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Generation and characterization of virus-neutralizing bovine monoclonal antibodies to bovine herpesvirus 1 glycoproteins gB, gC, and gD

Randall L. Levings
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Generation and characterization of virus-neutralizing bovine monoclonal antibodies to 
bovine herpesvirus 1 glycoproteins gB, gC, and gD

by

Randall L. Levings

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

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:
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Iowa State University
Ames, Iowa
2012

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ABSTRACT

Three bovine monoclonal antibodies (MAb) which neutralized bovine herpesvirus 1 (BHV1) were generated using a unique ‘self-refusion’ method. Each was determined to be directed to one of the three major neutralization targets of BHV1 (gB, gC, and gD), and was capable of passive immunization in a laboratory rabbit intravenous challenge model. They were found to be specific for the immunizing BHV1 or for BHV1 and the closely related BHV5. The epitope specificities of two were identified using characterized murine MAb. The previously unidentified combination of epitope and virus reactivity of the two bovine MAb suggest a sequence-structural difference in gB between BHV1.1 and BHV5, and an epitope adjacent to a known epitope for the anti-gD bovine MAb. Anti-idiotypic antibodies were generated against one of the bovine MAb for possible use in vaccination. Bovine MAb may offer unique advantages for research and application in diagnostics, vaccine development, and in better understanding the bovine immune response to infectious disease.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

In 1984 Georges J.F. Köhler and César Milstein received the Nobel Prize in Physiology or Medicine (with Niels K. Jerne) for the development of hybridoma technology and its resultant monoclonal antibodies (MAb). The technology became key to molecular biology and found wide use in research, diagnostics, and treatment. The conventional and popular application employed the laboratory mouse, allowing inexpensive syngeneic animal work on diseases affecting humans and veterinary species. Further, exacting modifications were implemented to ‘humanize’ the MAb products for use as licensed human therapeutics, which have not been generalized to veterinary species.

Host species MAb, because of their combination of specificity, immortality, and scalability on the one hand (reflecting hybridoma technology) and their host species evolution, reflection of the natural immune response, and host therapeutic potential on the other (reflecting host species origin), can provide unique insights into the host species immune system, the infectious agent, and the host response to the infectious agent.

The bovine immune system is of interest because of the economic importance of cattle to pastoral communities and commercial enterprises globally. Its similarities to and differences from the better studied mouse and human immune systems are only beginning to be understood. Bovine herpesvirus 1 (BHV1) primarily causes production losses in cattle worldwide, and is the subject of expensive eradication programs and clinical syndrome control efforts in many countries. Adaptation of host species origin hybridoma technology to the study of the bovine immune response to such an important pathogen, including characterizing and applying the resultant antibodies, would be helpful in understanding the bovine immune system, the key antigens of the virus, and the natural (bovine) host response to the virus infection.
Dissertation organization

The dissertation consists of the general introduction, five chapters consisting of manuscripts intended for submission to peer-reviewed journals, and a general conclusion. Randall L. Levings is the primary author on all the manuscripts. They include a literature review on the bovine immune response to BHV1, a description of the generation and preliminary characterization of three bovine MAb to major BHV1 glycoproteins, a report on BHV1 passive immunization trials using the bovine MAb, an account of characterization of the MAbs’ epitopes, and a description of the generation of anti-idiotypic antibodies to one of the bovine MAb.
CHAPTER 2. IMMUNOLOGY OF BOVINE HERPESVIRUS INFECTION

A paper to be submitted to Animal Health Research Reviews

Randall L. Levings¹, James A. Roth²

Abstract

Bovine herpesvirus 1 (BHV1), like other alphaherpesviruses, causes rapid lytic infections in some cell types and latent infections in others. It causes a variety of diseases and is globally distributed. Although similar in many respects to the human immune response to the herpesvirus type species human herpesvirus 1, the differences in the bovine virus proteins, immune system, physiology, and lifestyle mean the bovine immune response to BHV1 is unique. The innate immune system is the first to respond to the infection, with type I interferons, inflammatory cytokines, killing of infected host cells, and priming of a balanced adaptive immune response. Cell mediated immunity, including cytotoxic T lymphocyte killing of infected cells, is critical to recovery from infection. Humoral immunity, including neutralizing antibody and antibody-dependent cellular cytotoxicity, is important to prevention or control of (re-) infection. The virus possesses a variety of immune evasion strategies, including inhibition of type I interferon production, suppression of major histocompatibility presentation of viral antigen, helper T-cell killing, and latency. BHV1 immune suppression contributes to the severity and economic impact of its disease manifestations and to the bovine respiratory disease complex. A variety of conventional and molecular BHV1 vaccines have been developed as a result of the extensive study of BHV1 diseases, the virus, and the immune response to it. They are used to prevent or reduce clinical signs in many parts of the world, and marker vaccines have been used in the eradication of BHV1 disease in some European countries.

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1. Introduction

Bovine herpesvirus 1 (BHV1) causes a variety of diseases (Gibbs and Rweyemamu, 1977) and infection is world-wide (Beer, 2012). The diseases it causes are costly both in direct disease effects and in lost trade. The immunosuppression BHV1 causes potentiates secondary infections, and it is a major component of the bovine respiratory disease complex (BRDC), which has a large economic impact on the cattle industry in the US (Jones and Chowdhury, 2007).

BHV1 has been found to infect a number of artiodactyl species, and is closely related to viruses infecting other domestic and wild ungulates (Thiry et al., 2006). It is considered the prototype herpesvirus (HV) species of ruminants (Robinson et al., 2008). BHV1 is also similar to the (human) type species of its genus (Varicellovirus) and subfamily (alphaherpesvirinae, $\alpha$HV), and demonstrates similar life-cycle events. The human HV, and the $\alpha$HV of veterinary importance such as BHV1, have been extensively studied.

BHV1 infection is commonly diagnosed serologically. Serosurveys have been conducted in Africa (Straub, 1990; El Hussein et al., 2005), South Asia (Nandi et al., 2009), East Asia (Kampa et al., 2004; Yan et al., 2008), Australia (St. George et al., 1967; Smith et al., 1995), North America (Kahrs et al., 1964; Elazhary et al., 1984), South America (Straub 1990), and Europe (Wuyckhuise et al., 1994). Serological testing and removal of infected animals has been successfully used to eliminate BHV-1 from Denmark, Switzerland and Austria (Ackermann and Engels, 2006).

BHV1 disease is widely vaccinated against, on multiple continents. A variety of vaccines have been employed -- replicating and non-replicating, conventional and those produced through new technologies (Turin et al., 1999). Many of the vaccines have had problems or issues in application, including virulence, immunosuppression, reactivation, or failure to protect. In North America the aim is disease suppression, while in many EU countries vaccination is used in eradication campaigns (van Drunen Littel-van den Hurk, 2006). Because of BHV1’s large genome size, it has been tested as a viral vector for vaccination against other cattle diseases (Kit et al., 1991; Schrijver et al., 1997; Kweon et al., 1999).
The molecular characterization, ease of production, and likelihood of application of new tools for BHV1 have led to its use as a model for vaccine and other technologies, in spite of the complexity of the viral lifecycle and immune response. The impact of the disease and the promise of mitigation mean an understanding the bovine immune response to BHV1 is important and relevant.

2. BHV1 Life-cycle

2.1. Classification

BHV1 is a member of the family herpesviridae, whose type species is human herpesvirus 1 (HHV1), also known as herpes simplex virus 1 (HSV1). Membership in the family is based on virion architecture – a core containing a linear double-stranded DNA genome, an ~100nm icosahedral capsid of 162 capsomers, an amorphous tegument, and an envelope containing viral glycoprotein spikes (Pellet et al., 2007). HV specify a large number of enzymes for DNA synthesis, processing of proteins, and other functions. The genome synthesis and capsid assembly occurs in the nucleus. Production of infectious progeny results in the destruction of the host cell. All HV are able to remain latent in their hosts (Pellet et al., 2007).

Further, it is a member of the sub-family alphaherpesvirinae, whose type species is also HHV1. Members of the subfamily are classified based on variable host range, short reproductive cycle, lytic infection of cells, and ability to establish latency primarily in sensory ganglia (Pellet et al., 2007). BHV1 is a member of the genus varicellovirus, whose type species is human herpesvirus 3 (HHV3), also known as varicella-zoster virus (VZV). Membership in the genus is based on wide tissue tropism and genome arrangement (Cohen et al., 2007). The \( \alpha \)HV include numerous viruses of veterinary importance, including suid herpesvirus 1 (SHV1, pseudorabies virus, or PRV), equid herpesvirus 1 (EHV1, equine abortion virus), felid herpesvirus 1 (FHV1, feline viral rhinotracheitis, or FVR), and gallid herpesvirus 2 (Marek’s disease virus) (Davison et al., 2005). Such viruses may be studied as \( \alpha \)HV models and for disease control purposes (Mettenleiter, 1996; Pomeranz et al., 2005).
They are characterized by a short reproductive cycle, lytic infection of cells, and establishment of latency primarily in sensory ganglia (Pellet et al., 2007).

BHV1 was first isolated in the United States in 1956 (Madin et al., 1956). Subtypes (1.1, 1.2a, and 1.2b – and formerly including 1.3a and 1.3b, now a separate species, BHV5) were identified by genetic and antigenic analysis (Engels et al., 1981; Misra et al., 1983; Metzler et al., 1985; Brake and Studdert, 1985; Wyler et al., 1989) and were associated with geographic range and prevalence of clinical manifestations (Edwards et al., 1990; van Oirschot et al., 1995; D’Arce et al., 2002).

2.2. Virion structure

2.2.1. Genome

There are six sequence arrangements of the double-stranded DNA genomes of HV, based on the presence and location of repeats of terminal sequences. BHV-1 takes the D form, in which the terminal sequence is repeated in an inverted orientation internally. The genome segment between the repeats (unique short, or U$_S$) exists in two orientations relative to the unique long (U$_L$) segment (Pellet et al., 2007).

The genome of BHV1 was first mapped by Mayfield et al. (1983), and later sequenced by an international consortium (Schwyzer and Ackermann, 1996). The genome maps of BHV1.1 and 1.2 (Mayfield et al., 1983) and BHV5 (Engels et al., 1986) were determined and percent identity of BHV1.1 to BHV1.2 (95%) and BHV1.1 to BHV5 (~85%) calculated.

Seventy-three open reading frames (ORFs) were identified in the 135301 bp genome (Glazov et al., 2010). Of those, 33 were found to be essential to in vitro replication, 36 were not essential, with the status of 2 dual-copy genes inconclusive (Robinson et al., 2008). Most of the genes of BHV1 conform to HHV1 homologs in location, sequence (Whitbeck et al., 1994) and replication requirements (Robinson et al., 2008), but not all. Of the 71 BHV1 proteins, 67 are conserved in each of HHV3 and HHV1 (Davison 2010). Eight of 73 genes (spread among regulatory, capsid, tegument, and membrane proteins) differed from HHV1 in their requirement for in vitro replication (Robinson et al., 2008). Four ORFs are unique to
BHV1 – Circ, UL0.5, UL3.5, and US1.5 (Schwyzer and Ackermann, 1996). Some genes are conserved across all HVs, including DNA polymerase, major capsid protein UL19 (virus protein [VP] 5), tegument protein UL7, and envelope glycoproteins B, H, L, M, and N. Others are conserved at the subfamily level – for αHV, examples include latency-associated genes and glycoprotein D (gD) (Pellet et al., 2007). Genes and products of various HVs were named for positions of their restriction endonuclease fragments on gels, gene position on mapped genomes, sequence of expression or identification, and HHV1 homologue. This can be particularly confusing when the genome position in a virus is not the same as the ‘genome position name’ of the HHV1 homologue.

In addition to the ORFs identified in the BHV1 genome, 10 micro RNA (miRNA) genes have been identified, encoding 12 mature miRNAs with 14 miRNA-encoding loci. Two are located close to the origin of replication.

2.2.2. Core

For HHV1, there is no specific protein coating the DNA in the core. There are polyamines which are suggested to neutralize the DNA for better packing within the capsid (Roizman et al., 2005).

2.2.3. Capsid

The capsid of HHV1 is made up of 162 capsomers with T=16 icosahedral symmetry. The capsid is composed of an outer layer and an intermediate shell, with potential channels between the core and outside of capsid. The outer shell is composed of four proteins. VP5 is the major capsid protein, with 5 copies in each penton capsomere and 6 in each hexon capsomere (Roizman et al., 2007). VP5 is conserved across αHV (Davison 2010) and BHV1. VP5 is an essential gene (Robinson et al., 2008).

2.2.4. Tegument

The space between the envelope and the surface of the capsid is mostly unstructured in HHV1, but contains a variety of viral proteins (Roizman et al., 2007) differentially located in an inner and outer layer. They play a wide variety of roles, from capsid transport during
entry and egress to regulation of transcription, translation, and apoptosis (Kelly et al., 2009). In BHV1, the tegument protein VP8 (UL47) is the most abundant protein in the virion (Carpenter and Misra, 1991) and appears to act as an RNA-transporting protein (Verhagen et al., 2006). VP22 of BHV1 is similar to that of HHV1, but has some differences in cellular localization (Harms et al., 2000, Zheng et al., 2005). VP22 activities include microtubule reorganization and intracellular trafficking. UL41 encodes the viral host shutdown (vhs) protein of BHV1 – it is conserved in αHVs (Muylkens et al., 2007). In other αHVs studied, vhs is an mRNA-specific RNase that triggers rapid shutoff of host cell protein synthesis (Smiley, 2004). It degrades both viral and host mRNA, but the viral mRNA continues to accumulate (Roizman and Taddeo, 2007).

The tegument also contains cellular and viral transcripts (Roizman et al., 2007), as well as non-coding RNA (Amen and Griffiths, 2011). The RNA may be structural, or may code for an immune-regulatory protein as is known for HHV8. miRNAs are also known to be packaged in the virion (Amen and Griffiths, 2011).

