a surface antigen found on most gamma delta T cells (Hanby-Flarida et al., 1996).

Mitogen-stimulated B cells generally increased expression of activation markers, but proliferative responses to the mitogens varied. The percentage of B
cells expressing IL2r increased due to all mitogens, but only Con A, PWM, and anti-CD3 MAb caused significant increases in IL2r fluorescence intensity. Since over 99% of unstimulated B cells expressed MHC II, no increase could be seen with mitogen stimulation; however, all four mitogens caused increased MHC II fluorescence intensity. B cells proliferated significantly only in response to Con A, which changed the B cell distribution of generations and significantly increased the B cell PI. PWM, PHA, and anti-CD3 caused no significant proliferation of B cells.

The results of this study appear to conflict with dogma regarding bovine lymphocyte subset activation by mitogens. Con A and PHA have been reported to stimulate bovine T cells, but not B cells, as determined by blastogenesis (Schultz, 1981; Usinger et al., 1981). However, both Con A and PHA stimulated increased expression of both IL2r and MHC II in B cells when used at concentrations and incubations times optimal for blastogenesis. Con A was the only mitogen to cause noticeable proliferation of B cells. PWM treatment, which has been reported to induce B cell blastogenesis by a T cell dependent mechanism (Schultz, 1981), increased expression of activation molecules on B cells, but caused no detectable B cell proliferation.

Activation of B cells in response to mitogens appears to be at least partly an indirect effect. Anti-CD3 MAb, which should stimulate T cells but not B cells, induced small but significant increases in B cell expression of activation markers. However, anti-CD3 did not cause significant B cell proliferation. PWM and PHA also induced variable upregulation of B cell activation markers, but did not cause
proliferation. When non-purified populations of mitogen-stimulated lymphocytes are incubated together, there may be enough T cell cytokine secretion or cell-cell interactions to stimulate B cells to increase IL2r, but not enough to cause proliferation after three days of incubation. Con A may have some direct effect on B cells. It caused upregulation of IL2r and MHC II like the other mitogens; unlike the other mitogens, Con A induced measurable proliferation of B cells.

Differences were seen between expression of activation molecules and proliferation of some cell types, particularly gamma delta T cells and B cells. These differences may be due to delayed kinetics of proliferation of some subsets under the conditions in the study. It is also possible that incorporation of \(^{3}H\)-thymidine, expression of activation molecules, and cell division are inter-related, but do not necessarily provide interchangeable information. Expression of IL2r appears to be necessary for lymphocytes to be able to proliferate (Miyawaki et al., 1982), but human lymphocytes may upregulate IL2r without undergoing proliferation (Rubin et al., 1985). As observed with anti-CD3 stimulation of gamma delta T cells, upregulation of IL2r may be a necessary step towards activation, but additional signals may be required to enter the cell cycle and proliferate (Hanby-Flarida et al., 1996).

Optimal conditions for blastogenesis, activation marker expression, and proliferation may differ for lymphocyte subsets. It has been reported that mitogen-stimulated ovine CD4\(^{+}\), CD8\(^{+}\), and gamma delta T cells have different times of maximum IL2r expression (Bujdoso et al., 1993). Preliminary studies in our
laboratory indicated that day 3 of mitogen stimulation was optimal for lymphocyte blastogenesis. Con A induced CD3⁺ T cell expression of IL2r was maximal on days 2-4 of incubation (data not shown). Our intent was to compare activation marker expression and proliferation to our standard blastogenesis procedures, so optimal conditions for all measured parameters for all lymphocyte subsets were not determined.

The results of this study indicate that when non-separated populations of bovine lymphocytes are incubated with Con A under conditions similar to those used here, resulting blastogenesis values may be attributed to proliferation of CD3⁺, CD4⁺, CD8⁺, and gamma delta T cells, with a small contribution from B cells. Blastogenesis values of complete lymphocyte populations stimulated with PHA may be due to proliferation of CD3⁺, CD4⁺, CD8⁺, and gamma delta T cells, with no contribution from B cells. PWM blastogenesis values may be attributed to proliferation of CD4⁺ and CD8⁺ T cells, with no contribution from gamma delta T cells or B cells. Anti-CD3 MAb stimulated blastogenesis results may be due to proliferation of CD3⁺, CD4⁺, and CD8⁺ T cells, with no contribution from gamma delta T cells or B cells.

Two-color flow cytometric detection of lymphocyte subset expression of activation molecules and PKH2 detected proliferation are useful methods to study activation of bovine lymphocyte subsets. Both techniques can provide information regarding the responding cell types, and avoid the use of radioactive materials needed for blastogenesis. Both methods can detect stimulation of various lymphocyte subsets,
and can measure differences in response of a given subset to different stimuli. Measurement of increased IL2r and MHC II expression gave similar results for T cell subsets. Measuring expression of MHC II may be of little value in determining the activation status of B cells since even unstimulated cells express MHC II on their surface.

Acknowledgements

The authors would like to thank Tom Skadow for technical assistance. Thanks to Bruce Pesch for his expertise with PKH2 flow cytometry. Thanks to Kristi Harkins and Donghui Cheng for assistance with flow cytometric analysis. This study was partially funded by a Post-Doctoral Fellowship generously provided by the Merck Foundation.

References


Hanby-Flarida, M.D., Trask, O.J., Yang, T.J. and Baldwin, C.L., 1996. Modulation of WC1, a lineage-specific cell surface molecule of Gamma delta T cells, augments cellular proliferation. Immunology, 88:116-123.


Table 1. Monoclonal antibodies used in this study.

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Captions for figures

Figure 1. Lymphocyte blastogenesis after 3 days of incubation without stimulant (control), or with 2.5μg/ml Con A, 1:500 final dilution of PWM, 0.8μg/ml PHA, or anti-CD3 MAb. Figure 1a shows mean (n=8) counts per minute for control and stimulated PBMC. Figure 1b shows mean stimulation indexes for mitogen induced lymphocyte blastogenesis. Error bars represent standard error of the means. (* = P<0.01, the level of statistical significance of the difference between the indicated value and the value for non-stimulated control samples).

Figure 2. CD3+ T cell surface expression of IL2r and MHC II when incubated 3 days without stimulant, or with Con A, PWM, PHA (n=5), or anti-CD3 (n=6). Figure 2a depicts the percentage of CD3+ T cells expressing IL2r or MHC II. Figure 2b shows the mean phycoerythrin fluorescence intensity (IL2r or MHC II density) of CD3+ T cells. Figure 2c shows the IL2r and MHC II expression indexes for CD3+ T cells. Error bars represent standard errors of the means. (* = P<0.05, the level of statistical significance of the difference between the indicated value and the value for non-stimulated control samples).

Figure 3. CD4+ T cell expression of IL2r and MHC II when stimulated with Con A, PWM, PHA (n=5), or anti-CD3 (n=6). The percentage of CD4+ cells expressing
IL2r or MHC II is shown in figure 3a. CD4+ T cell mean fluorescence intensity of IL2r or MHC II expression is shown in figure 3b. CD4+ cell expression indexes for IL2r and MHC II are shown in figure 3c. Error bars represent standard errors of the means. (* = P<0.05, the level of statistical significance of the difference between the indicated value and the value for non-stimulated control samples).

Figure 4. CD8+ T lymphocyte expression of IL2r and MHC II due to stimulation with Con A, PWM, PHA, (n=5), or anti-CD3 (n=6). The percentage of CD8+ T cells expressing IL2r and MHC II is shown in figure 4a. Mean fluorescence intensity of IL2r and MHC II expression is shown in figure 4b. CD8+ T cell IL2r and MHC expression indexes are shown in figure 4c. Error bars represent standard error of the means. (* = P<0.05, the level of statistical significance of the difference between the indicated value and the value for non-stimulated control samples).

Figure 5. Gamma delta T lymphocyte expression of IL2r and MHC II due to stimulation with Con A, PWM, PHA (n=5), or anti-CD3 (n=6). The percentage of gamma delta T cells expressing IL2r and MHC II is shown in figure 5a. Mean fluorescence intensity of IL2r and MHC II expression is shown in figure 5b. Gamma delta T cell IL2r and MHC II expression indexes are shown in figure 5c. Error bars represent standard error of the means. (* = P<0.05, the level of statistical significance of the difference between the indicated value and the value for non-stimulated control samples).
Figure 6. B lymphocyte expression of IL2r and MHC II when cultured for three days with Con A, PWM, PHA (n=5), and anti-CD3 (n=6). The percentage of B cells expressing IL2r and MHC II is shown in figure 6a. The mean fluorescence intensity of IL2r and MHC II expression is shown in figure 6b. B cell Expression indexes for IL2r and MHC II are shown in figure 6c. Error bars represent standard errors of the means. (* = P<0.05, the level of statistical significance of the differences between the indicated value and the value for non-stimulated control samples).