2.2.5. Envelope

The αHV envelope consists of a lipid bilayer acquired from host cellular membrane, with virus-encoded proteins imbedded in it (Roizman et al., 2007). Twelve envelope proteins have been described for BHV1 -- 10 are glycosylated, 2 (gN and US9) are not (Jones and Chowdhury, 2007). The 10 glycoproteins (gp) are named gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM (Turin et al., 1999). gB, gC, and gD are considered “major” or more abundant gp, and others (e.g., gE and gH) as ‘minor’ gp (Baranowski et al., 1996). Most gp are homologous in function and structure to those specified by HHV1 but there are clear differences in sequences and roles (Turin et al., 1999). There are striking differences between HHV3 and BHV1 – gE is essential in HHV3, but not BHV1, and gD is essential in BHV1, but not present in VZV (Robinson et al., 2008; Davison, 2010). The gN of HHV1 and SHV1 is glycosylated, whereas the gN is a ‘false gp’ in BHV1 (Muylkens et al., 2007). Glycoprotein complexes of BHV1 were variously named by their positions in PAGE gels, by their molecular weights, by apparent homology with the glycoproteins of other HV including HHV1, and finally in accordance with the homologous HHV1 glycoprotein. The three major
BHV1 gps can serve as examples: gB (named GVP 6/11a/16, 130K/74K/55K, gII, then gB); gC (named GVP 3/9, 180K/91K, gIII, then gC); and gD (named GVP 11b, 150K/77K, gIV, then gD).

2.3. Virus entry

The ‘portals of entry’ for BHV1 are the mucous membranes of the upper respiratory tract, the genital tract, or the conjunctiva (Muylkens et al., 2007). Direct nose-to-nose contact or aerosol over short distances can result in infection (Mars et al., 1999; 2000). Genital infection can result from mating, or via infected semen (Muylkens et al., 2007). It has been proposed that the first cells infected with HHV3 are the epithelium and T cells (Abendroth et al., 2010; Arvin et al., 2010).

Although BHV1 subtypes were associated with different routes of infection, this may have been due to geographical isolation and common transmission. Each subtype will infect by the less-common route (Muylkens et al., 2007), and no difference in tropism was found using ovine respiratory and genital mucosal explants (Steukers et al., 2011). However, it should be noted that an anti-gC MAb was described that failed to react with all genital isolates tested (Rijsewick et al., 1999), and gC in HHV1 and 2 does influence binding properties (Muylkens et al., 2007).

2.4. Infected of the cell

2.4.1. Attachment

Five envelope glycoproteins are involved in HHV1 attachment and entry, as well as fusion of infected cells: gB, gC, gD, gH, and gL (Spear et al., 2000; Rey, 2006). It is believed similar mechanisms apply to all αHV except those lacking gD, e.g., HHV3. gC first binds non-specifically (possibly electrostatically, Cohen et al., 2007) to the host cell membrane glycosaminoglycans. Binding by other glycoproteins (e.g., gB non-specifically to those same receptors, or gD specifically to its receptors) can contribute to binding, and gC is not required for attachment. This is followed by the homodimer gD binding to one of the
three cellular receptors that vary by cell type and species, although they are usually homologous (Connolly et al., 2001). The three types of receptors are: herpesvirus entry mediator A (HveA); members of the nectin family; and 3-O-sulfated heparin sulphate. The use of receptors is specific for each of the closely related varicelloviruses studied (HHV1, HHV2, SHV1, BHV1) (Campadelli-Fiume et al., 2000; Spear, 2004). The use of multiple receptors by any one αHV may be due to the receptors’ presence and absence on various cell types (e.g., epithelium vs. T cells) and their presence on various cell surfaces (e.g., apical for primary infection, tight junctions for cell-to-cell spread) (Spear, 2004).

2.4.2. Fusion with the cell membrane

The differences in gB and gD among the αHV make comparisons difficult. It seems all HVs require gB and gH/gL for entry and cell-cell fusion, and some (including HHV1 and BHV1) also require gD (Atanasiu et al., 2010, Spear et al., 2006). gD of HHV1 has a receptor binding face and a fusion activation face. The nectin 1 and HVEM binding sites are distinct, and the amino- and carboxy-terminal peptides of gD play a role in covering or revealing binding sites (Di Giovine et al., 2011). gD assumes a different conformation in the absence of receptor, bound to HVEM, and bound to nectin-1 (Spear et al., 2006). It is believed that gD-receptor binding results in a displacement of the gD C-terminal region, triggering virus envelope – cell membrane fusion by gB or gH/L (Krummenacher et al., 2005).

‘Lead roles’ have been assigned to each of gB and gH. gB is a homotrimer with fusion domains similar to the vesicular stomatitis virus fusion glycoprotein (Heldwein et al., 2006). Homologues within the HV are highly conserved. A furin protease site is present on almost all gB homologues (including BHV1 gB), but not on HHV1. Because gH/gL did not resemble any documented viral fusion protein at a structural level, Atanasiu et al., (2010) proposed that receptor-activated gD altered gH/gL, which in turn up-regulated the fusogenic potential of gB. Conversely, Roizman et al., (2007) proposed fusion is due to a fusion peptide (Tu and Kim, 2008) and heptad repeats of gH, possibly activated by gB and conformationally-altered gD. In this model gL may block exposure of the repeats if not activated.
2.4.3. **Endocytosis**

Whole HV virions can also be internalized by endocytosis. It is believed the capsid is released into the cytoplasm by envelope fusion with the endosomal vesicle membrane, similarly to envelope-cell membrane fusion (Roizman et al., 2007).

2.4.4. **Capsid transport to the nucleus and entry of DNA**

It has been demonstrated that inner tegument proteins of HHV1 recruit microtubule motors of opposing directions, including the inbound dynein motor complex (Wolfstein et al., 2006; Radtke et al., 2010). The net effect is that after the capsid enters the cytoplasm, it and associated tegument proteins are transported to the nucleus. Other proteins such as VP16 (α-TIF) may localize to the nucleus independently.

In HHV1, VP1-2 is a tegument protein closely associated with the capsid and is likely responsible for DNA release at the nuclear pore. The cellular protein importin B also plays a role (Roizman et al., 2007).

2.4.5. **Transcription**

Host RNA polymerase (pol) II is responsible for viral DNA transcription (Roizman et al., 2007). Genes overlap, and are not spliced (Pellet et al., 2007). Viral gene transcription is temporally regulated, in immediate early (IE, or α), early (E, or β), early/late (leaky late, γ1), and true late (γ2) phases (Seal et al., 1992; Roizman et al., 2007).

IE genes are defined as those transcribed in the absence of de novo viral protein synthesis. In HHV1, IE transcription is induced by the tegument protein alpha gene trans-inducing factor (α-TIF, or VP 16), occurs in the first 2-4 hours after infection, and includes transcripts for six proteins (Roizman et al., 2005). Several of these encode regulatory proteins that stimulate expression of E and late (L) genes (Smiley, 2004), and one (ICP0) is involved in blocking host cell silencing. ICP0 induces a modification of the nuclear domain 10 protein of promyelocytic leukemia (PML) nuclear bodies, disrupting their inhibition of viral replication (Tavalaei and Stamminger, 2009).
In BHV1, α-TIF also stimulates IE gene transcription (Misra 1995), by a different mechanism. Two BHV1 IE transcription units 1 and 2 (IEtu1 and 2) encode: homologues of HHV1 ICP0 and ICP4, plus circ; and ICP22, respectively (Jones and Chowdhury, 2007). bICP0 is a transactivator for all viral promoters and the bICP0 transcript is constitutively expressed during productive infection (Jones and Chowdhury, 2007). bICP0 apparently does not bind to specific DNA sequences, suggesting it activates by interacting with cellular transcriptional machinery (Jones and Chowdhury, 2007). bICP0, 4, and 22 activate E genes.

E or β gene transcription occurs 4-8 hours after HHV1 infection. β gene proteins include enzymes and DNA-binding proteins involved in DNA replication. γ1 genes are only moderately stimulated by DNA replication, and can be difficult to differentiate from β (and from γ2). gB and gD genes are γ1 in HHV1 (Roizman et al., n2007) and in BHV1 the proteins are produced as early as 2-4 hours after infection (before DNA replication) (Baranowski et al., 1996). γ2 genes are defined as having almost no production without DNA replication, and are largely involved in virion assembly. gC is a γ2 protein. Most capsid, tegument, and envelope glycoproteins are encoded by γ genes.

2.4.6. DNA replication

It is believed the circular viral DNA genome serves as a template for a first round of theta replication, starting from origins of replication (ori) sequences. Two such sequences have been identified in each of the repeats flanking the Us of BHV1. The theta replication then switches to a rolling circle mechanism, yielding concatamers. The concatamers are cleaved to unit-length genomes as part of their packaging into capsids (Muylkens et al., 2007).

2.4.7. Capsid assembly

A procapsid is first assembled with the formation of the capsid shell and an internal scaffolding structure. Then, the procapsid is converted into a mature nucleocapsid structure - the internal scaffolding protein is released and replaced by the viral DNA genome and the capsid shell undergoes a major conformational change (Liu and Zhou, 2007, Mettenleiter et al., 2009).
2.4.8. Egress and envelopment

There are two models for how nucleocapsids acquire a tegument and envelope: the single nuclear envelopment model, involving a nuclear tegument and a nuclear membrane envelope; and the double envelopment model, with the final virion containing a cytoplasmic tegument and golgi membrane envelope (Campadelli-Fiume, 2007). The double envelopment model is currently most cited. It holds that capsids are first enveloped by budding at the inner leaflet of the nuclear membrane, with translocation of capsids into the cytoplasm by primary envelope fusion with the outer leaflet of nuclear membrane. Select tegument proteins (inner tegument) associate with the capsid, while others (outer tegument) associate with the cellular membrane (Mettenleiter, 2006). Final envelopment occurs by budding into vesicles of the trans-Golgi, and mature virions are released after fusion of the vesicles with the plasma membrane (Mettenleiter, 2002).

2.5. Dissemination in the host

Intracellular BHV1 virions were detected at 10 hours post-infection, with transmission to adjacent cells occurring at that time (Babiuk et al., 1975). gE (Rebordosa et al., 1996), gI, and gG are important to cell-to-cell spread of HHV1. gD, gB, and gH/L are required for cell to cell spread by BHV1, with gE and gG contributing (Muylkens et al., 2007). It has been noted that microvesicles secreted by HHV1-infected cells (L-bodies) contain tegument proteins that can prepare cells for infection (Meckes and Raab-Traub, 2011).

Extracellular BHV1 virions were detected at 12-13 hours post-infection (Babiuk et al., 1975), which would allow infection of adjacent and non-adjacent cells. The virus may spread by viremia, leading to e.g., abortion or systemic disease. Viremia may be cell-free (Kaashoek et al., 1996a), but is more likely via infected lymphocytes (Nandi et al., 2009).
2.6. Latency

Neurons of the peripheral nervous system are infected by cell-to-cell spread (Jones and Chowdhury, 2007). In a BHV1 respiratory infection this involves the trigeminal ganglia (TG), usually only first order neurons. BHV1 does not invade the central nervous system via the olfactory pathway as BHV5 does, due to differences in gE (Al-Mubarak et al., 2004, Chowdhury 2006). High levels of virus gene expression and infectious virus are detected in the TG 1-6 days after infection (Jones and Chowdhury, 2007). Then lytic gene expression and infectious virus levels drop, but viral genomes can be detected, and latency-related (LR) and ORF-E transcripts are produced at high levels. LR transcripts are detected early after neuron infection (Devireddy and Jones, 1999) and may have a role in determining the outcome of neuronal infection (Jones and Chowdhury, 2007).

BHV1 LR gene products inhibit cell proliferation, bICP0 RNA expression, and apoptosis (Lovato et al., 2003; Jones et al., 2006). BHV1 LR protein appears to prevent cell cycle progression in neurons, with enhanced survival of infected neurons (Schang et al., 1996). The LR gene is antisense to bICP0, which is a transactivator for all viral promoters. Expression of the BHV1 LR gene alone promotes survival in cell cultures stimulated to enter programmed cell death (Ciacci-Zanella et al., 1999). The LR gene contains two ORFs, and the LR-RNA is alternatively spliced in TG at day 7 (the transition to latency). The alternate transcript codes for a fusion of one ORF and part of the other. The resulting protein binds two host cell proteins that can induce apoptosis, including Bid. It also binds an ‘enhancer-binding protein’ (C/EBP-α), which stimulates lytic gene transcription in other HVs. ORF-E is a small ORF within the LR promoter (and antisense to LR). It may maintain neuron function after infection (Jones and Chowdhury, 2007).

In HHV1, miRNAs are expressed during latency that target ICP0 and ICP4, lytic genes (Boss and Renne, 2010). One of the 12 mature miRNAs encoded in BHV1 is antisense to the LR gene (Glazov et al., 2010).

BHV1 latency may also be established in cells of lymphoid origin. BHV1 DNA has been detected in lymphoid tissues when infectious virus was undetectable (Jones and
Upon reactivation, bICP0 expression is stimulated, likely due to host entities (E2F family members) acting on early promoters (Workman and Jones, 2010). LR and ORF-E expression drop, and other (lytic) gene expression is readily detected (Jones and Chowdhury, 2007). Dexamethasone treatment can trigger reactivation. It stimulates expression of cellular transcription factor C/EBP-α cited above, which interacts with the early promoter of bICP0 (Workman and Jones, 2010). Upon reactivation, aHV can spread from the infected neuron to adjacent cells at the axon synapse and along the axon’s length (Tomishima and Enquist, 2002).

2.7. Transmission from the host

Virus is excreted from the host for 7-10 days after infection (Jones and Chowdhury, 2007), with some reports of 10-17 days with $10^{10}$ TCID$_{50}$ (Straub, 1990). Nose-to-nose contact, aerosol, breeding contact with infected prepuce or vaginal epithelium, artificial insemination with infected semen, and even mechanical transmission by ticks has been reported (Straub, 1990).

2.8. Consequences of infection

Diseases caused by BHV1 include infectious bovine rhinotracheitis (IBR, McKercher et al., 1955), conjunctivitis (McKercher et al, 1959), infectious pustular vulvovaginitis (Kendrick et al., 1958), infectious pustular balanoposthitis (Huck et al., 1971), and abortion (Ormsbee, 1963) in adult cattle, as well as encephalitis (French, 1962a, 1962b), enteritis (Gratzek et al., 1966), and generalized disease (Van Kruiningen and Bartholomew, 1964) in calves. BHV1 is also a significant initiator of and contributor to “shipping fever” pneumonia (Yates, 1982). It does this by increasing susceptibility to secondary bacterial infection through injury to the respiratory tract, as well as local and more generalized immunosuppression (McChesney and Oldstone 1987; Tikoo et al., 1995a; Babiuk et al., 1996). Some experiments have measured specific immunosuppressive effects relative to secondary bacteria. BHV1 depressed blastogenic responses to Pasteurella multocida, and
neutrophil chemotactic response, although the antibacterial activity of the neutrophils was not significantly affected (Filion et al., 1983). Bovine respiratory disease complex has been estimated to cost the US cattle industry $3 billion annually (Jones and Chowdhury, 2007). The cost of BHV1 disease led to an expensive national BHV1 eradication program in Switzerland using a (serology) test-and-remove strategy (van Drunen Littel-van den Hurk, 2006).

3. The Bovine Immune Response to BHV1

The most studied mammalian immune systems are those of mice and humans. Aspects have been studied in other species due to zoonotic diseases, the species’ economic importance, as disease models, or to discern origins or commonalities. Some features appear to be fundamental and are conserved among mammals (Hirano et al., 2011), allowing useful generalizations or extrapolations. However, there are also differences in strategies (e.g., for generation of diversity of lymphocyte antigen receptors and immunoglobulins), and differences in members, sequences and so possibly modes of action (e.g., interleukins) between orders and species.

It has been noted that “Cattle specific evolutionary breakpoint regions have a higher density of species-specific variations in genes having to do with lactation and immune responsiveness” (Bovine Genome Sequencing and Analysis Consortium et al., 2009). Investigations of bovine-specific immune phenomena have been hampered by a lack of reagents (Rouse and Babiuk, 1978; Boysen et al., 2006), which is being addressed by the U.S. Veterinary Immune Reagent Network and the European ‘Immunological Toolbox’ (Entrican et al., 2009). The interactions of stress, nutrition, and fertility with the innate and adaptive immune systems are important for cattle (Lippolis, 2008). The innate immune system (particularly phagocytic cell function), is susceptible to stress and nutrition impacts in cattle (Roth and Perino, 1998), and stress including social factors may impact their adaptive immune system (Salak-Johnson and McGlone, 2007).

Most of what is known about immunity to αHV was first elucidated in the HHV1-mouse system, and then confirmed or expanded in HHV1/2-human and other systems, e.g.,
SHV1-mouse or -swine. Studies of beta- and gamma-HV (\(\beta\)HV and \(\gamma\)HV) have also been instructive, but revealed differences in viral strategies, e.g., \(\gamma\)HV employ more molecular mimickry than do \(\alpha\)HVs.

In most cases, cattle are able to overcome a primary BHV1 infection, so the primary immune response provides valuable information for primary, secondary, and passive immunity. The subject has been well reviewed at intervals (Rouse and Babiuk, 1978; Wyler et al., 1989; Tikoo et al., 1995a; Babiuk et al., 1996; Engels and Ackermann, 1996; Muylkens et al., 2007). Briefly, the first insult results in interaction with non-specific soluble factors (constitutive and induced), which recruit non-specific immune cells to the site and activate them. These immune cells secrete more cytokines, kill virus-infected cells, and bridge to the adaptive response, including by presenting antigen to lymphocytes. Starting at day 5 and peaking day 7-10, helper T cells activate macrophages and NK cells, and promote the proliferation of specific CTLs. Finally, beginning at day 10 and peaking after the infection is largely resolved, neutralizing and other antibodies are detectable. They likely help with clearing extracellular virus and with cellular cytotoxicity. They can then protect the host from reinfection (by reactivation or another exposure), and can protect the neonate via colostrum. The main immune response to the virus and virus-infected cells is to the viral envelope glycoproteins gB, gC, and gD.

3.1. Non-immune barriers

The organism can protect itself from infection through avoidance of infected cohorts or materials (Medzhitov et al., 2012). Mucous and cilia on epithelia, enzymes in tears and saliva, and tight junctions between epithelial cells protect the host from infection (Roth and Perino, 1998; Keele and Estes, 2011). The host must also have the correct receptors to be infected by a virus – i.e., humans are not infected by many non-human animal or plant viruses (Mayer 2012). Once infected or colonized, the host may tolerate the foreign organism (Medzhitov et al., 2012). Non-specific components of inflammation such as fever and the low pH of infiltrates may hamper viral infection (Mayer 2011). Intracellular repression, i.e., cellular silencing of transcription (Roizman et al., 2005), stress-induced shut-
down of translation (Buchkovich et al., 2008), are additional, non-immune responses to infection.

3.2. Innate immune system

The first response to viral infection involves the innate immune system, which is able to recognize and resist or kill foreign organisms. Should the infection continue, the innate response will have primed the more powerful adaptive response (Iwasaki and Medzhitov, 2010; Shetnten and Medzhitov, 2011), which in turn uses many of the tools in the innate system. Innate cells and adaptive cells have a complex interaction in αHV infections (Schuster et al., 2011).

3.2.1. Infected Cells

Infection of non-immune (e.g., mucosal epithelial) cells triggers molecular signals for the infected and neighboring cells, including interferons. Many of the same triggers and signals are used in innate immune cells. Pathogens express signature molecules, known as pathogen-associated molecular patterns (PAMPs), essential to their survival and pathogenicity (Kawai and Akira, 2006; Meylan and Tschopp, 2006; Kumar et al., 2011). These are recognized by conserved, germline-encoded host sensors known as pathogen recognition receptors (PRRs). Several families of PRRs are known to play a role in host defense, including toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytosolic DNA receptors (Kumar et al., 2011).

The distribution of TLRs that recognize the PAMPs of herpesviruses varies by cell type and by species (Carty and Bowie, 2010; Paludan et al., 2011). Bovine TLRs have been sequenced, revealing 66-86% nucleotide or amino acid sequence identity with their human/murine homologues (Werling et al., 2006). Natural TLR variants enhance the risk of severe infections in cattle (Seabury et al., 2010). Four TLRs have been shown to play a role in HSV resistance in mice – 2/6 heterodimer, 3, and 9 – they act through MyD88 (Chew et al., 2009). TLR2 in association with TLR1 (Paludan et al., 2011) or TLR6 (Chew et al., 2009).
2009) recognizes glycoproteins upon attachment. Once the capsid is internalized, viral ds DNA is recognized by TLR9 in the endosome. When the viral DNA is transcribed, higher order (ds, stem-loop) RNA molecules are recognized by TLR3 (Paludan et al., 2011). When activated, the TLRs induce different signaling cascades depending on the adaptor protein, ultimately leading to the activation of the transcription factors NF-κB, AP-1, and interferon-regulatory factor (IRF)-3 (Martinon et al., 2009). HHV1 infection results in MyD88-dependent and TRIF-dependent signaling (Vandevenne et al., 2010). TLR activation results in the production of antimicrobial peptides, inflammatory cytokines and chemokines, tumor necrosis factor (TNF)-α, and costimulatory and adhesion molecules, as well as in the upregulation of major histocompatibility complexes (MHCs) (Martinon et al., 2009).

Two RNA helicases, RIG-I and MDA5, were identified as cytoplasmic, viral RNA sensors (Martinon et al., 2009). The higher order RNA molecules produced after viral transcription are also recognized by RIG-I (products of Pol III) or MDA5 (replication intermediate) in the cytoplasm (Paludan et al., 2011). Upon viral stimulation of the two RLRs, NF-κB and IRF3/7 are activated and, in turn, induce the transcription of type I interferon (IFN).

NLRs are categorized in subfamilies and variably distributed on innate immune cells and epithelia. HHV1 is believed to trigger NALP3 (Chew et al., 2009). NLRs signal through caspases (Martinon et al., 2009). HHV1 viral dsDNA is recognized in the cytoplasm by DNA-dependent activator of IFN-regulatory factors (DAI) (Paludan et al., 2011), which results in induction of type I IFN and other genes involved in innate immunity (Takaoka et al., 2007).

3.2.2. Type I interferons

The interferon (IFN) family of cytokines is grouped into 3 types, I, II, and III. There are five human type I IFN: IFN-α (13 subtypes); -β; -ε; -κ; and –ω. There is one type II IFN, IFN-γ, and three type III (lambda) IFN, IFN-λ1–3 or IL-28A/B and IL-29. Type I and III IFNs are expressed in many cell types but II is expressed in NK and T cells (Paladino and Mossman, 2009).
Bovine IFN-α class 1 (10-12 members) and class 2 (15-20 members) were identified – each class shows greater sequence homology with their human homologues than with the other bovine class (Ohmann et al., 1987). Five bovine IFN-β genes were identified, unlike the one in humans. The bovine IFN-γ is encoded by one gene with introns, similar to other species (Ohmann et al., 1987). A bovine type III IFN (bovine IFN-λ3) was identified and characterized, including characterization of its anti-viral activity (Segundo et al., 2011). The receptor for IFN-λ (IL-28Rα) is expressed by a limited range of cells, but includes epithelium, so mucosal epithelium can respond (Perez-Martin et al., 2012).

Type I IFN (IFNα or β) induces resistance to viral infection, increases MHC-I expression and antigen presentation, activates dendritic cells and macrophages (Mph), and activates natural killer (NK) cells to kill virus-infected cells (Murphy et al., 2008). IFNβ signals result in production of IFNα subspecies and other interferon-stimulated genes (ISG) including IRF-7. IRF-7 activation results in up-regulation of IFN type 1 and in a full range of ISG. IFN λ stimulation has much the same effect, but in a more limited set of cells (Perez-Martin et al., 2012).

In BHV1 infection type 1 IFN is present within five hours post-infection. It is induced in the infected cell and cells recruited to the site, and reaches peak levels in nasal secretions and blood by 36-72 h post infection. Type I IFN levels remain elevated until virus replication ceases (Babiuk et al., 1996). IFN-α regulates polymorphonuclear neutrophils (PMN), NK, and Mph effector activities and influences T-cell trafficking (Tikoo et al., 1995a). Locally induced IFN after aerosol BHV1 infection was reported as providing partial protection from a second infection with BHV1 or other viruses (MacLachlan and Rosenquist, 1982; Cummins and Rosenquist 1982; 1984; Ohmann et al., 1987). Intranasal and intramuscular treatment with recombinant bovine IFN-α1 reduced clinical signs but not virus shedding of BHV1 (Babiuk et al., 1987a). Correlation of IFN genotype and BHV1 infection clinical outcome has been demonstrated (Ryan and Womack, 1997).

Six HHV1 proteins inhibit IFN expression or action - ICP0, ICP27, ICP34.5, US11, vhx, and US3 (Paladino and Mossman, 2009). HHV1 ICP0 blocks IFN regulatory factor 3 (IRF-3) and prevents IFNβ transcription. BHV1 ICP0 inhibits IFN signaling by reducing
IRF-3 protein levels, likely through degradation (Sairi et al., 2007). This leads to reduced IFN-β promoter activity. In addition, bICP0 inhibits the ability of IRF7 to activate IFN-β promoter activity (but does not reduce IFR7 protein levels) (Jones and Chowdhury, 2007; Jones, 2009).

3.2.3. Interleukins and TNF-α

Bovine interleukins (IL) and TNF-α homologous to human and murine members have been described, with varying degrees of sequence similarity. These include: IL 1α and 1β (Maliszewski et al., 1988); IL-2 (Cerretti et al., 1986), IL-6 (Droogmans et al., 1992), IL-7 (Cludts et al., 1992), IL-10 (Hash et al., 1994), IL-12 (Zarlenga et al., 1995), IL-18 (Shoda et al., 1999), and TNF-α (Cludts et al., 1993). Their functions appear to be similar to the human/murine homologues, as measured by response to similar stimuli (White et al., 2002). The major pro-inflammatory cytokines that are responsible for early responses are IL1-α, IL1-β, IL6, and TNF-α. The balance of these with anti-inflammatory cytokines (for example IL4, IL10) determines the status of the inflammation.

In BHV1 infection, pro-inflammatory cytokines (produced by infected cells and Mph) cause an influx of PMNs, and induce ICAM-1 on epithelial cells, so leukocytes adhere to them. With increased vascular permeability, immune cells migrate to site of infection. IL-1 and IL-6 stimulate GM-CSF production, contribute to Mph differentiation, and prime Mph to release other molecules such as TNF-α (Babiuk et al., 1996). IL-2 supports the growth and differentiation of antigen-activated T cells. IL1b and IL-2 have each been shown to enhance anti-BHV1 responses when administered to infected calves (Turin et al., 1999).

3.2.4. Chemokines

Chemokines are a family of low molecular weight chemoattractant cytokines. Chemokine expression may result in monocyte or lymphocyte (LC) homing to the site of infection, where the cells can differentiate or be activated. Bovine chemokines and chemokine receptors (homologues to human members) have been identified and similarities but also differences noted (Son and Roby, 2006; Widdison and et al., 2010; Widdison and
Coffey, 2011). BHV1 gG is a chemokine-binding protein, blocking activity (Bryant et al., 2003) and preventing LC homing (Jones and Chowdhury, 2007).

3.2.5. Complement

The complement (C) system is well conserved across vertebrates (Zhu et al., 2005), although the bovine C5a receptor of cattle has differences from that of the human or mouse (Nemali et al., 2008). The C cascade can be activated by three pathways – alternate (spontaneous), lectin, and classical (antibody). The latter will be discussed with the adaptive immune system. Complement can neutralize virus particles either by direct lysis or by preventing viral penetration of host cells. HHV1-infected cells are killed by direct C lysis (Ohmann and Babiuk, 1988). BHV1 infected cells were killed by complement-dependent neutrophil mediated cytotoxicity (CDNC, Ohmann and Babiuk, 1988).