Figure 7. Distribution of generations of lymphocyte subsets incubated with Con A, PWM, PHA, or anti-CD3 MAb. For each series of bars, the bar on the left represents the percentage of cells in the parent, or undivided, generation. Successive bars to the right represent the percentages of cells in generations 2-6. (n=8)

Figure 8. Calculated proliferation indexes of lymphocyte subsets incubated with Con A, PWM, PHA, or anti-CD3. (n=8) (* = P<0.05, the level of statistical significance of the difference between the indicated value and the value for control non-stimulated samples.) ^A
A. blastogenesis, mean counts per minute

- Control: 0
- Con A: 140,000
- PWM: 120,000
- PHA: 100,000
- Anti-CD3: 80,000

B. blastogenesis mean stimulation indexes

- Con A: 350
- PWM: 300
- PHA: 250
- Anti-CD3: 200

Figure 1
A. \% of CD3 T cells expressing IL2r and MHC II

B. CD3 T cell fluorescence intensity of IL2r and MHC II

C. CD3 T cell IL2r and MHC II expression indexes

Figure 2
A. % of CD4 T cells expressing IL2r and MHC II

B. CD4 T cell fluorescence intensity of IL2r and MHC II

C. CD4 T cell IL2r and MHC II expression indexes

Figure 3
A. % of CD8 T cells expressing IL2r and MHC II

B. CD8 T cell fluorescence intensity of IL2r and MHC II

C. CD8 T cell IL2r and MHC II expression indexes

Figure 4
A. % of gamma delta T cells expressing IL2r and MHC II

B. gamma delta T cell fluorescence intensity of IL2r and MHC II

C. gamma delta T cell IL2r and MHC II expression indexes

Figure 5
A. % of B cells expressing IL2r and MHC II

B. B cell fluorescence intensity of IL2r and MHC II

C. B cell IL2r and MHC II expression indexes

Figure 6
Figure 7

A. distribution of CD3 T cell generations

B. distribution of CD4 T cell generations

C. distribution of CD8 T cell generations
Figure 7 (continued)
A. CD3 T cell proliferation indexes

B. CD4 T cell proliferation indexes

C. CD8 T cell proliferation indexes

* indicates significant difference from the no stim group.
gamma delta T cell proliferation indexes

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B cell proliferation indexes

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Figure 8 (continued)
CHAPTER 4. ANTIGEN SPECIFIC UP-REGULATION OF INTERLEUKIN-2 RECEPTOR ON T LYMPHOCYTE SUBSETS OF CATTLE HYPERIMMUNIZED WITH BOVINE VIRAL DIARRHEA VIRUS

A paper to be submitted to Veterinary Immunology and Immunopathology

Mark J. Quade, Steve R. Bolin, Julia F. Ridgway and James A. Roth

Abstract

Up-regulation of interleukin-2 receptor (IL2r) on mononuclear cells from cattle hyperimmunized with bovine viral diarrhea virus (BVDV) was detected after in vitro stimulation with BVDV. Cattle were hyperimmunized by repeated challenge with different isolates of BVDV genotype 1 and genotype 2. Peripheral blood mononuclear cells were isolated from the hyperimmunized cattle and incubated in vitro for six days with or without live BVDV strain 890. Lymphocytes were analyzed by two-color flow cytometry. Monoclonal antibodies (MAb) labeled with fluorescein isothiocyanate specific for T lymphocyte surface molecules (CD3, CD4, CD8, and gamma delta T cell receptor (TCR)) were used to identify T cell subsets. Antigen specific activation of T cell subsets was determined by quantification of surface expression of IL2r using phycoerythrin-labeled MAb specific for IL2r. CD3^+^, CD4^+^, CD8^+^, and gamma delta TCR^+^ T cells from hyperimmunized cattle significantly increased expression of IL2r when incubated with BVDV, compared to cells from hyperimmunized cattle incubated in medium without BVDV. T lymphocyte subsets
from BVDV negative control cattle did not have measurably increased expression of IL2r when incubated with BVDV.

**Introduction**

Bovine viral diarrhea virus (BVDV) is a ubiquitous pathogen of cattle, causing enteric, respiratory, and reproductive diseases. BVDV may be the most economically damaging viral infection of cattle in the United States (Baker, 1995). Much is known regarding antibody responses of cattle to BVDV, but little is reported regarding antigen specific T cell responses. It has been reported that T lymphocytes from cattle infected with BVDV recognize virus-specific antigen: lymphocytes isolated from BVDV infected cattle will proliferate when incubated *in vitro* with live BVDV or viral proteins (Larsson et al., 1992; Lambot et al., 1997), and virus-specific cytotoxic T lymphocytes (CTL) have been demonstrated (Beer et al., 1997).

The alpha subunit of interleukin-2 receptor (IL2r) is expressed on the surface of activated lymphocytes, but not resting cells (Abbas et al., 1994; Uchiyama et al., 1981; Minami et al., 1993). Activation of lymphocytes and expression of IL2r may be induced by mitogens, T cell specific monoclonal antibodies (MAb), or by specific antigen (Cantrell et al., 1984; Smith, 1988). Increased expression of IL2r may be detected by immunohistochemistry or flow cytometry using fluorescently labeled monoclonal antibody (MAb) against IL2r. When used in conjunction with MAb against various lymphocyte subset markers, e.g. CD4 or CD8, it is possible to detect
the subset(s) of lymphocytes activated by an antigenic stimulus (Begara et al., 1995; Bujdoso et al., 1993). Using two-color flow cytometry, the percentage of cells in a lymphocyte subset expressing activation markers may be calculated. The mean fluorescence intensity of cells expressing activation markers, which corresponds to the density of activation molecules on the surface of each cell (Shapiro, 1995), also may be measured. Two-color flow cytometry has been used to detect antigen specific in vitro upregulation of IL2r of T lymphocytes in humans (Ito et al., 1992), swine (Dillender et al., 1993), deer (Hesketh et al., 1993), and cattle (Isaacson et al., 1998).

In this study, we determined the T lymphocyte subsets from hyperimmunized cattle that become activated when exposed to viral antigen in vitro. BVDV positive animals were produced by repeated challenge exposure with different isolates of BVDV. Calves negative for BVDV and antibody against BVDV were used as controls. Peripheral blood mononuclear cells (PBMC) were isolated from hyperimmunized and control cattle and incubated in vitro with or without live BVDV for six days. Two-color flow cytometry was used to identify CD3+, CD4+, CD8+, or gamma delta T cell receptor+ (TCR) T lymphocytes, and surface expression of IL2r. The magnitude of activation of each subset was determined by calculating the percentage of cells expressing IL2r, by measuring the mean fluorescence intensity of IL2r expression, and using these two parameters to calculate an IL2r expression index.
Materials and methods

Animals

Six Holstein steer calves which were hyperimmunized in order to produce antibody for another experiment were used as BVDV positive animals. Two 4-6 month old steers, numbers 8 and 9, were hyperimmunized with BVDV as follows: animals received live BVDV strain NY-1 (genotype 1, non-cytopathic) intranasally, followed four weeks later by live strain 1373 (genotype 2, non-cytopathic) intranasally, followed four weeks later by Singer strain (genotype 1, cytopathic) soluble antigen (Coria et al., 1984) subcutaneously. One 3-4 month old steer, number 10, was hyperimmunized with BVDV strain 28508-5 (genotype 2, non-cytopathic) intranasally, followed four weeks later by strain NY-1 intranasally. Two 3-4 month old steers, numbers 11 and 12, were hyperimmunized intranasally with strain 28508-5 , followed four weeks later by strain NY-1 intranasally, followed four weeks later by strain NADL (genotype 1, cytopathic) intranasally. One 3-4 month old steer, number 13, was hyperimmunized with strain 28508-5 intranasally, followed by strain 1373 intranasally, followed by strain NADL intranasally. Seven colostrum-deprived calves raised in isolation and negative for BVDV and antibody against BVDV were used as negative control animals. Beginning four weeks after the last virus challenge exposure, PBMC were isolated from the cattle for testing. PBMCs from each animal analyzed on two occasions.
Virus

BVDV strain 890 (genotype 2, non-cytopathic) was used for in vitro stimulation of PBMC. Virus was propagated in bovine turbinate cells grown in McCoy’s medium supplemented with 10% fetal bovine serum. Culture flasks were freeze/thawed, and culture medium centrifuged at 200 x g for 10 minutes to pellet cell debris. The supernatant was passed through a 0.45μm filter, aliquoted, and stored at -80°C. The resulting suspensions contained 1 x 10^7 live BVDV/ml.

Monoclonal antibodies

Mouse monoclonal antibodies (MAb) that bind to the following bovine antigens were purchased from VMRD (Pullman, WA): CD3 (MM1A), CD4 (CACT138A), CD8 (CACT80C), gamma delta TCR (GB21A), alpha chain of IL2r (also known as CD25)(CACT108A). Anti-CD3, -CD4, and -CD8 were IgG1 isotype. Anti-gamma delta TCR was IgG2b isotype. Anti-IL2rα was IgG2a isotype. Affinity purified goat anti-mouse isotype specific secondary antibodies were purchased from Caltag (Burlingame, CA): phycoerythrin (PE) -labeled IgG2a (M32204), fluorescein isothiocyanate (FITC) -labeled IgG1 (M32101), and FITC-labelled IgG2b (M32501). Mouse monoclonal antibodies of irrelevant specificity used as isotype controls were purchased from Caltag: IgG1 (MG100), IgG2a (MG2a00), and IgG2b (MG2b00).
**Isolation and culture of PBMC:**

PBMC were isolated using a previously described method (DeBey et al., 1996). Briefly, citrated blood was centrifuged, the buffy coat was collected, and contaminating erythrocytes lysed by hypotonic flash lysis. PBMC were washed twice in HBSS without Ca\(^{++}\) and Mg\(^{++}\) (HBSS) (Celox), and resuspended at 2 x 10^6/ml in 5ml RPMI 1640 (10% fetal bovine serum, 0.1% tissue culture penicillin/streptomycin) in wells of 6-well tissue culture plates. Control cells were incubated in medium only. Isolated cells were >95% viable by trypan blue exclusion. Virus treated PBMC were incubated with live BVDV strain 890 at a multiplicity of infection (moi) of 1:2. PBMC incubated with 2.5\(\mu\)g/ml concanavalin A and analyzed for up-regulation of IL2r on CD3\(^+\) T cells only were used as positive controls. All assays were done in duplicate. Plates were incubated for 6 days in humidified 5% CO\(_2\) atmosphere at 39°C.