Cells infected with BHV1 (and HHV1) express gC on the cell surface, which can function as a receptor for the cleavage product of C3b (Ohmann and Babiuk, 1988; Favoreel et al., 2003). It has been proposed that CDNC is due to cross-linking of C3b between the viral gC on the virus-infected cell and the receptor on the PMN (Babiuk et al., 1996). The C3b receptor has also been proposed to prevent C action on the virus or the infected cell (Muylkens et al., 2007). In addition, it has been suggested SHV1 incorporates host complement regulators (to regulate the spontaneous activation of the alternate pathway) in its viral envelope (Favoreel et al., 2003).

3.2.6. Macrophages, neutrophils, and plasmacytoid dendritic cells

Innate immune system cells include phagocytic and other cells which express PRR that can recognize PAMPs. They do not have memory, but can be primed in some cases. Included in this category are Mph, PMN, and dendritic cells (DC).

Mph have TLRs, scavenger receptors, and other PPRs on their surfaces, and engulf extracellular pathogens. They are important in BHV1 infection, as shown by transfer experiments (Rouse and Babiuk, 1977). Early in the infection (3-4 days p.i.) they are a primary contributor of IFN-α production, believed to be important in limiting viral spread (Tikoo et al., 1995a). Later they are stimulated by IFN-γ from T cells to kill virus-infected
cells in a non-MHC restricted way (Campos et al., 1989, Babiuk et al., 1996). The activity is detectable as early as 2 days after infection in lung parenchymal cells and 5-7 days after infection in peripheral blood (Tikoo et al., 1995a). BHV1 infects Mph, interfering with functions (Roth and Perino, 1998) such as TNF and other cytokine production, and participation in ADCC (Tikoo et al., 1995a). BHV1 infection of peripheral blood mononuclear cells (PBMC) leads to their apoptosis (Muylkens et al., 2007). Epitopes on gC are similar to that of an Mph receptor, suggesting immune evasion through molecular mimickry (Fitzpatrick et al., 1990).

Neutrophils have PRRs and C receptors, and are the principle cell engulfing pathogens (Murphy et al., 2008). It was observed that neutrophils prevented BHV1 plaque growth without Ab, in a way that did not require contact (Rouse and Babiuk, 1977). PMNs were the most effective cells in ADCC assays, destroying infected cells more quickly and completely, with less antiserum (Grewal et al., 1977). BHV1 interferes with lung PMN activities (Roth and Perino, 1998; Muylkens et al., 2007), and PMNs from BHV-1 infected animals had reduced anti-bacterial functions such as reduced chemotactic and phagocytic capacity (Tikoo et al., 1995a). Epitopes on gC also cross-react with epitopes on PMNs, again suggesting immune evasion through molecular mimickry (Fitzpatrick et al., 1990).

Plasmacytoid DCs (pDCs) express TLR7 and TLR9 in endosomes, with which they sense viral nucleic acids (Gilliet et al., 2008). They internalize antigen (including by means of FcγIIα, Lanzavecchia and Sallusto, 2007) and rapidly produce large amounts of type I IFNs when stimulated (Barchet et al., 2005). pDCs produce 1000 times the IFNI of other cells, and when stimulated can produce TNF-α and (in mice) IL-12, and can present antigen. So they are key bridges from the innate immune response to the adaptive one (Reizis et al., 2011). pDCs have been identified in cattle - they generated high levels of type-I IFN in response to the TLR-9 agonist CpG (Reid et al., 2011). pDCs have been described as the ‘professional producers’ of IFNI in response to all HVs tested (Baranek et al., 2009).

3.2.7. Natural killer cells

NK cells are derived from a common lymphoid progenitor with T cells and B cells, but have been categorized as an innate immunity cell. They mediate cytotoxicity as CTL do...
(by degraluating), but the killing is not MHC-restricted. Cytotoxic granules are released onto the surface of the bound target cell, and the granule contents (perforin and granzymes) penetrate the cell membrane and induce programmed cell death. NK cells have multiple receptor types – killer lectin-like receptors (KLRs), killer cell immunoglobulin-like receptors (KIRs), and natural cytotoxicity receptors (NCRs) (Murphy et al., 2008). NK cells can undergo a clonal-like expansion following virus infection in humans and mice and previously primed NK cells can mediate secondary memory responses in mice in spite of not having RAG- recombinase-dependent clonal antigen receptors (Sun et al., 2011, Paust and von Andrian, 2011).

Bovine NK cells have been identified as constitutively expressing homologues of the human NK receptors NKp46, CD244, and CD94, and the granule proteins granulysin and perforin (Endsley et al., 2006). Multiple receptors have been identified on NKp46 (CD335) expressing, CD3(-) lymphocytes, including multiple KIRs and a single Ly49 (Boysen and Storset, 2009). NK cells produce IFN-γ (Boysen and Storset, 2009). Two sub populations (CD2+ and CD2-) were distinguished, both cytotoxic, both producing IFN-γ and transcripts for KIR, CD16, CD94 and KLRJ (Boysen et al., 2006).

NK-like cells (CD-2+, CD4-, CD8-) were stimulated by cytokines to kill BHV1 infected cells without MHC restriction (Babiuk et al., 1996). NK killing was dependent on antigen expression, with gB and gD being primary targets and gC of lower importance (Babiuk et al., 1996). NK cells scan host cells for both stimulatory and inhibitory signals. The reduction in MHC production many αHV cause should increase NK targeting. Some HV target both signals for reduction using miRNAs, but this activity is not among those listed for αHV when summarized by Griffin et al., (2010). Some αHVs do internalize gB, which should reduce NK targeting (Deruelle and Favoreel, 2011). Blocking of apoptosis of cells infected by BHV1 and other αHVs is described in the section on CTLs.

3.2.8. Interferon gamma (IFN-γ)

This cytokine is produced predominantly by NK and natural killer T (NK T) cells as part of the innate immune response, and by Th1 CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells as part of the adaptive immune response (Schoenborn and Wilson,
IL12 produced by antigen presenting cells stimulates NK and T cells to produce IFN-\(\gamma\) (Jaime-Ramirez et al., 2011). The bovine IFN-\(\gamma\) is encoded similarly to other species (Ohmann et al., 1987).

Type II IFN is involved in the immune response to HHV1 (Paladino and Mossman, 2009). It is ‘a predominant response after BHV-1 infection’ (Campos et al., 1989) and is necessary for the activation of non-MHC restricted cytotoxic activities mediated by Mph. HHV1 US3 modifies the IFN-\(\gamma\) receptor post-transcriptionally, resulting in inhibition of ISG induction (Paladino and Mossman, 2009).

3.3. Innate-like intermediates

Four innate-adaptive evolutionary intermediates have been described for humans and mice: \(\gamma\delta\) T cells, B-1 cells, NK T cells, and natural antibodies (Murphy et al., 2008).

In the human and mouse, those T cells expressing \(\alpha\beta\) and \(\gamma\delta\) T-cell receptors (TCRs) are said to perform non-overlapping roles in the immune response. \(\alpha\beta\) T cells are located primarily in secondary lymphoid organs, recognize peptides in association with class I and II MHCs, and respond by facilitating the production of antibody or by lysing infected target cells. \(\gamma\delta\) T cells represent a small percent of cells in the thymus and secondary lymph tissue, are abundant at epithelial surfaces and use fewer gene segments (to encode the TCR) to recognize a wider variety of antigens, including non-classical MHCs, heat shock proteins, and lipids (Lee et al., 2010). Some \(\gamma\delta\) T cells appear to recognize antigen without presentation (Murphy et al., 2008). The \(\gamma\delta\) T cells in bovine have different characteristics, which are described in the adaptive immune response section.

B-1 cells are a separate lineage of B cells (distinct from conventional, or B-2 cells) that produce large quantities of multi-reactive IgM, IgG3 and IgA (natural antibodies) (Tarakhovsky, 1997; Hardy, 1992). Such CD5+ cells are found in various proportions and locations by species, and CD5 expression in cattle may represent activation (Haas and Estes, 2001). Naessens (1997) suggested all bovine B cells are of the B-1 lineage because they lack IgD.
NK T cells express TCRs using one invariant α chain, paired with one of a few β chains, and produce cytokines rapidly (Murphy et al., 2008). It has been posited that cattle don’t have NK T cells based on their lack of a functional CD1d gene and a failure to react to a potent NK T stimulus (Van Rijhn et al., 2006).

3.4. Adaptive immune response

The adaptive immune response is characterized by: 1) the specificity of T and B lymphocyte receptors due to gene segment rearrangement and assembly, mutation, and clonal selection; and 2) the memory of the response (Bonilla and Oettgen, 2010). B cells recognize surface epitopes with the immunoglobulin B-cell receptor (BCR). T cells, by means of the TCR, recognize peptides that are the products of protein breakdown in another cell and displayed on that cell’s surface in a complex with an MHC (Murphy et al., 2008). The adaptive response is commonly described as having two ‘arms’, cell-mediated and humoral, enabled by T-helper 1 and 2 responses, respectively. The involvement of T cells in both ‘arms’ means the adaptive response is “MHC-restricted”, unlike the innate response.

MHC restriction describes the phenomena of T cells only being stimulated by peptides bound to ‘self’ MHC. They only kill infected cells with the same MHC type I, or proliferate when presented with antigen by cells of the same MHC type II. This has been demonstrated in cattle using multiple viral systems, including studies of genetic variation in strength and character of immune response to pathogens, and determination of key amino acids in MHC binding pockets for vaccine design (Collen and Morrison, 2000; Glass, 2004; Gerner et al., 2009; Baxter et al., 2009, Glass et al., 2012).

However, for lymphocytes to proliferate, become effector cells, and generate memory cells, a ‘second signal’ beyond antigen recognition by BCR or TCR is needed, such as binding by a co-receptor and stimulation by cytokine. A third signal is also proposed to be needed for efficient stimulation.

The bovine response to BHV1 is balanced, including generation of cytotoxic T-cells (CTLs) and neutralizing antibodies (Ab). Ab are considered important in the prevention of
BHV1 (re-) infection, CTLs for virus clearing of and recovery from an infection (Babiuk et al., 1996).

3.4.1. Antigen Presenting Cells

Dendritic cells, Mph, and B-cells can serve as antigen-presenting cells (APC), because in addition to presenting antigen peptides on MHC I or II, when activated during an infection they express the co-stimulatory molecules needed to activate T cells (Renjifo et al., 1999; Murphy et al., 2008). They migrate to the local draining lymph node to do so. DCs have the unique ability to sensitize (prime) naïve T cells. Mph and B cells present engulfed and soluble Ag (respectively) to already-primed effector T cells (Murphy et al., 2008).

Conventional DCs (cDCs) are so named to differentiate them from plasmacytoid DCs (pDCs), which have a different origin and distribution in tissues. cDCs, also known as myeloid DCs, include migratory cells and lymphoid-resident cells (Freer and Matteucci, 2009). cDCs: 1) have specialized mechanisms for Ag capture and processing; 2) migrate to defined sites in lymphoid organs to initiate immunity; and 3) rapidly mature in response to a variety of microbial and other (e.g., cytokines produced by innate immune cells) stimuli (Steinman and Hemmi, 2006). After activation, cDCs produce IL-12 and IL-15 that stimulate IFN-γ secretion by NK cells, and promote differentiation of CD4+ and CD8+ T cells (Lambotin et al., 2010). So, they serve as a major link between innate and adaptive immunity. cDCs are continuously produced and positioned at the skin, mucosal surfaces and the blood, so they are likely to rapidly encounter and be activated by invading pathogens (Murphy et al., 2008). cDCs can be infected by viruses themselves, can phagocytose infected cells, or can micropinocytose antigen. Migrating cDCs may also transfer antigen to lymph-node resident DCs (Murphy et al., 2008; Singh and Cresswell, 2010).

They are equipped with a set of varied PPRs, such as TLRs in the endosome and MDA and RIG-I in the cytosol. Damage-associated molecular patterns (DAMPs) may also activate immature DCs (Nace et al., 2012). Stimulation changes the chemokine receptors on the cDC, which in turn results in their ability to migrate to the peripheral lymphoid tissue to activate naïve T cells (Murphy et al., 2008). Activated DCs also present many peptide-MHC
complexes and co-stimulatory molecules, such as B7.1 (CD80) or B7.2 (CD86), for which T-cells express complementary CDs (e.g., CD28) (Murphy et al., 2008).

cDCs comprise 2 main subsets: CD8-, which are efficient at presenting exogenous Ag on MHCII to CD4+; and CD8+, which present Ag on MHCII to CD8+ cells (Reizis et al., 2011). Presentation to naïve CD8+ Tcells is known as cross-priming, and presentation to stimulated ones is known as cross-presentation (Singh and Cresswell, 2010). Cross-presentation is important for the response to viruses that don’t infect APCs directly. The dominant mechanism for cross-presentation is translocation of Ags to the cytosol, where proteosomal degradation generates peptides, which are then transported via the TAP and bind to newly synthesized MHCI (Singh and Cresswell, 2010). DCs can also regulate T cell differentiation with interleukins (Freer and Matteucci, 2009). cDCs produce IL6, IL-8, IL-12, and TNFa (Murphy et al., 2008). DCs performed better than monocytes as APC for BHV1 (measured by stimulation of T-cell proliferation in vitro). The DCs were not BHV1-infected (Renjifo et al., 1999).

Macrophages from BHV1 infected cattle were shown to express increased levels of MHC II (Tikoo et al., 1995a), and antigen presentation by bovine alveolar macrophages were shown to stimulate proliferation of T cells in vitro. Bovine alveolar Mph and monocytes are permissive to BHV1 infection (Renjifo et al., 1999), resulting in the impairments described in the innate immunity section.

B-cells can internalize antigen bound to the BCR, and process it in the endosome (triggering TL7 and TLR9, a third signal for the B cells), leading to presentation of antigen on MHC II (Lanzavecchia and Sallusto, 2007).

3.4.2. Lymphocytes

Lymphocytes are the effector cells of the adaptive immune system. Study of leukocyte differentiation molecules has shown that many of those identified in humans and mice (e.g., CD2, 3, 4, 8) are highly conserved in structure and function across mammalian species (Davis and Hamilton, 1998).
3.4.3. T lymphocytes

T cell receptors are constituted of two chains, each of which is coded by recombined gene segments (resulting in high diversity). The gene segments are variable (V), junction (J), diversity (D), and constant (C). The proteins are made by recombination of VJC (α and γ chains) and VDJC (β and δ chains) genes (Murphy et al., 2008). Nucleotide deletion and substitution at the V(D)J junction by exonuclease and terminal deoxynucleotide transferase activity increases the diversity achieved during recombination. Consequentially much of the variability is focused in the complementarity determining region (CDR) 3, encoded by the V(D)J junction (Connelley et al., 2008). The CDR3s of both chains are central in the binding site and central to antigen recognition (Murphy et al., 2008).

In humans (and mice) most TCR are α–β. There are 40-70 variable α or β gene segments, many J segments, and the D gene for the β chain is frequently read in three frames. The pairing, junctional, and P/N nucleotide diversity together lead to a diversity of $10^{18}$ (Murphy et al., 2008). As has been noted, the contribution of γδ TCR to TCR diversity in humans is minimal.

For cattle it was assumed that the high levels of γδ diversity observed meant αβ diversity was likely to be low, but this appears not to be the case. Over 400 genes have been observed in the α–δ locus (Reinink and Van Rhijn, 2009). Further, 48 functional Vβ genes of 17 subfamilies were identified. Clonal expansions were distributed over a large number of Vβ subfamilies, although a limited number of clonotypes dominated the response (Connelley et al., 2008).

3.4.4. Bovine γδ T cells

Unlike in humans and mice, γδTs are a major population of T cells in cattle, particularly in calves, where WC1+ γδTs can represent 50% of peripheral blood leukocytes (PBLs) (MacHugh et al., 1997). There is more gene diversity (VDJC γ; VJC δ) in ruminants and some other species than in mice and humans (Reinink and Van Rhijn, 2009), and multiple γ genes are used (Guzman et al., 2012). γδTCRs interact with non-classical MHCs
in mice and humans – it is believed unlikely that γδ TCR interact with classical MHC in cattle (Reinick and Van Rhijn, 2009).

Two populations of γδ Ts have been found (MacHugh et al., 1997) – WC1+, CD2-, CD4-, CD8-, and WC1-, CD2+ CD8+/- . WC1 is encoded by a large, multi-gene family, part of the group B scavenger receptor cysteine rich (SRCR) superfamily (Herzig and Baldwin, 2009; Herzig et al., 2010). WC1+, CD2-, CD4-, CD8- cells are present in peripheral blood, marginal zones of the spleen, dermal and epidermal layers of the skin and lamina propria of the gut. The majority of WC1-, CD2+ CD8+/- cells are localized in the red pulp of the spleen. The two populations may use different families of TCR genes (MacHugh et al., 1997). A population of WC1+ γδ T cells increased expression of MHCII, processed antigen, and demonstrated NK cell-like killing in response to infection with foot and mouth disease (Toka et al., 2011). A large population of CD8+ T cells in cattle are γδ T cells (MacHugh et al., 1997), and a subset of CD8+ γδTs home to mucosal tissues due to selective expression of adhesion molecules and chemokine receptors (Wilson et al., 2002).

PAMPs prime bovine γδ T cells, as observed by an increase in receptors in the absence of IFN-γ secretion (Jutila et al., 2008). A population of peripheral blood γδ T cells increased rapidly upon inoculation with or exposure to BHV1 (Amadori et al., 1995). Vaccination with one dose of modified live BHV1 generated γδ (as well as CD4 and CD8) T cells in the peripheral blood of cattle that became activated in response to live BHV-1 in culture (using CD25 as a marker) (Endsley et al., 2002). Of two populations of bovine γδ T cells studied (CD2- and CD2+), one (CD2-/D62L+) was reduced after vaccination with product containing inactivated BHV1 and other viruses (Vesosky et al., 2003).

3.4.5. CD8, CD4 and T cell types
Double-positive thymocytes that have been positively selected develop into either CD4+ or CD8+ T cells, as determined by the MHC-restriction specificity of their TCR (Singer et al., 2008). CD8+ cells become cytotoxic T cells. CD4+ cells can differentiate into T-helper 1 (Th1) or T-helper 2 (Th2) (or T17 or T regulatory [Treg]) cells (Murphy et al., 2008). IL-12, IL-18, TNF-α and IFN-α are associated with skewing naïve T cells to Th1. Th2 are produced in the absence of such cytokines and in the presence of IL-19. TGF-β promotes the
generation of Treg cells, while IL-6 inhibits the generation of Treg and induces T helper 17 cells (Freer and Matteucci, 2009). Th1 activate Mph, including increasing their ability to kill intracellular pathogens (such as BHV1). Th2 help provide help in B-cell activation and class switching. Th17 enhance neutrophil response, and Treg suppress the T cell response (Murphy et al., 2008).

Bovine CTL (Hogg et al., 2011), Th1, and Th2 have been characterized. Although a strict Th1/Th2 dichotomy was not observed, a biased immune response was indicated when the cytokines expressed by cloned Th cells with different antigen specificities were compared (Brown et al., 1998). There is evidence for bovine Treg activity in populations of CD4+CD25+ and WC1+, CD4+, CD25+ γδ T cells (Coussens et al., 2012).

3.4.6. CD8

CD8+ T cells predominantly recognize peptide-MHC-class I complexes (because CD8 binds best to MHC-I) and kill the cells that bear them. The peptides are bound primarily at the ends of the MHC binding groove. MHC-I are present on all cells and are normally loaded by self-peptide fragments (generated by proteasome) via TAP (Murphy et al., 2008). IL-12 and IFN-I have been proposed as the third signal for human CD8 (Curtsinger et al., 1999; Curtsinger and Mescher, 2010). Cytotoxic T lymphocytes (CTL) kill by releasing perforin (which helps deliver granzymes into the target cell), granzymes (which are pro-proteases that are activated intracellularly to trigger apoptosis in the target cell), and granulysin (in humans). They also carry the membrane-bound effector molecule Fas ligand (CD178). When this binds to Fas (CD95) on a target cell it activates apoptosis in the Fas-bearing cell. This may be less important for virus-infected cell killing than for killing lymphocytes after the response is over (Murphy et al., 2008).

Granzymes trigger apoptosis by activating caspasess. For example, granzyme B cleaves and activates caspase-3, which triggers a cascade ending in DNAse. The DNAse degrades both cellular and viral DNA. Granzyme B also triggers apoptosis through actions that result in the release of apoptosis-inducing molecules, including cytochrome c (Murphy et al., 2008). Bovine CD8+ T cells express perforin (increasing with age) (Hogg et al., 2011) and have demonstrated MHC-I restricted killing in vitro (Guzman et al., 2008).
BHV1-encoded proteins appear on the cell surface to serve as targets within 3-4 hours after infection (Babiuk et al., 1975; 1996). gC and gD were demonstrated targets for CD8+ CTL (Denis 1993), although when cells were infected with vaccinia expressing BHV1 gB, gC, or gD, memory T cell populations did not react with them (Hart et al., 2011). Bovine CTL killing was MHCI restricted and BHV1-specific (Splitter et al., 1988; Hart et al., 2011). Cell mediated immunity (CMI) responses peaked 7-10 days after infection and correlated with recovery (Babiuk et al., 1996). CTL likely play a role in control of reactivation from latency in αHVs (Jones and Chowdhury, 2007).

HVs have multiple mechanisms to evade CTL killing (Ploegh, 1998), and in some cases even closely related viruses such as αHV use different molecules for the same mechanism. It should be noted that although in BHV1 infection CD4+ T cells are killed preferentially, CD8+ numbers decreased in PBMC in infection, resulting in decreased CMI (Winkler et al., 1999).

The gN homologue encoded by BHV1 ORF 49.5 (Liang et al., 1993) inhibits transporter-associated antigen processing (TAP)-mediated transport of cytosolic peptides into the endoplasmic reticulum (ER), which consequently blocks the assembly of MHC-I – peptide complexes in vitro in virus-infected cells (s-Lalic et al., 2005; Verwij et al., 2011). Furthermore, the BHV-1 gN targets the TAP complex for proteosomal degradation (Koppers-Lalic, 2005). Sequences within the gN transmembrane domain are likely to interact with TAP (Jones and Chowdhury, 2007). The homologues in HHV1 (Koppers-Lalic, 2007) and HHV3 do not possess this activity, but those in SHV1, EHV1, and EHV4 do (Deruelle and Favoreel, 2011).

Other BHV1 factors inhibit CTL killing. BHV1 gG is a chemokine-binding protein that prevents homing of LCs to sites of infection (Jones and Chowdhury, 2007). The BHV1 UL41.5 protein inhibits CD8+ T-cell recognition of infected cells by preventing trafficking of viral peptides to the surface of cells (Jones and Chowdhury, 2007). BHV1 vhs shuts down synthesis of MHC-I (and MHCII), reducing antigen presentation (Gopinath et al., 2002; Muylkens et al., 2007). The LR alternate transcript binds Bid, which is specifically cleaved by granzyme B. In this way LR proteins impair the CTL-induced death of infected neurons (Jones and Chowdhury, 2007).
Other αHV activities may be assumed for BHV1, have not yet been demonstrated. Despite low aa similarity, the US3 homologues show “substantial functional conservation” (Deruelle and Favoreel, 2011). HHV1 US3 has multiple immune evasion activities, and many of these have also been observed in SHV1. It interferes with fas-mediated apoptosis. It interferes with MHC1 presentation of Ag (as do its HHV3 homologue ORF66 and SHV1 US3). It results in endocytosis of gB (in HHV1, not shown for BHV1) (Deruelle and Favoreel, 2011). The HHV3 US3 homologue ORF66 retains mature MHC class I complexes in the cis/medial Golgi (Griffin et al., 2010). HHV1 gD blocks apoptosis (Roizman and Taddeo, 2007).

In other cases, αHV anti-CTL or anti-apoptosis factors have no homologue in BHV1. HHV1 gJ blocks CTL (Roizman and Taddeo, 2007), but has no homologue in BHV1 (Schwyzer and Ackermann, 1996; Schmitt and Keil, 1998). HHV1 ICP47 (IE12) inhibits MHC1 expression (Bauer and Tampé, 2002), but has no homologue in BHV1 (Ambagala et al., 2004). HHV1 ICP34.5 interacts with Beclin 1 (Cavignac and Esclatine, 2010; Taylor et al., 2011), but there is no BHV1 homologue (Henderson et al., 2005). Finally, HHV1 US11 interacts with PKR, both inhibiting autophagy and presentation of gps on the cell surface (Cavignac and Esclatine, 2010; Taylor et al., 2011), but there is no homologue in BHV1 (Schwyzer and Ackermann, 1996; Schmitt and Keil, 1998).

BHV1 infection leads to programmed cell death, with p53 and caspases activated (Devireddy and Jones, 1999). Penetration of the cell is not needed (Hanon et al., 1999). The induction or blocking of apoptosis is a matter of timing for the host and αHV (Srikumaran et al., 2007). Early in the cell infection, apoptosis destroys viral components (including progeny DNA), obviating their assembly and release. Thus, when danger signals and immune cells induce apoptosis, there is an advantage to the host. After assembly, however, apoptosis may be advantageous to release of the virus (Nguyen and Blaho, 2009). The balance may also be cell type dependent.

3.4.7. CD4

CD4+ T cells predominantly recognize peptide-MHC-class II complexes (because CD4 binds best to MHCII) and are activated by or activate the cells that bear them. MHCII
are borne primarily by APC, and bind proteasome-degraded peptides along their length (Murphy et al., 2008). IL-1 has been proposed as the third signal for human CD4 (Curtsinger et al., 1999; Curtsinger and Mescher, 2010). CD4+ Th1 can bear Fas ligand, which triggers death of the Fas-bearing cell (Murphy et al., 2008).

During BHV-1 infection, CD4+ T cells are considered to be essential for virus clearance in vivo. CD4 T cells, but not γδ T cells or CD8 T cells, were identified as the limiting cell type in antigen-induced proliferation in BHV1 infection (Denis et al., 1994). They are required for the generation of antibody-producing cells, class II-restricted CD4+ cytotoxic T lymphocytes (Wang and Splitter, 1998), and other cytotoxicity activity (Renzjifo et al., 1999). Th1 secrete IL-2, IL-12, IFN-g and Th2 secrete IL-4, IL-5, IL-6 and IL-10 to drive the antibody response (Campos et al., 1994). CD4+ T cells were cytotoxic against Mph pulsed with BHV1 peptides, acting through Fas and in an MHCII-restricted fashion (Wang and Splitter, 1998). The association of BHV1 antibody response and MHCII genotype has been studied (Juliarena et al., 2009).

BHV1 gB, gC, gD, and VP8 are recognized by CD4 T helper cells from immune cattle (Hutchings et al., 1990; Leary and Splitter, 1990). gE, gI, and gG were shown not to be significant for lymphoproliferative responses (Denis et al., 1996). T cell heterohybridomas specific for gB, gC, and gD have been generated (Nataraj and Srikumaran, 1994), and T cell epitopes have been mapped on BHV1 gB (Gao et al., 1999) and gD (Tikoo et al., 1995b).