**Preparation of cultured PBMC for flow cytometry:**

At the end of the culture period, plates were chilled on ice. Cells were recovered by aspirating medium and non-adherent cells, which were placed into test tubes. Wells were rinsed with cold HBSS, and the HBSS with cells was added to the cells and medium initially recovered. Wells were then treated with 1 ml cold trypsin/EDTA for 3-4 minutes and rinsed with cold HBSS, which was added to
previously recovered cells and media. Cells harvested from duplicate wells were pooled for further processing. A small percentage of cells remained adhered and could not be recovered by this method.

Recovered cells were washed once with HBSS, then washed once in phosphate buffered saline containing 1% bovine serum albumin and 0.1% sodium azide (PBS++) and resuspended in PBS++ at 5 x 10⁶/ml. Cell suspension (200μl) was placed in wells of a 96-well u-bottom microtiter plate. Cells were pelleted by centrifugation at 200 x G for 1 minute, and the supernatant discarded. All cells were incubated with two primary MAb: MAb against a lymphocyte phenotype marker and MAb against IL2r. Cells in replicate wells were resuspended in 25μl anti-IL2r (10μg/ml PBS++) followed by 25μl of MAb (10μg/ml in PBS++) against either CD3, CD4, CD8, or gamma delta TCR (anti-CD4 was used at 20μg/ml). Duplicate wells containing cells used as isotype controls were resuspended in 25μl IgG2a isotype control MAb (control for anti-IL2r) and either 25μl of IgG1 isotype control MAb (control for anti-CD3, -CD4, and -CD8), or IgG2b MAb (control for anti-gamma delta TCR), all at 10μg/ml PBS++. Cells and primary antibodies were incubated at 4°C for 30 minutes. Cells then were washed four times with PBS++, and resuspended in dilutions of two isotype specific secondary antibodies. All cells were resuspended in 25μl of PE-labeled anti-IgG2a, followed by either 25μl FTC-labeled anti-IgG2b or 25μl FITC-labeled anti-IgG1 in appropriate wells. All secondary antibodies were diluted to 10μg/ml PBS++. Cells and antibodies were incubated for 30 minutes at 4°C in the dark. Cells were washed five times with
HBSS, then fixed in 1% paraformaldehyde in PBS for flow cytometric analysis. Cell fluorescence was analyzed on an Epics XL-MCL flow cytometer (Coulter) equipped with System II (version 1.0) software. Excitation was at a wavelength of 488nm using an argon laser. FITC fluorescence was measured in the FL1 channel, PE fluorescence was measured in the FL2 channel.

Data Analysis

For each lymphocyte subset marker, the percentage of PBMC staining positive for IL2r was calculated. The mean fluorescence intensity (PE) of IL2r positive cells also was determined. The IL2r expression index (EI) for each T cell subset was calculated using this equation:

\[
\frac{\text{(% of cells IL2r+) x (mean fluorescence intensity of IL2r+ cells)}}{\text{for PBMC incubated with BVDV}}
\]

\[
\frac{\text{(% of cells IL2r+) x (mean fluorescence intensity of IL2r+ cells)}}{\text{for same animal PBMC incubated without BVDV}}
\]

Results are expressed as means of repeated trials. Statistical significance of differences was determined by analysis of variance using software from SAS Inc. (Cary, NC)
Results

When PBMC from hyperimmunized cattle were incubated with BVDV strain 890, the percentage CD3$^+$ T cells, CD4$^+$ T cells, and gamma delta TCR$^+$ T cells expressing IL2r increased significantly (P<0.05) compared to PBMC incubated without virus (figures 1a, 1c, and 1g). CD3$^+$ T cells, CD4$^+$ T cells, and gamma delta TCR$^+$ T cells from hyperimmunized cattle did not significantly (P>0.05) increase IL2r fluorescence intensity when incubated with BVDV (figures 1b, 1d, and 1h). Incubation with BVDV did not cause CD3$^+$, CD4$^+$, or gamma delta TCR$^+$ T cells from control animals to measurably increase (P>0.05) the percentage of cells expressing IL2r (figures 1a, 1c and 1g) or IL2r fluorescence intensity (figures 1b, 1d, and 1h).

The percentage of CD8$^+$ T cells expressing IL2r increased significantly when PBMC from hyperimmunized cattle (P<0.05) but not from control cattle (P>0.05) were incubated with BVDV 890 compared to PBMC incubated in medium alone (figure 1e). CD8$^+$ T cells from hyperimmunized cattle (P<0.05) but not from control cattle (P>0.05) significantly increased IL2r fluorescence intensity when incubated with BVDV when compared with cells incubated in medium alone (figure 1f).

Figure 2 shows IL2r EI for T lymphocyte subsets. Expression indexes for T cells from hyperimmunized cattle were significantly higher than for T cells from control cattle for CD3$^+$ (P<.01), CD4$^+$ (P<0.05), CD8$^+$ (P<.01), and gamma delta TCR$^+$ T cells (P<0.01).
Con A treated CD3+ T cells of both hyperimmunized and control cattle significantly increased (P<0.05) the percentage of cells expressing IL2r (figure 3a) and IL2r fluorescence intensity (figure 3b) compared to unstimulated cells. IL2r EI for CD3+ T cells were not significantly different (P>0.05) between infected and control groups (figure 3c).

T cell subset IL2r EI for individual control animals are shown in figure 4a. Mean values are represented by columns, error bars represent the range of the two values for IL2r EI obtained for each T cell subset of each animal. Mean values for controls are generally below 3, with only one mean value higher than 5. Two individual values exceed 5. T cell subset IL2r EI for infected animals are presented in figure 4b. Animals 8, 9, 11, and 12 have most subset averages or individual values in excess of 5. Animal 13 has individual values above 5, with most average subset IL2r EI below 5. Animal 10 has no EI values above 4.

Discussion

Few reports of BVDV-specific T cell responses have been published. Antigen specific lymphocyte blastogenesis responses have been reported from cattle naturally (Larsson et al., 1992) and experimentally infected (Lambot et al., 1997) with BVDV. A BVDV non-structural protein, p80, has been shown to induce blastogenesis of PBMC from infected cattle (Lambot et al., 1997). Measuring blastogenesis of lymphocytes that have not been separated into subsets does not identify
subpopulations of lymphocytes activated by antigen. Lymphocytes may be separated into subsets, then tested in blastogenesis assays in an attempt to determine the types of lymphocytes that are stimulated by specific antigens (Denis et al., 1994). However, if lymphocytes are separated into subsets, the interaction of subsets cannot be studied. The blastogenic responsiveness of the various subsets to antigen may be different when they are separated as compared to when incubated together in a mixed population. Cytokines produced by commingled subsets, and cell-cell interactions, may influence the activation and blastogenic responses of individual lymphocyte subsets.

BVDV-specific CTL have been detected in PBMC from BVDV immunized cattle (Beer et al., 1997). Antigen specific cytotoxic activity generally is associated with CD8$^+$ T lymphocytes. However, gamma delta T cells, which represent a prominent percentage of circulating lymphocytes in cattle (Hein et al., 1991), are capable of cytotoxicity (Lang et al., 1995; Kaufmann, 1996). Even CD4$^+$ T cells may be cytotoxic under some conditions (Choi et al., 1994). Hence, demonstrating cytolytic activity does not identify the T cell subset responsible for this function.

During primary infection of gnotobiotic calves with BVDV, in vivo depletion of CD4$^+$ T cells with anti-CD4 MAb resulted in extended viremia and increased nasal shedding of virus, while depletion of CD8$^+$ or gamma delta TCR$^+$ T cells did not increase viremia or nasal shedding (Howard et al., 1992). Although these data demonstrated that CD4$^+$ T cells are important in limiting a primary BVDV infection in gnotobiotic calves, it is possible that CD8$^+$ and/or gamma delta TCR$^+$ T memory
cells may be generated by primary infection or by vaccination, and that these subsets may be important in responding to second exposure to the same strain or a heterologous strain of BVDV.

In this study, two-color flow cytometry was used to detect activation of BVDV specific lymphocytes by measuring up-regulation of IL2r on T cell subsets. Interleukin-2 receptor consists of 3 subunits, alpha, beta, and gamma. The beta and gamma subunits are expressed on resting lymphocytes and have a low affinity for IL-2; high concentrations of IL-2 are required for sufficient binding to the beta-gamma complex to occur and induce lymphocyte proliferation. When lymphocytes are activated by binding of specific antigen to the TCR, IL2r alpha (CD25) is rapidly upregulated. IL2r alpha complexes with the beta and gamma subunits, forming a high-affinity receptor; antigen-activated lymphocytes expressing the alpha-beta-gamma IL2r complex are responsive to significantly lower concentrations of IL-2 than resting cells (Abbas et al., 1994).

The results of this study demonstrate that cattle infected with BVDV generate memory CD3+, CD4+, CD8+, and gamma delta TCR+ T lymphocytes that recognize BVDV antigens and respond by upregulating IL2r. It is unknown whether the antigen responsive T cells were functionally active or proliferating. Wild-type BVDV and vaccine strains of BVDV have been shown to inhibit lymphocyte proliferation and suppress some leukocyte functions (Roth et al., 1983; Brown et al., 1991; Atluru et al., 1992). Expression of IL2r may be necessary for lymphocytes to enter the cell cycle and divide (Miyawaki et al., 1982), but does not mean that T cells are
proliferating. Human T lymphocytes may increase surface expression of IL2r without proliferating (Rubin et al., 1985), and mitogen stimulated bovine lymphocytes, particularly gamma delta T cells and B lymphocytes, may upregulate IL2r expression without proliferating (Quade et al., 1998). Flow cytometric detection of activated lymphocytes combined with proliferation assays and functional assays, such as antigen specific cytokine secretion or cytotoxicity, may lead to a better understanding of BVDV pathogenesis and the roles and interactions of the various lymphocyte subpopulations in the immune response of cattle to BVDV.