BHV1 infects and results in apoptosis of CD4+ T cells, including activated ones (Griebel et al., 1990; Eskra and Splitter, 1997; Winkler et al., 1999). CD4+ but not CD8+ T cells were shown to be infected, and gD but not gC (γ2) transcripts were detected, indicating a non-productive infection (Winkler et al., 1999). UV-irradiated BHV1 suppressed IL-2 and (heterologous) antigen-induced proliferative responses (Hutchings et al., 1990). Anti-gB or gD antibody was able to block this effect. BHV1 has other mechanisms of reducing CD4 responses. BHV1 vhs (UL41) causes a decrease of MHCII (and MHCII) presentation (Muylkens et al., 2007). Light (L)-particles (Dargan et al., 1995) have been observed in BHV1 infected MDBK cells and are believed to be involved in immune evasion (Meckes and
They do this by shuttling HLA-DR (MHCII) to the exosomal secretion pathway instead of the cell surface.

### 3.4.8. B lymphocytes

Naive B cell activation is dependent on three signals: 1) B cell receptor (BCR) binding by antigen; followed by 2) cognate interaction with helper T cells through an immunological synapse; and 3) TLR stimulation (Ruprecht and Lonzavecchia, 2006; Lonzavecchia and Sallusto, 2007; Murphy et al., 2008). The B-cell ‘co-receptor complex’ includes CD21 (Complement receptor 2), CD19, and CD81. So if the cleaved complement fragment C3d is bound to the antigen, the complement can bind CR2, the Ag can bind BCR, and the complex of the two can result in augmented signal (Murphy et al., 2008). Some repeating antigens (T-cell independent Ags) and anti-idiotypes are able to provide multiple signals by cross-linking BCR.

BCR binding up-regulates TLRs (Ruprecht and Lonzavecchia, 2006) and MHCII (Ratcliff and Mitchison, 1984), key to subsequent signals. Specific activation of the B cell by its cognate T-cell (a helper T primed by the ‘same’ antigen) consists of ILs and ligand (T cell CD40L to bind B cell CD40) (Murphy et al., 2008). The T cells must recognize Ag on the B cell in association with MHC (Ratcliffe and Mitchison, 1984). The T cell – B cell immunological synapse is enriched in center for TCR-MHC-peptide and CD40-CD40L, and ‘sealed’ at the periphery by interaction of T cell LFA-1 and B cell ICAM-1 (Murphy et al., 2008). The T and B cells polarize their secretory and endocytic/exocytic machinery, respectively, toward the synapse (Duchez et al., 2011). Th2 provide help in B-cell activation and secrete the B cell growth factors IL-4, IL-5, IL-9, and IL-13. In cattle, IL-2 was observed to drive the antibody response, but other factors may drive it to one class or another (e.g., IgG1 with IL-4 or IgG2 with IFN-γ) (Estes and Brown, 2002; Estes, 2010). The roles of cytokines in the mouse were not found to extrapolate well to cattle.

### 3.4.9. Immunoglobulins

Immunoglobulin (Ig) generation, classes and subclasses, and strategies for use may vary between mammalian species. For example, the ileal Peyer’s patch is a likely bursa
equivalent in cattle (Meyer et al., 1997). The concentration of different Ig classes in milk and colostrum varies considerably according to species, breed, age, stage of lactation, and health status. In many species, absorption of Igs is selective and receptor mediated. In ruminants, absorption is non-selective during the first 12-36 hours after parturition (Marnila and Korhonen, 2011). Ig subclasses do not match between species because the species diverged before the classes or subclasses subdivided (Butler, 1995). IgG1 is the primary secretory Ig in cattle.

Diversity of antigen specificity is generated by five main mechanisms: 1) combinations of different variable-light (VL) and –heavy (VH) domains; 2) combinations of different V, diversity (D), and J genes; 3) addition and deletion of nucleotides at junctions of V, (D), and J genes during recombination; 4) somatic hypermutation; and 5) gene conversion. Different species have been found to use different strategies to generate diversity (reviewed in Butler, 1997). Primates and rodents express a large number of V, D, and J genes and emphasize combinatorial mechanisms as well as templated (antigen-driven) somatic hypermutation (mutations in ‘hotspots’ while the B-lymphocyte is in the germinal center, Teng and Papavasiliou, 2007). Artiodactyls, lagomorphs, and chickens, conversely, express few V, D, and J genes and emphasize untemplated somatic mutation and gene conversion.

Bovine Ig genes (C, then V, then J and D) were located on chromosomes (Zimin et al., 2009), using homology to mouse and human genes and the identification of flanking, conserved recombination signal sequences (RSS) (reviewed in Butler, 1995; 1997; Levings and Roth, 2012). It was determined that cattle express one VH family (Saini et al., 1997, Niku et al., 2012). Bovine light chains are predominantly lambda type, with only a few sub-families of genes, and only a few sub-sub-families used (Sinclair et al., 1995). One J gene is predominantly expressed in each of H (Saini et al., 1997; Zhao et al., 2003) and L (Pasman et al., 2010) chains. Three D genes have been identified, with varying lengths that contribute to varying length H chain CDR3, including the extremely long ones found in IgM only (Shojaei et al., 2003).

Ig effector function is in the Fc, or constant domains. Key Ig effector functions in the immune response to BHV1 include neutralization, C fixation, and antibody dependent cellular cytotoxicity (ADCC). These functions are important late in the immune response,
and protect the host from further primary, or later re-infection. They are effective against virions and infected cells.

### 3.4.10. Neutralization

Neutralization of animal virus infectivity can occur by multiple mechanisms (Reading and Dimmock, 2007). Antibody (Ab) may aggregate virions and reduce the number of infectious centers. Ab that mimic a cell receptor can bind virions and lead to premature virion steps, e.g., release of the genome. It may inhibit virion attachment by blocking receptor engagement. It may inhibit fusion, either at the cell membrane or in an endocytotic vesicle. Antibody can bind to a cell-surface protein and result in the transduction of a signal into the cell that aborts the infection. Post-entry neutralization can occur by transmission of a signal via the virus surface protein to the virion core. Transcytosing IgA antibodies may neutralize virus when their respective vesicles fuse. Antibody may bind nascent virions and block their budding or release from the cell surface (Reading and Dimmock, 2007).

In the bovine immune response to BHV1, Ab is key to binding glycoproteins and preventing attachment. It can do that late in primary infection to prevent extracellular virus from infecting, it can do that during re-activation to do same thing, it can do that on secondary exposure, and it can coat the virus that is being shed (Pastoret et al., 1979).

In the primary response, gB, gC, and gD are the primary inducers and targets of neutralizing Ab (Turin et al., 1999). The response is expanded in recrudescence or secondary exposure – it is elevated against the major gps, and responses to minor ones like gE ‘become detectable.’ Dubuisson et al. (1992) examined the mechanisms of neutralization of monoclonal Ab (MAb) to gB, gC, and gD. The majority of MAb did not prevent attachment. Few Mab to gB were effective. Anti-gD MAbs worked as well after attachment as before, which was likely due to gD’s role in penetration. C enhanced the activity of almost all of the gB and gC MAb, but not the gD MAb. The conformational change of HHV1 gD when it binds receptor provides a new neutralization site (Lazear et al., 2012). Ab protected against fatal multi-systemic BHV1 disease in newborns (Turin et al., 1999), but did not prevent initial viral replication, resulting in latency. This results in latently infected seronegative animals after the maternal Ab declines (Lemaire et al., 2000a; Nandi at al., 2009). Passive
transfer of Ab did not protect completely, although it prevented death from challenge (Marshall and Letchworth, 1988). The primary immune response or vaccination is able to successfully control re-excretion (Muylkens et al., 2007).

αHV evade neutralizing Ab using three mechanisms (Favoreel et al., 2006): Fc receptor Ab binding (by gE/gI, which is not apparent for BHV1 (Whitbeck et al., 1996); endocytosis of gps, or Ag-Ab complex internalization by same mechanism; hiding from Abs through intracellular retention of viral proteins and directed egress to intimate cell-cell contacts. The synapse can be seen as an example of the latter (Favoreel et al., 2006). In HHV1, cell-to-cell transmission depends on gE-gI, which bind to components of cell junctions (while gD localizes to apical surface) (Dingwell and Johnson, 1998). BHV1 gC includes Ig-related domains. The low gC reactivity of bovine antisera may be explained by molecular mimickry (Fitzpatrick et al., 1989; 1990). Finally, syncytial strains of HHV1 avoid neutralization by not using extracellular virus to infect neighboring cells. This is said not to occur with wild-type viruses, however (Roizman et al., 2007).

3.4.11. Antibody-Dependent Cellular Cytotoxicity (ADCC)

Ab binding to determinants on virus-infected cells may lead to those cells being killed in a non-MHC restricted manner. PMNs are the most effective mediators of ADCC. Macrophages also contribute, and lymphocytes do not (Rouse et al., 1976, Grewal et al., 1977). Interferon and complement enhance the activity (Rouse and Babiuk, 1977). IgM is inactive in ADCC alone, but can enable ADCC-C-mediated lysis, which may be important early in the humoral response. BHV1 infection of Mph limits their ability to perform ADCC (Ohmann and Babiuk, 1986). The FcγR of HHV1 blocked ADCC (Lubinski et al., 2011).

3.4.12. Other antibody activities

Abs label antigens on virions and virus-infected cells for activity by complement, phagocytes, and NK cells (Favoreel et al., 2006). Ab to viral antigens can trigger the classic pathway of complement activation on virions and infected cells. It is not believed this is important early in infection because high amounts of each were needed for activity in vitro (Babiuk et al., 1975, Rouse and Babiuk, 1977). Cattle have differences from humans and
mice in their FcR (particularly Fcγ2R), possibly because of the different role of IgG re: mucosal surfaces (Kaeskovics, 2004). NK and other immune cells bear FcR. Antibodies can also neutralize the immunosuppressive effects induced by BHV1 against T cells (Hutchings et al., 1990).

The BHV1 evasion methods for these activities would be the same or similar to those cited for neutralization or innate complement activation, including viral FcR and C3bR. Fc receptors can serve to shield the antigen with normal Ig, or do Ig bridging (Ag-Ab-Fc) to prevent C activation. SHV1 can shed or internalize Ab-Ag-C complexes (Favoreel et al., 2003).

3.4.13. Immune response in latency and reactivation

The role of the immune system in preventing reactivation from latency is controversial. There is a chronic inflammatory (immune) response in latently infected TGs, with elevated CD8+ and cytokine/chemokine expression. This was interpreted as maintaining viral latency and suppressing reactivation of HHV1 (Theil et al., 2003). This role in control of reactivation from latency in αHVs was noted and believed potentially due to viral protein expression in rare cells in the TG (Jones and Chowdhury, 2007). This has been called “spontaneous molecular reactivation”. IFN-γ was also believed to play a role (Jones, 2003). However, it has been reported that LAT of HHV1 is responsible for CD8+ CTL functional exhaustion in TGs (Chentoufi et al., 2011). Also, CD8+ T cells surround only a small proportion of LAT+ neurons, but miRNAs are present in all of the LAT+ cells (Held et al., 2011).

3.4.14. Mucosal immunity

The selective localization of mucosal lymphocytes to specific tissues is due to their expression of chemokine receptors and the differential expression of cognate chemokines and tissue-specific addressins by epithelial cells (Czerkinsky and Holmgren, 2012). T cells (CD4+ and CD8+) primed by DCs in the local LN are influenced to home, based on receptors (Ciabattini et al., 2011). The chemokine/chemokine receptor pairs CCL25/CCR9 and CCL28/CCR10 have been shown to be important to trafficking of antibody secreting
cells to mucosal tissues. The expression of these molecules is different in cattle than in humans and mice, suggesting different mechanisms for accumulation in specific mucosal tissues (Distelhorst et al., 2010).

4. Vaccination

4.1. General BHV1 vaccinology

Nucleosidic antiviral drugs have been used to treat human herpesviral infections since the 1970s, and have been tested and applied for limited applications in veterinary species, including for herpesvirus infections (Rollinson et al., 1988; Wilkins et al., 2003; van der Meulen et al., 2006; Henninger et al., 2007). However, widespread clinical use of antiviral drugs is not common in veterinary medicine (Kahn et al., 2005). Administration of interferon (Cummins and Hutcheson, 1993) or interferon inducers (Theil et al., 1971) to reduce the clinical signs of BHV1 infection has been limited to experimental trials. Anti-herpesviral immunomodulators such as host defense proteins (Jenssen, 2009), double-negative “intracellular immunization” (Mühlbach et al., 2009), and gene therapy (Bunnell and Morgan, 1998) are not currently used in food animal medicine. Rather, biosecurity and vaccination are the primary control measures for the diseases caused by BHV1.

BHV1 is a good candidate for conventional and new vaccines (van Drunen Littel-van den Hurk, 2006). Although there are subtypes of BHV1 (Metzler et al., 1985), the subtypes are broadly immunologically cross-reactive and there is limited antigenic variation within a geographic region. Also, BHV1 is a stable virus, has a limited host range, and has a viremic phase (van Drunen Littel-van den Hurk, 2006). In natural infection there is a strong, long-lasting (possibly due to persistent infection, Kaashoek et al., 1996b) and well-balanced (Th1/Th2) immune response to protective Ags. There is also a significant response to other viral proteins that can serve as markers. The virus is easily grown (rapidly, to high titers) in cell cultures, facilitating production of vaccine virus.

Conventional MLV and KV BHV1 vaccines have been used for many years (Kendrick et al., 1957; Kolar et al., 1972). However, problems due to the nature of the virus
(e.g., MLV immunosuppression) or vaccine technologies (KV efficacy) encouraged the use of new technologies to develop “second generation veterinary viral vaccines” (reviewed in Meeusen et al., 2007; Zhao and Xi, 2011). The emphasis has been on delivery of major gps, and on use of major or minor gps as negative markers (Baranowski et al., 1996; Babiuk et al., 1996; Turin et al., 1999). The goal of vaccination is a well-balanced immune response, similar to that of natural infection.