Two-color flow cytometric analysis of lymphocyte phenotype and IL2r expression is a useful method to detect BVDV specific T cells from a population of hyperimmunized cattle. No published reports of T cell responses generated by BVDV vaccines could be found; this technique may provide a way to study the ability of modified-live virus and killed virus vaccines to induce BVDV specific T cell subsets. The ability of T cell subsets generated against a given strain of BVDV to recognize and protect against a heterologous strain of BVDV needs further study; the technique described in this study may be useful to examine T cell responses to heterologous and homologous strains of BVDV within genotypes 1 and 2 as well as between genotypes. Variability between animals and day to day variability for the same animal may require testing of multiple animals and multiple sampling of individual animals.
Acknowledgements

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References


Captions for figures

Figure 1. T lymphocyte subset IL2r expression when PBMC of uninfected control cattle (n=7) and BVDV hyperimmunized cattle (n=6) are incubated in vitro for six days in medium only or with live BVDV. The percentage of CD3^+ T cells expressing IL2r is shown in figure 1a. Figure 1b depicts the IL2r fluorescence intensity of CD3^+ T cells. The percentage of CD4^+ T cells expressing IL2r is shown in figure 1c, and CD4^+ T cell IL2r fluorescence intensity is shown in figure 1d. The percentage of CD8^+ T cells expressing IL2r is shown in figure 1e and the CD8^+ T cell IL2r fluorescence intensity is shown in figure 1f. The percentage of gamma delta TCR^+ T cells expressing IL2r is shown in figure 1g, and the gamma delta TCR^+ T cell IL2r fluorescence intensity is shown in figure 1h. (* = P<0.05, the level of statistical significance of the difference between the indicated value and the value for cells incubated without BVDV).

Figure 2. T cell subset IL2r expression indexes when PBMC of uninfected control cattle (n=7) and BVDV hyperimmunized cattle (n=6) are incubated in vitro for six days in medium only or with live BVDV. (** = P<0.01, * = P<0.05, the level of statistical significance of the difference between the value for infected animals and the value for control animals).
Figure 3. CD3+ T cell IL2r expression when PBMC of uninfected control cattle (n=7) and BVDV hyperimmunized cattle (n=6) are incubated in vitro for six days in medium only or with Con A. Figure 3a shows the percentage of CD3+ cells expressing IL2r. Figure 3b shows the IL2r fluorescence intensity of CD3+ T cells. Figure 3c shows CD3 T+ cell IL2r expression indexes for hyperimmunized and control cattle. (* = P<0.05, the level of statistical significance between the indicated value and the value for unstimulated cells.)

Figure 4. T cell subset expression indexes against BVDV 890 of individual uninfected control cattle and cattle hyperimmunized with BVDV. Figure 4a shows T cell subset IL2r expression indexes for control cattle, and figure 4b shows T cell subset IL2r expression indexes for hyperimmunized cattle. Each group of columns represent IL2r expression indexes for one animal; the first column represents mean IL2r EI for CD3+ T cells, followed by CD4+, CD8+, and gamma delta TCR+ T cell mean IL2r EI. Error bars represent the range of the two IL2r EI values for each T cell subset from each animal.
Figure 1

A. 

% of CD3* T cells expressing IL2r

- Control
- Hyperimmunized

B. 

CD3* T cell IL2r fluorescence intensity

- Control
- Hyperimmunized

C. 

% of CD4* T cells expressing IL2r

- Control
- Hyperimmunized

D. 

CD4* T cell IL2r fluorescence intensity

- Control
- Hyperimmunized
Figure 1 (continued)
Figure 2
A. Con A stimulation, % of CD3⁺ T cells expressing IL2r

B. Con A stimulation, CD3⁺ T cell IL2r fluorescence intensity

C. Con A stimulation, CD3⁺ T cell IL2r expression indexes

Figure 3
A. control cattle, T cell subset IL2r expression indexes

![Graph showing IL2r expression indexes for control cattle.]

B. BVDV hyperimmunized cattle, T cell subset IL2r expression indexes

![Graph showing IL2r expression indexes for BVDV hyperimmunized cattle.]

Figure 4
CHAPTER 5. ANTIGEN SPECIFIC IN VITRO ACTIVATION OF T LYMPHOCYTE SUBSETS OF CATTLE IMMUNIZED WITH A MODIFIED-LIVE BOVINE HERPESVIRUS 1 VACCINE

A paper to be submitted to Viral Immunology

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Abstract

Bovine peripheral blood mononuclear cells from cattle immunized with a modified-live virus bovine herpesvirus 1 (BHV1) vaccine and from BHV1 negative cattle were incubated in vitro with inactivated BHV1 for six days. Antigen specific activation of T lymphocyte subsets was measured by two-color flow cytometric analysis of T cell phenotype and surface expression of interleukin-2 receptor (IL2r). Vaccinated animals, but not unvaccinated animals, had CD3^+, CD4^+, and gamma delta T cells that significantly (P<0.05) increased expression of IL2r when incubated with BHV1. CD8^+ T cells from vaccinated animals did not consistently increase IL2r expression when incubated with inactivated BHV1.

Introduction

Bovine herpesvirus type 1 (BHV1) is a major cause of respiratory and reproductive disease in cattle. Humoral immune responses to BHV1 are relatively
well known, while T cell immune responses are less well characterized. Antigen-specific lymphocyte blastogenesis (Griebel et al., 1988) and cytokine secretion in vitro (Tikoo et al., 1995) have been demonstrated. Antigen specific CD4+ T cells (Tikoo et al., 1995), and cytotoxic T cells (Leary et al., 1992) have also been reported. Depletion and enrichment of lymphocyte subsets have been used to try to determine which T cell subsets are responding to BHV1 antigens (Griebel et al., 1988; Denis et al., 1994). Single color flow cytometry has revealed that actively infected cattle have reduced numbers of CD8+ T cells in the blood (Griebel et al., 1988), and that interleukin-2 receptor expression may be increased on peripheral blood mononuclear cells (PBMC) isolated from infected animals (Lan et al., 1996). Some viral gene products recognized by T lymphocytes have been identified (Denis et al., 1993; Leary et al., 1992; Tikoo et al., 1995).

Interleukin-2 receptor (IL2r) is expressed on the surface of activated lymphocytes, but not resting cells (Uchiyama et al., 1981; Minami et al., 1993). Activation of lymphocytes and expression of IL2r may be induced by mitogens, T cell specific monoclonal antibodies (MAb), or by specific antigen (Cantrell et al., 1984; Smith, 1988). Increased expression of IL2r may be detected by immunohistochemistry or flow cytometry using fluorescently labeled MAb against IL2r. When used in conjunction with MAb against various lymphocyte subset markers, e.g. CD4 or CD8, it is possible to tell which subset(s) of lymphocytes are activated by an antigenic stimulus (Begara et al., 1995; Bujdoso et al., 1993). Using two-color flow cytometry, the percentage of a given lymphocyte subset expressing the
activation marker IL2r may be calculated. The mean fluorescence intensity of cells expressing activation markers, which corresponds to the number of activation molecules on the surface of each cell (Shapiro, 1995), may also be measured. Two-color flow cytometry has been used to detect \textit{in vitro} activation of antigen-specific T lymphocytes in humans (Ito et al., 1992), swine (Dillender et al., 1993), deer (Hesketh et al., 1993), and cattle (Isaacson et al., 1998).

In this study, we have attempted to characterize T lymphocyte subset responses of cattle to BHV1 following immunization with a modified live virus vaccine. PBMC isolated from vaccinated cattle and from unvaccinated control cattle negative for BHV1 antibody were incubated with or without inactivated BHV1 \textit{in vitro}. T lymphocytes (CD3\(^+\)) and T lymphocyte subsets CD4\(^+\), CD8\(^+\), and gamma delta T cell receptor (TCR)\(^+\) were analyzed by two-color flow cytometry to determine the degree of activation of each subset. Activation was measured by determining the percentage of cells of each subset expressing IL2r, measuring the mean fluorescence intensity of IL2r expression, and calculating an IL2r expression index (EI) for each subset.
Materials and methods

Animals

Five Holstein steers 3-4 months old were immunized with a commercially available vaccine (Bayer, Shawnee Mission, KS) containing modified live (MLV) BHV1, bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), and parainfluenza type 3 virus (PI-3). A booster dose was given 14 days later, as recommended on the label for immunity against BRSV. Ten calves that tested negative for BVDV and BHV1 antibody were used as a pool of negative control animals. PBMC were isolated from vaccinated calves on the day of vaccination (day 0). Control cattle were not sampled on day 0. Beginning two weeks following initial vaccination, PBMC were isolated once per week until seven weeks after vaccination from all vaccinated cattle and three control cattle.

Virus

Stock BHV1 was propagated in Madin Darby Bovine Kidney (MDBK) cells cultured in Dulbecco's modified Eagle's medium (Sigma) containing 2% fetal bovine serum and gentamicin (50μg/ml) for 24 hours at 37°C in humidified 5% CO₂ atmosphere. Culture flasks were freeze-thawed and medium and cells were briefly sonicated to disrupt any remaining cells. Culture medium was centrifuged at 500 x G
for ten minutes and passed through a 0.45μm filter to remove cell debris. The remaining medium containing BHV1 virus was heated at 56°C for 30 minutes to inactivate virus, aliquoted and stored at -80°C. Virus concentration was determined by plaque assay. The virus preparation contained 1 x 10⁸ plaque forming units/ml.