There is such a wide variety of BHV1 vaccines (in practice and in the literature), that it can be helpful to describe them as belonging to categories. The most common divisions are: conventional and molecular; replicating and non-replicating; and marker and non-marker. Vaccines can also be categorized by route (intranasal, intramuscular, etc.) or administration technique (e.g., aerosol, injection, ‘gene gun’). The divisions are not absolute -- e.g., some molecular vectors (e.g., canarypox in mammals or alphavirus replicons) do not replicate in the host but non-productively infect cells and express antigens on the cell surface similar to live vaccines (Taylor et al., 1995; Vander Veen et al., 2012). In some cases vaccines are best used in combination regimens, called ‘prime-boost,’ e.g., MLV and KV gene-deleted vaccines (Muylkens et al., 2007), or DNA and subunit vaccines (van Drunen Littel-van den Hurk et al., 2008).

The ‘differentiating infected from vaccinated animals’ (DIVA) strategy employs a vaccine that is missing an antigenic marker (usually, although a positive marker can also be added, Chowdhury, 1996), and a complementary diagnostic assay for that marker. A diagnostic assay for the (protective) vaccine antigen that is present in both the vaccine and field virus is also employed. Marker vaccines can range from single-gene mutation or deletion live virus to single glycoprotein subunit vaccines. A desirable negative marker protein is one: not needed for in vitro production; not critical for protection; present in all wild-type viruses; and that induces a rapid, strong, long-lasting response in both naïve and vaccinated animals (Kaashoek et al., 1996c; van Drunen Littel-van den Hurk, 2006). Also, the companion diagnostic should be sensitive and specific. Widely employed BHV1 marker companion diagnostics have occasionally demonstrated problems with each of these characteristics (Muylkens et al., 2007; van Oirschot et al., 1999).
4.2. Non-replicating vaccines

4.2.1. Conventional

Conventional KV have been used for decades (Kolar et al., 1972). They have the advantage of safety, especially in pregnant cattle. However, typically two immunizations are needed, the immune response is primarily humoral, and the duration of immunity is shorter than for modified live vaccines (Tikoo et al., 1995a; van Drunen Littel-van den Hurk, 2007). The adjuvants commonly added to increase immunogenicity can introduce problems of their own.

The conventional killed virus BHV1 vaccine is produced through physico-chemical inactivation of cell culture fluids. Agents used have included formalin, beta-propiolactone, binary ethylene amine, ethanol, UV irradiation, and heat (Haralambiev, 1976; Levings et al., 1984; van Drunen Littel-van den Hurk, 2006). The vaccine includes all components of the virus (and cell culture), but there is the concern that inactivation could damage key epitopes (Jones and Chowdhury, 2007). A marker vaccine can be produced using the same inactivation methods when the production virus is gene-deleted (e.g., gE-, Kaashoek et al., 1995; Strube et al., 1996).

4.2.2. Subunit

Subunit vaccines containing the major gps (gB, gC, gD) have been proven effective. These have included detergent extracts of virus preparations (to solubilize envelope gps) (Lupton and Reed, 1980), including incorporation of the extracts into ISCOMs (Trudel et al., 1988). Individual gps have also been purified from such extracts for vaccine use using affinity chromatography (Babiuk et al., 1987b). gB, gC, and gD subunit vaccines were all protective, with gD eliciting the highest titers and best protection (Babiuk et al., 1987b).

The gps for subunit vaccine use have also been produced using various expression systems. Vaccinia and adenovirus systems in mammalian cells, and baculovirus systems in insect cells yielded protective gps due to their glycosylation. E.coli systems produced partial protection (van Drunen Littel-van den Hurk et al., 1993). A truncated, secreted version of gD was produced in a bovine cell line (Kowalski et al., 1993) and shown protective (van
Drunen Littel-van den Hurk et al., 1994). When the adjuvant CpG was incorporated into the vaccine, no virus was shed after challenge (Ioannou et al., 2002).

4.2.3. Anti-idiotype

Anti-idiotype (anti-Id, or Ab2) immunizations for herpesviruses (Kennedy et al., 1984; Gurish et al., 1988; Tsuda et al., 1992; Zhou and Afshar, 1995), and BHV1 in particular have been reported. Srikumaran et al. (1990), Hariharan et al. (1991), and Orten et al. (1991) used neutralizing murine monoclonal antibody (MAb) as Ab1 to generate bovine polyclonal antibody (PAb), bovine MAb, or rabbit PAb Ab2 respectively, which in turn were used to elicit neutralizing Ab3 in mice. Orten et al. (1993) immunized calves with an Ab2 (rabbit PAb anti-Id to murine anti-gB and gD MAb), resulting in a slight reduction of clinical signs and one calf producing BHV1-neutralizing antibodies.

4.3. Replicating vaccines

4.3.1. Modified live (attenuated)

Modified live vaccines (MLV) have been used for BHV1 disease since 1956 (Kendrick et al., 1957). MLV in general are generated by passage in cell culture, sometimes in heterologous cell culture (Quinlivan et al., 2011). This allows for mutations or deletions in genes important to viral fitness, but that are not essential to in vitro replication. The main advantage of MLV is that they replicate in the host’s target cells, so antigen is presented on MHCI (eliciting CTL), as well as on MHCII (eliciting humoral immunity) (van Drunen Littel-van den Hurk, 2007). After one dose of MLV, when PBL were exposed to live BHV1, CD25 was increased in CD4, CD8, and γδ T cells (Endsley et al., 2002). BHV1 MLVs also typically elicit substantial duration of immunity (van Drunen Littel-van den Hurk, 2007).

BHV1 conventional MLV problems have included those specific to BHV1 disease. These include virulence (e.g., in small calves, or pregnant animals) (Whetstone et al., 1986; Bryan et al., 1994; Jones and Chowdhury, 2007; O’Toole et al., 2012), latency (Pastoret et al., 1980; Whetstone et al., 1986), and immunosuppression, including a reduction in the response to another vaccine administered simultaneously (Harland et al., 1992). Other
problems common to all MLVs can also occur. These include reversion to virulence (Belknap et al., 1999), lack of efficacy due to over-attenuation, and adventitious agents. The latter is particularly likely if the vaccine is produced in host cells or with host ingredients (Wessman and Levings, 1999; Falcone et al., 2003), but can occur even if the vaccine is produced with non-host cell or ingredients (Wilbur et al., 1994). A temperature-sensitive MLV was generated using chemical mutagenesis (Tikoo et al., 1995a) which was safe for pregnant animals.

4.3.2. Gene deleted

Although gene deletions may occur using conventional attenuation (Kaashoek et al., 1994), the design of mutations or deletions can be more controlled with genetic engineering. There are typically two goals in constructing gene-deleted live vaccines: 1) remove/reduce virulence or other undesirable disease trait; and/or 2) remove (or add) a marker detected by a companion diagnostic, usually a serologic marker (which can also be detected on a viral isolate). In the case of BHV1, deletions in the thymidine kinase, gC, gE, gG, gI, Us9, and LR genes have been made to reduce virulence (Kit et al., 1985; Chowdhury, 1996; Kaashoek et al., 1998) and/or recrudescence (Kaashoek et al., 1998; Inman et al., 2001). It has been suggested gN be targeted to reduce immunosuppression (Jones and Chowdhury, 2007). Viral envelope gps have been targeted for serologic markers, including gC and gE due to the host’s strong serologic responses to these non-essential proteins.

Disadvantages of gene-deleted live vaccines are under- or over-attenuation (Kaashoek et al., 1998), depending on the genes chosen. Because virulent isolates are usually the starting material for deletion work, recombination can also be an issue (reviewed in Thiry et al., 2005). BHV1 recombination in vivo between two gene-deleted strains, leading to wild-type virus was demonstrated (Schynts et al., 2003). In addition, recombination leading to a virulent marker (gE-) BHV1 virus was shown (Muylkens et al., 2006; 2006a), a situation that could confuse eradication campaigns. Such recombination of gene deleted vaccines has been demonstrated for other αHV (Henderson et al., 1991, Lee et al., 2012).
4.3.3. Live virus vectored

Vaccination using live vectors for BHV1 gps have resulted in virus neutralizing (VN) Ab, CMI responses, and/or partial protection. These have included vaccinia-vectored gB and gC (VN, van Drunen Littel-van den Hurk et al., 1989), bovine adenovirus 3 expressing gD (VN and CMI, Zakhartchouk et al., 1999), human adenovirus 3 or 5 expressing gC or gD (VN, Gupta et al., 2001), and Newcastle disease virus-vectored gD (partial protection, Khattar et al., 2010). Although an αHV chimeric veterinary vaccine has been developed (Cochran et al., 2000; 2001), no chimeric BHV1 vaccine has been reported.

4.3.4. DNA vaccines

DNA vaccines for BHV1 have also been used in trials. DNA vaccines provide certain advantages over conventional MLV, including safety, stability, and efficacy in the presence of maternal antibodies (Donnelly et al., 1997). They result in antigen presentation by both MHCI and MHCII, similar to live vaccines (Gurunathan et al., 2000), although they typically elicit a Th1 response. Although replicating, they can be made specific to one or a few antigens. A disadvantage at this time is their mode of delivery, e.g., veterinary use of gene gun is not currently practical (Loehr et al., 2001). In most reported trials, complete protection was not achieved.

BHV1 gp (gB, gC, and gD) DNA has been administered by a variety of routes. Trials include gB, gC, and gD individually (Cox et al., 1993), gD (van Drunen Littel-van den Hurk et al., 1998), gC with ubiquitin (Gupta et al., 2001), secreted gD (Castrucci et al., 2004), a combined secreted gB-gD, (Caselli et al., 2005), gB (Huang et al., 2005), and gD with CpG (van Drunen Littel-van den Hurk et al., 2008).

4.3.5. BHV1 as a vector

The use of BHV1 as a vector of other proteins has a variety of advantages, including knowledge of the molecular biology of the virus, existing systems for vaccine production, and the already-widespread use of BHV1 vaccines (so there are few or no new safety or serosurveillance concerns) (Jones and Chowdhury, 2007). The virus has been used to express IL-1 β (Raggo et al., 1996), IL-2, IL-4 (Kühnle et al., 1996), IFN-g (Raggo et al.,
and to display IFN-α (Keil et al., 2010). Expression of cytokines could provide an adjuvant effect for BHV1 vaccination. Protective immunogens of other bovine viruses have been expressed in BHV1. A foot-and-mouth disease (FMD) virus VP1 epitope was inserted as the N-terminal sequence of a BHV1 gC fusion protein, was expressed on the surface of virions and infected cells, and elicited protective levels of Ab to FMD, while protecting against BHV1 (Kit et al., 1991). The G protein of bovine respiratory disease virus (BRSV) was expressed and the vaccine provided the same degree of protection to BHV1 and BRSV in calves as a multi-valent vaccine (Schrijver et al., 1997). Bovine viral diarrhea (BVD) virus E2 was expressed in BHV1 and the vaccine virus elicited VN Ab to BVD (Kweon et al., 1999). Parainfluenza 3 fusion (F) and hemagglutinin (HN) genes were inserted into BHV1 (Cochran et al., 2000). In addition, insertion of an influenza HA1 sequence resulted in HA1 being expressed with gG as a fusion protein on the outside of virions and infected cells (Keil et al., 2010). αHV have also been proposed vectors for other virus’ vaccine production (Epstein and Manservigi, 2004) and as episomal systems for gene therapy (Macnab et al., 2008).

4.4. Routes

BHV1 infects via mucosal epithelium, so stimulating immunity for those surfaces would be desirable. However, most of the conventional vaccines are parenterally administered and may result in systemic rather than mucosal immunity. In contrast, mucosal immunization induces mucosal as well as systemic immunity (Loehr et al., 2000). Immunization of mucosal surfaces results in good antigen detection, and B and T lymphocytes stimulated in the mucosa home to mucosa in general and to the immunized mucosal tissue specifically (Neutra and Kozlowski, 2006). A variety of routes have been employed or suggested for viral (including αHV) vaccines, such as oral, nasal, vaginal, ocular, sublingual, and anorectal (Shiau et al., 2001; Pavot et al., 2012; Czerkinsky and Holmgren, 2012).

Temperature-sensitive BHV1 vaccine administered intranasally (IN) was shown to induce secretory IgA and a CMI response (Frerichs et al., 1982). Israel et al. (1992)
demonstrated mucosal immunity to BHV1 subunit vaccine using cholera B subunit as an adjuvant. Use of conventional BHV1 IN vacc in one of the regimes tested was shown to confer rapid protection (Roth and Carter, 2000; Endsley et al., 2002). Intravaginal vaccination with gD DNA (Loehr et al., 2000; 2001) protected against IN BHV1 challenge. Oral vaccination with BHV1 in utero stimulated mucosal immunity (Gerdts et al., 2002). A gD DNA vaccine was administered IN with reduction in challenge virus shedding (Castrucci et al., 2004), and a gB DNA vaccine administered vulvovaginally elicited partial protection from genital lesions (Huang et al., 2005).

4.5. Application

The ultimate goal of BHV1 vaccination would be to prevent infections, which can in turn lead to latency/recrudescence and spread. Although this may occasionally be achieved (Israel et al., 1992), it is not routinely practical.

A challenge for vaccination in cattle is immunizing stressed animals (vaccines are often administered as part of movement, and with multiple other treatments). Such stressors impact immune function (Kelley, 1980) and have been demonstrated to be associated with increased blood cortisol levels. High cortisol levels can impair the phagocytic cell function, decrease CMI, and decrease Ab response to primary vaccination (Roth and Perino, 1998). Vaccinating young animals includes the difficulty of vaccinating in the face of passive immunity (Menantau-Horta et al., 1985), and young animals may mount poor Th1 responses (van Drunen Littel-van den Hurk, 2006). Use of CpG adjuvants or DNA vaccines may help with the younger animal immunization.

In the United States, BHV1 vaccines are currently used as an aid in the prevention of disease. Between 150 and 200 million doses are produced annually (Anon, 2011; personal communication), all of the conventional types (modified live and killed virus vaccines). In some countries of the EU, (gE-) marker vaccines are used in eradication programs (Kahrs, 2001; van Oirschot et al., 1996, Ackermann and Engels, 2006; van Drunen Littel-van den Hurk, 2006). Because vaccines cannot prevent infection, vaccination must be frequent to keep recrudescence low, and culling based on DIVA serology employed. A significant issue
for control and eradication is seronegative cattle that can re-excrete after a stress (Hage et al., 1998). It has been shown that young animals infected while protected by passive immunity can remain seronegative, and recrudesce at a later time (Lemaire et al., 1995; Lemaire et al., 2000a; 2000b).