Monoclonal antibodies

Mouse monoclonal antibodies (MAb) that bind to the following bovine antigens were purchased from VMRD (Pullman, WA): CD3 (MM1A), CD4 (CACT138A), CD8α (CACT80C), delta chain of gamma delta T cell receptor (GB21A), and alpha chain of IL2r (also known as CD25)(CACT108A). Anti-CD3, -CD4, and -CD8, were IgG1 isotype. Anti-IL2r was IgG2a isotype. Anti-gamma delta T cell receptor (TCR) was IgG2b isotype. Affinity purified goat anti-mouse isotype specific secondary antibodies were purchased from Caltag (Burlingame, CA): phycoerythrin(PE)-labeled IgG2a (M32204), fluorescein isothyocyanate (FITC)-labeled IgG1 (M32101), and FITC-labeled IgG2b (M32501). Mouse monoclonal antibodies of irrelevant specificity used as isotype controls were purchased from Caltag: IgG1 (MG100), IgG2a (MG2a00), and IgG2b (MG2b00).
Isolation and culture of PBMC:

Peripheral blood mononuclear cells were isolated using a previously described method (DeBey et al., 1996). Briefly, citrated blood was centrifuged, the buffy coat was collected, and contaminating erythrocytes lysed by hypotonic flash lysis. PBMC were washed twice in HBSS without Ca^{++} and Mg^{++} (HBSS) (Celox), and resuspended at 2 x 10^6/ml in 5ml RPMI 1640 (Celox) containing 10% fetal bovine serum (tested negative for BVD virus and antibody) (Hyclone) and 0.1% tissue culture penicillin/streptomycin (RPMI+) in wells of 6-well tissue culture plates. Isolated cells were >95% viable by trypan blue exclusion. Antigen stimulated PBMC were incubated with 25μl of heat-inactivated BHV1 preparation, a multiplicity of infection of 1:4. The optimal ratio of BHV1 antigen to PBMC was determined in preliminary trials using lymphocyte blastogenesis (data not shown). Unstimulated control cells were incubated in medium only. Cells incubated with 2.5μg/ml Concanavalin A (Con A, Sigma) were used as positive controls. All wells were set up in duplicate. Plates were incubated in humidified 5% CO_2 atmosphere at 39°C for six days.

Preparation of cultured PBMC for flow cytometry:

At the end of the culture period, plates were chilled on ice. Cells were recovered by aspirating medium and non-adherent cells, which were placed into test
tubes. Wells were rinsed with cold HBSS, and the HBSS with cells added to the cells and medium recovered initially. Wells were then treated with 1 ml cold trypsin/EDTA for 3-4 minutes and rinsed with cold HBSS, which was added to previously recovered cells and media. Cells harvested from duplicate wells were pooled for further processing. A small percentage of cells remained adhered and could not be recovered by this method.

Recovered cells were washed in HBSS, then washed and resuspended in phosphate buffered saline containing 1% bovine serum albumin and 0.1% sodium azide (PBS++) at 5 \times 10^6/ml. Cells (200\mu l) were placed in wells of a 96-well u-bottom microtiter plate, pelleted by centrifugation at 200 \times G for 1 minute, and the supernatant discarded. All cells were incubated with two primary antibodies: a T cell subset specific MAb and MAb against IL2r. Cells were resuspended in 25\mu l MAb against either CD3, CD4, CD8, or gamma delta TCR, all at 10\mu g/ml PBS++ (except anti-CD4 was 20\mu g/ml). Wells then received 25\mu l anti-IL2r (10\mu g/ml PBS++). Isotype control cells were resuspended in 25 \mu l IgG2a isotype control (control for anti-IL2r) and 25\mu l of either IgG1 isotype control (control for anti-CD3, -CD4, and -CD8) or IgG2b (control for anti-gamma delta TCR), all at 10\mu g/ml. Con A stimulated cells were stained for CD3 and IL2r expression only. Cells and primary antibodies were incubated at 4°C for 30 minutes. Cells were then washed four times with PBS++, and resuspended in dilutions of two appropriate isotype specific secondary antibodies at 10\mu g/ml PBS++; 25\mu l of phycoerythrin-labeled anti-Ig2a and 25\mu l FITC-labeled anti-IgG2b, or anti-IgG2a and anti-IgG1. Cells and antibodies
were incubated for 30 minutes at 4°C in the dark. Cells were washed five times with HBSS, then fixed in 1% paraformaldehyde in PBS for flow cytometric analysis.

Cell fluorescence was analyzed using an Epics XL-MCL flow cytometer (Coulter) equipped with System II (version 1.0) software. Excitation was by argon laser at 488μm, with FITC fluorescence measured in the FL1 channel, PE fluorescence measured in the FL2 channel. Lymphocytes were discriminated by forward and side light scatter properties. Ten thousand cells were analyzed for each sample. Settings for Quad-stat gates were determined using isotype control samples.

**Data Analysis**

For each T lymphocyte subset, the percentage of cells staining positive for IL2r (PE positive) was calculated. The mean fluorescence intensity (PE) of IL2r positive cells was also determined. The IL2r expression index (EI) was calculated using this equation:

\[
\frac{(\% \text{ of cells IL2r}^+ \times \text{mean fluorescence intensity of IL2r}^+ \text{ cells})}{\text{for cells incubated with BHV1}}
\]

\[
\frac{(\% \text{ of cells IL2r}^+ \times \text{mean fluorescence intensity of IL2r}^+ \text{ cells})}{\text{for cells incubated without BHV1}}
\]

Results are presented as means. Error bars represent standard error of the means. Significance of differences of percent IL2r expression, IL2r fluorescence intensity, and IL2r expression indexes were determined by analysis of variance using software from
SAS Inc. (Cary, NC). IL2r expression index results were converted to log base 2 for statistical analysis.

Results

The percentage of CD3⁺ T cells expressing IL2r when PBMC from vaccinated cattle were incubated with BHV1, compared to cells incubated without antigen, significantly increased (P < 0.05) at all times tested except on the day of vaccination, day 0 (figure 1a). For CD3⁺ T cells from unvaccinated cattle, the percentage of cells expressing IL2r when incubated with heat-inactivated BHV1 showed a small but statistically significantly increase compared to control cells only on week 6 (figure 1a). Mean fluorescence intensity of IL2r expression of CD3⁺ T cells for vaccinated cattle when incubated with BHV1 was significantly increased (P<0.05) from weeks 3-7 after immunization, compared to samples incubated without virus. IL2r fluorescence intensity of unvaccinated cattle CD3⁺ T cells was significantly increased (P<0.05) by incubation with BHV1 on weeks 3, 4, and 7, however the increase on these weeks was smaller than that observed for vaccinated animals (figure 1b). IL2r EI for CD3⁺ T cells from vaccinates were significantly higher (P<0.05) than unvaccinated animals from weeks 2-7 post-vaccination (figure 1c).

The percentage of CD4⁺ T cells expressing IL2r when PBMC from vaccinated cattle were incubated with antigen, compared to unstimulated cells, increased significantly (P < 0.05) compared to PBMC not incubated with antigen on weeks 2, 3,
4, 6, and 7 following vaccination. The percentage of CD4+ cells expressing IL2r when PBMC from control cattle were incubated with BHV1 did not significantly increase (P>0.05) at any time tested (figure 2a). Vaccinated animal CD4+ T cell IL2r fluorescence intensity increased significantly (P<0.05) when incubated with BHV1 from weeks 3-7, while control animal cells IL2r fluorescence intensity increased significantly only on week 4 (figure 2b). Vaccinated cattle CD4+ cell IL2r EI were significantly greater (P<0.05) than unvaccinated cattle on weeks 2, 3, 4, 6, and 7 (figure 2c).

The percentage of CD8+ T cells expressing IL2r when PBMC from vaccinated calves were incubated with BHV1 showed a small but significant (P<0.05) increase only on week 2, while the percentage of control cattle CD8+ cells expressing IL2r did not significantly increase (P>0.05) at any time (figure 3a). CD8+ T lymphocytes from vaccinates' had significant increases (P<0.05) in fluorescence intensity of IL2r expression on weeks 2, 3, 4, 6, and 7. Control animals CD8+ cells did not significantly increase IL2r fluorescence intensity at any time (figure 3b). Vaccinated calves' CD8+ T cell IL2r EI were significantly greater (P<0.05) than unvaccinated cattle only at week 2 (figure 3c).

The percentage of gamma delta T cells expressing IL2r when PBMC from vaccinated cattle were incubated with BHV1 antigen, compared to unstimulated cells, increased significantly (P<0.05) at all times post-vaccination. The percentage of unvaccinated control animal gamma delta T cells expressing IL2r increased significantly (P<0.05) only on week 6 (figure 4a). This increase was much smaller
than that observed for the vaccinated cattle. Gamma delta T cells from vaccinates had significantly increased (P<0.05) fluorescence intensity of IL2r expression from weeks 3-7, while control cattle gamma delta T cell fluorescence intensity increased only on week 4 (figure 4b). Gamma delta T cell IL2r EI for vaccinated cattle were significantly greater (P<0.05) than unvaccinated calves on weeks 2, 3, 4, 6, and 7 (figure 4c).

Concanavalin A stimulation significantly increased (P<0.05) the percentage of CD3+ T lymphocytes from vaccinated and unvaccinated cattle expressing IL2r and the fluorescence intensity of IL2r expression at all times tested (figures 5a and 5b). Con A stimulated IL2r EI for vaccinates and controls were not significantly different (P>0.05) at any time tested (figure 5c).

Figure 6 depicts IL2r EI values for lymphocyte subsets of individual cattle on each test day in order to show day to day and animal to animal variability of the assay. T cell IL2r expression indexes for vaccinates (figures 6a, 6b, 6c, 6d, 6e) showed week to week variability, with values often in excess of 10, and several values greater than 20 for CD3+, CD4+, and gamma delta T cells. Only two values for CD8+ T cells exceeded 10. Four of the five vaccinates (figures 7a, 7b, 7d, 7e) had two or more IL2r EI greater than 10 for both CD4+ and gamma delta T cells. Vaccinate #3 (figure 6c) had no CD4+ T cell IL2r expression indexes greater than 10, but all gamma delta T cell values exceeded 10. In contrast, vaccinate number 1 (figure 6a) generally had higher IL2r EI for CD4+ cells than for gamma delta T cells.
T cell subset IL2r EI for control cattle (figures 6f, 6g, 6h) were generally less than 4 for all subsets, with only 2 values greater than 10.

**Discussion**

BHV1-specific memory T cells may be detected in a number of ways. Delayed-type hypersensitivity (Bradshaw et al., 1996), lymphocyte blastogenesis (Griebel et al., 1988), antigen specific cytokine production (Tikoo et al., 1995), and cytotoxic T lymphocyte (CTL) assays (Leary et al., 1992; Campos et al., 1992) have been used to demonstrate T lymphocyte responses to BHV1 antigens in infected cattle. Vaccine induced BHV1-specific T cell-mediated immune responses have been detected using blastogenesis (Denis et al., 1994; Roth et al., 1998), CTL assays (Splitter et al., 1988), and cytokine production (Tikoo et al., 1995). These techniques have been used to help identify BHV1 epitopes to which T lymphocytes respond (Leary et al., 1992; Denis et al., 1993; Tikoo et al., 1995; Van Drunen Littel-Van den Hurk et al., 1995).

In this study, antigen-specific lymphocyte subset activation by BHV1 was measured by two-color flow cytometric detection of increased expression of IL2r. Interleukin-2 receptor consists of three subunits: alpha, beta, and gamma. The beta and gamma subunits are expressed on resting lymphocytes and have a low affinity for IL-2; high concentrations of IL-2 are required for sufficient binding to the beta-gamma complex to occur and induce lymphocyte proliferation. When lymphocytes
are activated by binding of specific antigen to the TCR, IL2r alpha (CD25) is rapidly upregulated. IL2r alpha complexes with the beta and gamma subunits, forming a high-affinity IL-2 receptor. Antigen activated lymphocytes expressing the alpha-beta-gamma IL2r complex are responsive to much lower concentrations of IL-2 than resting cells (Abbas et al., 1994).

Cattle immunized against BHV1 with a modified-live vaccine developed CD3⁺, CD4⁺, and gamma delta T cells that responded to inactivated BHV1 antigen in vitro by upregulation of IL2r. T lymphocytes from non-immunized BHV1 negative cattle did not increase expression of IL2r when incubated with BHV1 antigen. This method allows antigen-responsive T cell subsets to be identified without separating lymphocytes into subsets before exposure to antigen.

CD8⁺ T cells from vaccinated animals did not respond to BHV1. The percentage of CD8⁺ cells expressing IL2r did not increase when incubated with BHV1, but there were occasional small significant (P<0.05) increases in the fluorescence intensity of CD8⁺ T cell IL2r expression. This may be due to CD8⁺ T cells being activated by other antigens in vivo and expressing IL2r prior to isolation, then increasing the density of IL2r expression in vitro because of cytokine secretion by, or interaction with, BHV1 responsive CD4⁺ or gamma delta T cells.

It is possible that the modified-live vaccine did not generate BHV1-responsive CD8⁺ cells. It seems more likely that since the BHV1 preparation used for in vitro stimulation was heat inactivated the BHV1 antigens were not processed and presented appropriately on MHC I molecules to stimulate CD8⁺ T cells. The
inactivated BHV1 would most likely be processed by antigen-presenting cells via the exogenous antigen pathway and primarily be presented by MHC class II molecules, which would be recognized by CD4^+ T cells. CD8^+ T cells recognize antigen in the context of MHC I, which primarily presents antigens produced by a live virus replicating within the antigen-presenting cell and processed by the endogenous pathway. The heat inactivated BHV1 antigen used in vitro would probably not be processed by the endogenous pathway and be presented on MHC I molecules to a sufficient extent to stimulate CD8^+ T cells.

Our laboratory has attempted without success to demonstrate BHV1 specific T lymphocyte responses from infected and vaccinated animals using live BHV1 as an in vitro stimulant. No references could be found that describe using live BHV1 as an in vitro lymphocyte stimulant. Both live and inactivated BHV1 are reported to prevent proliferation and cause apoptosis of bovine PBMC (Hanon et al., 1996; Hanon et al., 1997), and live BHV1 preferentially causes apoptosis of CD4^+ lymphocytes but not CD8^+ or gamma delta T cells (Eskra et al., 1997). Additionally, live BHV1, but not inactivated virus, has been shown to inhibit lymphocyte expression of MHC I (Nataraj et al., 1997), which could interfere with CD8^+ T cell recognition of BHV1 antigens. Inhibition of MHC I expression and induction of apoptosis of CD4^+ cells may account for the inability to use live BHV1 as an in vitro stimulant. It may be necessary to transfect BHV1 genes into cell lines (Leary et al., 1992) to study presentation of endogenous antigen to CD8^+ T cells.
There was considerable day to day and animal variability in the pattern of T cell subset activation and in the amount of subset IL2r expression increase. All of the vaccinates developed antigen-responsive CD3^+ T cells. CD3^+ is a pan-T lymphocyte marker, and is found on CD4^+, CD8^+, and gamma delta T cells (Davis et al., 1993). Four of the five vaccinated animals generated CD4^+ T cells that responded to inactivated BHV1, and all five vaccinates developed gamma delta T cells that responded in varying degrees to BHV1 in vitro. One vaccinated animal (vaccinate #3, figure 6c) seemed to develop primarily gamma delta T cells that recognized BHV1, with little or no response from CD4^+ T cells. Conversely, one animal (vaccinate #1, figure 6a) developed a stronger CD4^+ T cell response, as compared to the gamma delta T cell response. T cell subsets from unvaccinated control animals did not increase IL2r expression when incubated with BHV1, except for a few isolated values. The few values that did increase were still generally lower than values generated from vaccinated animals, and probably reflect the inherent variability of the assay method. This technique can identify antigen-specific T cell subset activation in an immunized population, but variability may limit its usefulness for detection of infected or vaccinated individuals based on a one-time test.

T cells from BHV1 vaccinated animals were activated by heat-killed BHV1 in vitro to have increased surface expression of IL2r. Flow cytometric detection of T cell subset upregulation of IL2r identifies antigen-responsive lymphocytes, but does not determine the proliferative status of the responsive T cells. IL2r expression appears to be necessary for lymphocytes to proliferate (Miyawaki et al., 1982), but
bovine lymphocytes may upregulate IL2r without proliferating (Quade et al., 1998). Neither does IL2r expression determine the functional status of antigen responsive T cells. Perhaps a combination of IL2r expression, assays that identify which subsets are proliferating (Quade et al., 1998), and antigen-induced function, such as cytokine production, will yield the most complete picture of the bovine immune response to BHV1.

Two-color flow cytometry is a useful method to monitor T cell responses of a population to BHV1 vaccination. The technique provides information regarding the cell types that are responding, and avoids the use of radioactive materials needed for lymphocyte blastogenesis. It can detect differences in stimulation of various T cell subsets, and can measure individual differences in subset responsiveness between animals. It may be a useful technique to identify T cell epitopes and study the efficacy of new technologies that allow vaccine antigens to activate T lymphocytes or specific T cell subsets to direct specific immune responses (Bona et al., 1998).

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References


Captions for figures

Figure 1. CD3⁺ T lymphocyte surface expression of IL2r of vaccinated (n=5) and unvaccinated (n=3) cattle PBMCs incubated with or without inactivated BHV1 for 6 days. Cattle were vaccinated on day 0 and PBMCs isolated at indicated intervals after vaccination for testing. The percentage of CD3⁺ T cells expressing IL2r is shown in figure 1a. Figure 1b shows the IL2r mean fluorescence intensity of CD3⁺ T cells. CD3⁺ T cell IL2r expression indexes are depicted in figure 1c. Error bars represent standard errors of the means. (For figures 1a and 1b, * = P<0.05, the level of statistical significance of the difference between the indicated value and the value for cells incubated without BHV1. For figure 1c, * = P<0.05, the level of statistical significance of the difference between the indicated value for vaccinated cattle as compared to unvaccinated cattle.)

Figure 2. CD4⁺ T lymphocyte surface expression of IL2r for vaccinated (n=5) and unvaccinated (n=3) cattle PBMCs incubated with or without inactivated BHV1 for 6 days. The percentage of CD4⁺ T cells expressing IL2r is shown in figure 2a. CD4⁺ T cell mean IL2r fluorescence intensity is shown if figure 2b. IL2r expression indexes of CD4⁺ T cells is shown in figure 2c. Error bars represent standard errors of the means. (For figures 2a and 2b, * = P<0.05, the level of statistical significance of the difference between the indicated value and the value for cells incubated without
BHV1. For figure 2c, * = P<0.05, the level of statistical significance between the indicated value for vaccinated cattle as compared to unvaccinated cattle.)

Figure 3. CD8+ T lymphocyte surface expression of IL2r for vaccinated (n=5) and unvaccinated (n=3) cattle PBMCs incubated with or without inactivated BHV1 for 6 days. The percentage of CD8+ T cells expressing IL2r is shown in figure 3a. CD8+ T cell IL2r mean fluorescence intensity is shown in figure 3b. IL2r expression indexes of CD8+ T cells is shown in figure 3c. Error bars represent standard errors of the means. (For figures 3a and 3b, * = P<0.05, the level of statistical significance of the difference between the indicated value and the value for cells incubated without BHV1. For figure 3c, * = P<0.05, the level of statistical significance of the difference between the indicated value for vaccinated cattle as compared to the value for unvaccinated cattle.)

Figure 4. Gamma delta T cell surface expression of IL2r for vaccinated (n=5) and unvaccinated (n=3) cattle PBMC incubated with or without inactivated BHV1 for 6 days. The percentage of gamma delta T cells expressing IL2r is shown in figure 4a. Gamma delta T cell IL2r fluorescence intensity is shown in figure 4b. IL2r expression indexes of gamma delta T cells is shown in figure 4c. Error bars represent standard errors of the means. (For figures 4a and 4b, * = P<0.05, the level of statistical significance of the difference between the indicated value and the value for cells incubated without BHV1. For figure 4c, * = P<0.05, the level of statistical
significance of the difference between the indicated value for vaccinated cattle and the value for unvaccinated cattle.)

Figure 5. CD3⁺ T cell surface expression of IL2r for vaccinated (n=5) and unvaccinated (n=3) cattle PBMC incubated with or without Con A for 6 days. The percentage of CD3⁺ T cells expressing IL2r is shown in figure 5a. CD3⁺ T cell IL2r fluorescence intensity is shown in figure 5b. CD3⁺ T cell expression indexes are shown in figure 6c. Error bars represent standard errors of the means. (* = P<0.05, the level of statistical significance of the difference between the indicated value and the value for cells incubated without Con A.)

Figure 6. Individual calf IL2r expression indexes for all T cell subsets at each time tested. Values for vaccinated calves are shown in figures 6a-6e. Control animal values are shown in figures 6f-6h. For each T cell subset of vaccinates, the first column represents day 0 (day of vaccination) values, and successive columns to the right represent weeks 2-7 post-vaccination. For control cattle, columns represent weeks 2-7. (Data for week 6 are missing for vaccinate #4.)
A. % of CD3 T cells expressing IL2r

B. CD3 T cell mean fluorescence intensity of IL2r expression

C. CD3 T cell IL2r expression indexes

Figure 1
Figure 2
A. % of CD8 T cells expressing IL2r

![Graph showing percentage of CD8 T cells expressing IL2r over time.]

B. CD8 T cell mean fluorescence intensity of IL2r expression

![Graph showing mean fluorescence intensity of CD8 T cells expressing IL2r over time.]

C. CD8 T cell IL2r expression indexes

![Graph showing IL2r expression indexes over time.]

Figure 3
A. % of gamma delta T cells expressing IL2r

- control
- control + BHV1
- vacc
- vacc + BHV1

B. gamma delta T cell mean fluorescence intensity of IL2r expression

C. gamma delta T cell IL2r expression indexes

Figure 4
A. Con A stimulation, % of CD3 T cells expressing IL2r

B. Con A stimulation, CD3 T cell IL2r fluorescence intensity

C. Con A stimulation, CD3 T cell IL2r expression indexes

Figure 5
A. vaccinate #1, T cell subset IL2r El

B. vaccinate #2, T cell subset IL2r El

C. vaccinate #3, T cell subset IL2r El

D. vaccinate #4, T cell subset IL2r El

E. vaccinate #5, T cell subset IL2r El

F. control #1, T cell subset IL2r El

G. control #2, T cell subset IL2r El

H. control #3, T cell subset IL2r El

Figure 6
CHAPTER 6. RESEARCH SUMMARY AND GENERAL CONCLUSIONS

Summary of experimental results

This dissertation describes experiments investigating in vitro detection of lymphocyte subsets activated by stimulation with mitogens or specific antigens. The role of T cell mediated immune responses (CMI) in immunity to and pathogenesis of many diseases is poorly characterized. Some techniques for investigating CMI, such as lymphocyte blastogenesis, can detect activation of T lymphocytes, but cannot identify the subpopulation(s) of lymphocytes that are responding. In order to more fully understand how the immune system responds to infection or vaccination, methods must be devised to specifically identify the subsets of lymphocytes that respond to antigen and determine their function.

Stimulation of bovine peripheral blood mononuclear cells with mitogens

The initial step of this research was to adapt two-color flow cytometric analysis of lymphocyte phenotype and activation marker expression for use with bovine peripheral blood mononuclear cells (PBMC) in our laboratory. Stimulation of bovine PBMC with mitogens caused measurable upregulation of the activation markers Interleukin-2 receptor (IL2r) and major histocompatibility complex II (MHC II)
molecules to varying degrees on most lymphocyte subsets, thereby validating the ability of the technique to measure differences of activation of lymphocyte subsets.

In the course of developing the assay, it was noted that the percentages of lymphocyte subsets recovered from cultures of mitogen stimulated PBMC were different from subset percentages from unstimulated cells. It appeared that some subsets had proliferated and other subsets had been reduced in number compared to unstimulated PBMC. The patterns of apparent proliferation were contradictory to the reported effects of lymphocyte subset proliferation due to mitogens. It was decided to investigate these results further.

Others have determined the proliferative effects of mitogens on lymphocyte subsets by deleting certain lymphocyte populations before performing lymphocyte blastogenesis tests. Using these methods, it was concluded that Concanavalin A (Con A) and phytohemagglutinin (PHA) stimulate bovine T cells, and pokeweed mitogen (PWM) stimulates bovine T and B cells (Usinger et al., 1981). Mitogen effects on gamma delta T cells have been poorly characterized. Due to lymphocyte subset cytokine secretion and cell-cell interactions, lymphocyte subset responses to mitogens may be different when incubated together, compared to a purified population or a population depleted of certain subsets.

In order to directly measure proliferative responses of lymphocyte subsets incubated together, isolated PBMC were stained with PKH2 fluorescent membrane dye before being placed in incubation. Proliferating lymphocytes contain progressively less PKH2 and decreased membrane fluorescence with each successive
generation. At the end of incubation, recovered cells were incubated with subset specific monoclonal antibodies (MAb), and flow cytometry was used to detect cell phenotype and relative membrane PKH2 fluorescence.

The results of the comparison of mitogen induced lymphocyte subset activation marker expression and proliferation were interesting. While all the lymphocyte subsets responded to Con A, PHA, PWM, and anti-CD3 MAb with generally increased expression of IL2r and MHC II, subset proliferation varied. It was concluded that, under conditions similar to those used in the experiment, B cell proliferation is induced only by Con A. Con A and PHA induce proliferation of CD3⁺, CD4⁺, CD8⁺, and gamma delta T cells. Pokeweed mitogen stimulates proliferation of CD4⁺ and CD8⁺ T cells, but not gamma delta T cells. Pokeweed mitogen also failed to stimulate significant proliferation of CD3⁺ T cells since they gamma delta T cells comprise a large percentage of total T cells in cattle. Anti-CD3 MAb stimulates proliferation of CD3⁺, CD4⁺ and CD8⁺ lymphocytes, but not gamma delta T cells. Although neither PWM nor anti-CD3 MAb caused measurable proliferation of gamma delta T cells (a large percentage of the CD3⁺ cell population), anti-CD3 MAb caused significant proliferation of CD3⁺ T cells while PWM did not. Apparently, proliferation of CD4⁺ and CD8⁺ T cells due to anti-CD3 MAb is sufficiently greater compared to proliferation induced by PWM that the effect on the total CD3⁺ T cell population is detectable.

Mitogenic activation of B lymphocytes may be partly an indirect effect. Anti-CD3 MAb, which should stimulate T cells but not B cells, induced small but
significant increases in B cell expression of activation markers, but did not cause measurable B cell proliferation. PWM and PHA also induced variable upregulation of B cell IL2r and MHC II without causing proliferation. However, Con A may have some direct effect on B cells. Like the other mitogens, it caused increased expression of IL2r and MHC II; unlike the other mitogens, Con A induced measurable proliferation of B cells.

*Stimulation of PBMC with bovine viral diarrhea virus*

An experiment was designed to test the hypothesis that cattle infected with bovine viral diarrhea virus (BVDV) develop BVDV specific T lymphocytes, and that these antigen specific T cells can be detected *in vitro*. Calves that had been hyperimmunized (for other purposes) by repeated intranasal challenge with different strains of live BVDV served as BVDV infected animals; 2 calves were infected with BVDV strains NY-1, 1373, and Singer; four calves were infected with strains 28508-5, NY-1, and NADL. Colostrum deprived BVD virus- and antibody-negative calves raised in isolation were used as control animals. PBMC isolated from the animals were incubated with or without live heterologous BVDV (strain 890) *in vitro* for six days. CD3⁺, CD4⁺, CD8⁺, and gamma delta TCR⁺ T cells from the infected animals (n=6) increased expression of IL2r when incubated with BVDV, as compared to cells incubated without BVDV. T cells from control animals (n=7) did not increase IL2r expression when incubated with BVDV.
An experiment was designed to determine if a commercially available vaccine (Bayer) containing modified-live (MLV) BVDV, bovine herpesvirus 1 (BHV1), bovine respiratory syncytial virus, and parainfluenza 3 virus could generate detectable T cell subset responses when injected into cattle. Eight calves serologically negative for BVDV and BHV1 were divided into two groups; five calves were immunized with the MLV vaccine, and three calves were separated and received no immunization. PBMC were isolated from the animals at weekly intervals and incubated with or without live BVDV as described for the previous experiment, and with or without heat inactivated BHV1.

Lymphocytes from vaccinated animals increased expression of IL2r when incubated with BVDV, as compared to cells incubated without BVDV. Lymphocytes from the BVDV serologically negative control animals also increased IL2r expression when incubated with BVDV. Different BVDV serologically negative animals were obtained, and their lymphocytes also increased IL2r expression when incubated with BVDV. Colostrum deprived BVD virus and antibody negative calves were found to use as controls; their T cells did not become activated when incubated with BVDV. Altogether, ten different control animals were used, three of which (the colostrum deprived calves) proved to be unresponsive to BVDV. While this part of the experiment did not yield publishable results and is not reported in this dissertation, it lead to some interesting insights into the potential development of antigen specific T
lymphocytes in the presence of maternal antibody blockade of humoral immune response.

Useable data were obtained regarding BHV1 vaccination. CD3+, CD4+, and gamma delta T cells from vaccinated animals increased expression of IL2r when incubated with inactivated BHV1, compared to cells incubated without BHV1. CD8+ T cells from vaccinates were not activated when incubated with heat killed BHV1. T cells from the ten control animals (all serologically negative for BHV1) did not become activated when incubated with BHV1.

**Significance of research results**

The results of the mitogen stimulated activation marker expression experiments demonstrated that two-color flow cytometry is a useful method for detecting lymphocyte phenotype and activation status. The technique can measure differences of activation between lymphocyte subsets and between various stimuli.

The PKH2 fluorescent cell membrane staining technique is a useful method of detecting lymphocyte proliferation. Information regarding proliferation of various lymphocyte subsets may be obtained while all the subsets are incubated together and allowed to interact, and no radioactive materials are needed. Lymphocyte expression of activation markers should not be regarded as being synonymous with proliferation. Surface expression of IL2r may be necessary for lymphocytes to proliferate (Miyawaki et al., 1982), but another study demonstrated that human lymphocytes may increase
IL2r without proliferating (Rubin et al., 1985) and the results of this research extend that finding to bovine lymphocytes.

The subset proliferation results may clarify the situation regarding which lymphocyte subpopulations are proliferating in response to mitogen. When incubating complete populations of bovine lymphocytes under conditions similar to those in the study, blastogenesis results due to Con A stimulation may be due to activation of CD3⁺, CD4⁺, CD8⁺, and gamma delta T cells, with a small contribution from B cells. Blastogenesis results from PHA stimulated PBMC may be due to activation of CD3⁺, CD4⁺, CD8⁺, and gamma delta T cells. Blastogenesis results from PWM stimulated PBMC may be due to activation of CD4⁺ and CD8⁺ T cells. Blastogenesis results from anti-CD3 MAb stimulated PBMC may be due to activation of CD3⁺, CD4⁺, and CD8⁺ T cells.

There are few published reports of T cell mediated immune responses of cattle to BVDV. Antigen specific blastogenesis (Larsson et al., 1992) and cytotoxicity (Beer et al., 1997) have been reported. The manuscript entitled "Antigen specific up-regulation of Interleukin-2 receptor on T lymphocyte subsets of cattle experimentally infected with bovine viral diarrhea virus" is apparently the first report of BVDV specific T cell subset activation. Detecting the presence of antigen responsive T cell subsets may be an important beginning step toward a better understanding of bovine immune responses to BVDV. Combining this information with assays of lymphocyte function and subset proliferation may clarify the roles of each T cell subset in protective responses to BVDV.
This experiment also demonstrated that there is some ability of BVDV specific T cells to recognize heterologous strains of BVDV. T cells from most of the infected animals became activated when incubated with a strain of BVDV that the animals had not been challenged with. Flow cytometric detection of antigen specific T cells may facilitate the study of T cell immune responses to heterologous, as well as homologous, strains of BVDV. The technique may aid the identification of specific viral gene products or epitopes recognized by T cell subsets and help to identify conserved protective T cell epitopes shared by heterologous strains of BVDV.

The apparent BVDV specific activation of T cells from BVDV seronegative calves was frustrating because it led to data that were not publishable, but may open new avenues of research. It has been reported that when calves with colostrum derived antibody against BHV1 get exposed to the virus, the calves may develop BHV1 sensitive memory T cells (Bradshaw et al., 1996; Ellis et al., 1996) or become latently infected (Lemaire et al., 1995) without generating detectable antibody against BHV1. Flow cytometric detection of antigen specific T cell subsets may be a useful technique for confirming this observation, and for investigating the possibility of a similar phenomenon in calves exposed to BVDV. These observations also raise questions about the usefulness of serology to detect prior exposure to BVDV and BHV1 in cattle.

The detection of BHV1 specific T cell subsets following vaccination demonstrates that the two-color flow cytometric assay may be a useful way to examine BHV1 immune responses and the ability of different types of vaccines and
adjuvant combinations to generate CMI responses. While BHV1 specific CD4+ (Tikoo et al., 1995) and cytotoxic cells (Leary et al., 1992; Campos et al., 1992) have been described, this is apparently the first report of BHV1 responsive gamma delta T cells.

**Recommendations for future research**

**Cross-strain recognition of BVDV T cell epitopes by T cell subsets**

Chapter four of this dissertation describes evidence that there may be T cell epitopes that are conserved between different strains of BVDV. These findings should be expanded and more thoroughly investigated. BVDV negative cattle could be divided into groups, and each group challenged with a single strain of BVDV. Groups challenged with strains of genotype 1 and genotype 2 BVDV should be included. PBMC from animals of each group should be incubated *in vitro* with homologous and heterologous virus strains of both genotypes to determine if antigen specific T cell subsets generated by challenge with a given strain of BVDV recognize antigen from other strains. Similar methods could be used to study the ability of modified-live and killed BVDV vaccines to stimulate development of T cell subsets that recognize homologous and heterologous strains of virus.
The two-color flow cytometric method described in this dissertation can identify BVDV or BHV1 antigen specific T cell subsets, but the proliferative and functional properties of the responsive T cells remain unknown. Proliferative responses of BVDV or BHV1 specific T cell subsets could be determined by testing antigen specific IL2r expression and proliferation (determined by relative PKH2 fluorescence intensity) in parallel, similar to the experiment described in chapter two of this dissertation. For example, PBMC of BVDV infected animals would be isolated and divided into two samples. One sample from each animal would be incubated in vitro with or without BVDV and analyzed flow cytometrically for lymphocyte phenotype and IL2r expression. The other sample of PBMC from each animal would be stained with PKH2 prior to incubation with or without BVDV. At the end of the incubation period, PKH2 stained cells would be analyzed flow cytometrically for lymphocyte phenotype and proliferation. Alternatively, three-color flow cytometry of lymphocyte phenotype, IL2r expression, and relative PKH2 fluorescence intensity could be attempted.

Effector function of BVDV and BHV1 activated T cell subsets should also be investigated. Cytokine production by BVDV or BHV1 responsive PBMC could be determined in a number of ways. PBMC of BVDV infected cattle could be incubated with and without BVDV, and the supernatant assayed for a number of cytokines by ELISA or bioassay. Cytokine producing cells could be quantitated by
ELISPOT. Alternatively, cytokine mRNA of PBMC could be assayed by reverse transcription-polymerase chain reaction (RT-PCR) (Kee et al., 1997).

The phenotype of cytokine producing T cells could be determined by adapting previously described methods. PBMC from infected animals would be incubated with and without BVDV. After incubation, T cell subsets would be separated using immunomagnetic beads (or perhaps by fluorescence activated cell sorting) and T cell subset cytokine production detected by ELISPOT (Herr et al., 1996) or RT-PCR. Alternatively, PBMC from BVDV infected animals would be incubated in vitro with and without BVDV. After incubation, cells could be permeabilized and incubated with MAb against various cytokines as well as MAb against T cell subset markers. T cell phenotype and intracellular cytokine content would be analyzed flow cytometrically (Falchetti et al., 1995).

Can BVDV antibody negative calves have BVDV reactive T cells?

While conducting the experiment described in chapter four of this dissertation, several BVDV seronegative control animals had T cells that apparently became activated by BVDV. The possibility of BVDV antibody negative animals possessing BVDV specific memory T lymphocytes should be investigated for three reasons. First, as a practical matter, to discover a way to identify truly BVDV negative animals for use as control animals in future research. Second, to uncover the mechanism by which this might happen. Does the presence of colostral antibody against BVDV
inhibit seroconversion of calves without preventing generation of antigen specific T lymphocytes, as has been reported to occur with BHV1 (Bradshaw et al., 1996; Ellis et al., 1996)? Might these observations be evidence for colostral transfer of antigen specific T cell mediated immunity? Thirdly, the diagnostic and epidemiological implications of such a phenomenon may be important.

Neonatal calves could be given experimental colostrum. One group of calves would receive colostrum known to be free of BVDV or BVDV antibody. Another group of calves would receive colostrum containing known concentrations of BVDV antibody. Calves sera would be tested for presence of BVDV antibody. Calves would then be challenged with live BVDV. Serum antibody titers would be tested, and PBMC would be tested at regular intervals for the presence of BVDV specific T cells. A similar experiment could be done using BHV1 in order to corroborate previous reports. It would be possible to determine if live or killed vaccines differ in their efficiency of inducing different memory T cell subsets in the face of maternal antibody.

References