5. Summary/Conclusions

In summary, there is a delicate balance between viral infection, host response, and viral evasive measures in BHV1 infection and immunity in cattle. BHV1 has a rapid life-cycle and robust systems for entry, transcription, assembly and egress. The host responds with multiple tools, from infected-cell interferons to antibody-assisted infected cell killing. Like all αHV, BHV1 has multiple evasion strategies to blunt or delay the host response, including in some cases multiple measures for the same host effector mechanism. The timing of response vs. viral replication (and spread in animal and between animals) is therefore critical for disease outcomes. Maternal antibody (provision of humoral tools from the dam’s immune response), and vaccination (ensuring the response to infection will be a rapid, strong secondary immune response) can provide the host with the advantage needed to prevent severe disease on primary infection.

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CHAPTER 3. GENERATION BY SELF RE-FUSION OF BOVINE$^3$ X MURINE$^2$ HETEROHYBRIDOMAS SECRETING VIRUS-NEUTRALIZING BOVINE MONOCLONAL ANTIBODIES TO BOVINE HERPESVIRUS 1 GLYCOPROTEINS gB, gC, and gD

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Abstract

Seventy-eight heterohybridomas (HH) stably secreting bovine monoclonal antibodies (BoMAb) to Bovine herpesvirus 1 (BHV1) were produced by fusing lymph node cells from a hyperimmunized calf with 3 types of non-secreting fusion partners. Seven were produced through fusion with the murine x murine (murine$^2$) hybridoma SP2/0, 3 through fusion with bovine-murine$^2$ HH generated using cells from the same calf, and 68 through fusion with bovine$^2$-murine$^2$ HH generated by sequential fusions using cells from the same calf. The chromosome number of example HH increased with increasing numbers of input fusions. A variety of indirect fluorescent antibody assay patterns was observed using the BoMAb, suggesting diverse antigen specificity. Three bovine$^3$-murine$^2$ HH secreted IgG1 BoMAb neutralizing BHV1 without complement, and were chosen for further characterization. SDS-PAGE of detergent-solubilized BHV1 proteins bound to the 3 neutralizing BoMAb demonstrated their individual specificities for BHV1 envelope glycoproteins gB, gC, and gD. The 3 HH stably secreted the BoMAb in culture for over one year, and pilot-scale production of the BoMAb was accomplished by in vivo and in vitro methods. Self re-fusion was shown to be advantageous for efficiently producing hybridomas stably secreting host monoclonal antibodies. The BoMAb described should prove useful in studies of the host immune response to BHV1 and as reagents.

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1. Introduction

Bovine herpesvirus 1 (BHV1) causes diseases of global economic significance in cattle (Wyler et al., 1989) and is the subject of national control and eradication programs (Tikoo et al., 1995; van Drunen Littel-van den Hurk, 2006). Diseases caused by BHV1 include infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious balanopostitis (IPB), conjunctivitis, abortion, and others (Gibbs and Rweyemamu, 1977). It is also a significant initiator of and contributor to “shipping fever” pneumonia (Yates, 1982; Jones and Chowdhury, 2007). Subtypes (1.1, 1.2a, and 1.2b, as well as 1.3a and 1.3b, now a separate species) were identified by genetic and antigenic analysis (Metzler et al., 1985; Wyler et al., 1989) and were associated with geographic range and prevalence of clinical manifestations (Edwards et al., 1990; van Oirschot et al., 1995; D’Arce et al., 2002). BHV1 and the bovine immune response to it have been extensively studied (reviewed by Gibbs and Rweyemamu, 1977; Wyler et al., 1989; Tikoo et al., 1995; Babiuk et al., 1996; Muylkens et al., 2007), and many vaccines are commercially available in the U.S. (Anon, 2012) or globally for protection against IBR disease or eradication (van Drunen Littel-van den Hurk, 2006).

BHV1 is a member of the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus (Davison et al., 2009). Alphaherpesvirus envelope glycoproteins function in the attachment and penetration of the virus into the host cell (Spear, 2004; Rey 2006), and are the targets of protective cellular and humoral host immune responses (Norrild et al., 1979; Denis et al., 1996; Babiuk et al., 1996). BHV1 glycoprotein epitopes have been characterized using murine monoclonal antibodies (MAb) (Collins et al., 1984; Van Drunen Littel-Van den Hurk and Babiuk, 1985; Marshall et al., 1988, Hughes et al., 1988; Ayers et al., 1989). The use of bovine MAb (BoMAb) would have advantages in determining host-relevant epitopes, in other in vitro and in vivo studies of the bovine immune response, as sources of framework or antigen-binding sequences, and as diagnostic reagents.

Heterohybridomas (HH), also called heteromyelomas, inter-species hybridomas, or xenohybridomas have been successfully employed as a source of non-murine MAb for
macaque monkey, rabbit, guinea pig, cat, dog, mink, horse, pig, sheep, goat, and cattle (reviewed in Groves and Tucker, 1989; Groves and Morris, 2000). Fusions between bovine lymphocytes (b) and murine myelomas (m) or murine hybridomas (m²) have produced HH stably secreting bovine Ig (Srikumaran et al., 1983; 1984), Ig specific for the non-infectious agent immunogen (Anderson et al., 1986), and Ig specific for the infectious agent immunogen (Raybould et al., 1985; Guidry et al., 1986; Anderson et al., 1987; Kennedy et al., 1988). Srikumaran et al. (1990) produced b x m² HH secreting non-neutralizing BoMAb specific for BHV1 glycoprotein C (gC).

Fusions of non-murine lymphocytes with murine myeloma cells have often resulted in few stable lines due to loss of secretion as a result of rapid chromosome loss, but “re-fusion” has been reported to improve yields (Tucker et al., 1984). Bovine x m (Tucker et al., 1984; Anderson et al., 1986; 1987; Groves et al., 1987; 1988; Kennedy et al., 1988; Kemp et al., 1990) and b² x m aminopterin-sensitive HH (Anderson et al., 1986; 1987; Tucker et al., 1987) have been used for fusion with bovine lymphocytes. In some cases the selected HH fusion partners themselves secreted Ig, but in most cases clones not secreting Ig were selected for use. In each case the fusion partners and lymphocytes were derived from different individuals of the same species.

The aim of this study was to examine the effect of increasing degrees of re-fusion with lymphocytes from the same individual on HH generation rates, to exploit the increased rate of productive HH generation after re-fusion to derive HH producing neutralizing BoMAb to BHV1 glycoproteins, and to conduct basic characterization of the neutralizing BoMAb.

2. Materials and Methods

2.1. Virus

The immunizing virus was a virulent, low cell culture passage preparation. The Cooper strain (also known as Colorado) subtype 1.1 virus was received in 1970 from T. L. Chow of Colorado State University as fifth cell culture passage. The virus was passaged
once more, calves were exposed intranasally, and the virus was re-isolated by nasal swab. The virus used (free of bacteria, mycoplasma, and bovine viral diarrhea virus) was the second cell culture passage from the nasal swab. When 10^7 plaque forming units (pfu) was administered intranasally, it produced typical clinical signs of IBR in seronegative calves.

2.2. Immunization

A subadult Holstein breed cow was subcutaneously injected with 5 ml of a dilution (10^5 pfu/ml) of the live BHV-1 described previously. Virus preparation was injected (dosage divided and injected in multiple sites) into the area drained by a targeted superficial lymph node (prescapular or prefemoral). For many of the immunizations the live virus was emulsified with equal volumes of Incomplete Freund’s Adjuvant (Gibco, Grand Island NY). The target lymph node area was injected up to five times at 2-4 week intervals. At 3-4 days before fusion, unadjuvanted virus was injected in the area to increase numbers of dividing B cells. The lymph nodes were sequentially targeted, and surgically removed under local or general injectable anesthesia (xylazine, Rompun, Bayer, Shawnee Mission KS). All work was approved by the Institutional Animal Care and Use Committee (IACUC).

2.3. Fusions

All fusions were performed by a modification of a standard murine hybridoma protocol (Van Deusen and Whetstone, 1981; Van Deusen 1984). Briefly, the lymph nodes were sectioned and passed through an 80-mesh sieve. The extracted cells were washed and then controlled-rate frozen or immediately used for fusion. Fusions were done using polyethylene glycol 1540 and cells seeded into 96 well plates at 5 x 10^5 fusion partner cells/ml. All fusion, growth, cloning, freezing, etc. media used were as described in the protocols, except all serum was horse serum, and conditioned media was from fusion partner cultures appropriate to each fusion.

Three sets of fusions were performed, each using a different stimulated lymph node from the same animal. In all sets the SP2/0-Ag14 (SP2/0) line was included, and in the
successive sets heterohybridoma fusion partners were also used. The SP2/0 line had been
generated by fusing BALB/c spleen cells (from a mouse immunized with sheep RBCs) with
the P3X63Ag8 non-secreting myeloma (Shulman et al. 1978), so for this work it was
designated murine$^2$.

In the first set of fusions, two fusions - one (@BL5-0) with fresh lymph node cells
and one (@BL5-1) later with frozen lymph node cells - were done using SP2/0 in a 2:1 and
3:1 lymphocyte:myeloma ratio, respectively. The products would therefore be murine$^2$ x
bovine$^1$. Fusion @BL5-0 resulted in 0 HH secreting anti-BHV1 Ig (of 480 cultures). 72
primary cultures were selected for methotrexate sensitivity by passage in media containing 6-
thioguanine and 8-azaguanine, and frozen in pools of 3-8. Fusion @BL5-1 resulted in 2 HH
(@BL5-1.Y1B1 and @BL5-1.Y3E6) transiently secreting anti-BHV1 Ig (of 480 cultures).
They were selected for methotrexate sensitivity and frozen as individual cultures.

In the second set of fusions, three fusions were performed on the same day with fresh
lymph node cells in a 1:1 lymphocyte:myeloma ratio. Fusion @BL5A0 was done using
SP2/0 (murine$^2$, so products = murine2 x bovine$^1$), fusion @BL5A1 with an equal mixture of
BL5-1.Y1B1 and BL5-1.Y3E6 (murine$^2$ x bovine, so products = murine$^2$ x bovine$^2$), and
fusion @BL5A2 was done using a pool of six methotrexate sensitive primaries from @BL5-
0 (murine$^2$ x bovine, so products = murine$^2$ x bovine$^2$). The @BL5A0 fusion resulted in 13
HH secreting anti-BHV1 Ig (of 384 cultures) that quickly ceased secreting. The @BL5A1
fusion resulted in 4 HH (including @BL5A1.Y2C2 and @BL5A1.Y5All, of 576 cultures)
that temporarily secreted Ig to BHV1. Eight cultures including the 4 formerly secreting HH
were selected for methotrexate sensitivity. The @BL5A2 fusion also resulted in 4 HH (of
576 cultures) transiently secreting Ig to BHV1. Ten cultures including the 4 formerly
secreting HH were selected for methotrexate sensitivity.

In the third set of fusions, three fusions were performed on the same day with fresh
lymph node cells in a 2:1 lymphocyte:myeloma ratio. Fusion @BL5C0 was done using
SP2/0 (murine$^2$, so products = murine2 x bovine$^1$), fusion @BL5C1 with an equal mixture of
BL5-1.Y1B1 and BL5-1.Y3E6 (murine$^2$ x bovine, so products = murine$^2$ x bovine$^2$), and
@BL5C2 with an equal mixture of BL5A1.Y2C2 and BL5A1.Y5A11 (murine$^2$ x bovine$^2$, so
products = murine$^2$ x bovine$^3$).
2.4. Growth culture retention, cloning, and stability

Primary cultures with observed growth were tested using microtiter indirect fluorescent assay (IFA) and microtiter virus neutralization (VN) using duplicate test wells. Primary cultures from @BL5C0 and @BL5C1 fusions were preferentially tested relative to @BL5C2 (97-100% vs. ~60%) due to the relative rarity of cultures with growth for those fusions, and limiting culture expansion and testing capacity. Selected cultures positive by IFA (IFA+) from all three fusions were retained, expanded, and controlled-rate frozen, again with preference for @BL5C0 and @BL5C1 lines. All VN positive (VN+) cultures were also IFA+. All primary cultures selected for further study were cloned and re-cloned by limiting dilution and controlled-rate frozen at the clone and re-clone stage. Testing typically occurred on the culture before cloning, on the clones after cloning, on the culture before freezing, and on the remaining culture after freezing. All cloned and re-cloned cultures were tested by IFA (and VN if the culture was VN+ at the primary stage) and ~6 resulting cultures of each retained. Three HH (@BL5C2.870005, 870009, and 870016, hereafter referred to only as 870005, 870009, 870016) were chosen for special scrutiny because they secreted VN+ BoMAb (hereafter also referred to by the HH designations). They were passaged continuously for 1 year and assayed for secretion, and removed from frozen storage after more than 1 year and assayed for secretion. They were also selected for resistance to 6-thioguanine and oubain while monitoring for continued secretion.

2.5. Karyology

Cells and slides were prepared according to the initial steps in the Center for Veterinary Biologics (CVB, Ames IA) protocol ABRMPRO1101.03. Briefly, cells were passaged to maximize cells in metaphase, colcemid (Karyomax, Gibco, Grand Island NY) treated to stop cells in metaphase, fixed with methanol/acetic acid, and washed, then spread on a slide and dried. Chromosomes were stained with a trypsin-Giemsa staining technique (Seabright, 1971), karyotyped using a microscope (Axioplan 2, Zeiss, Thornwood NY).
coupled with a CCD camera (Photometrics, Huntington Beach CA), and images were captured with karyotyping software (Band View 5.5, Applied Spectral Imaging Inc., Vista CA). In those cases where chromosome morphology was too poor for analysis, fluorescence *in situ* hybridization (FISH) was used to help determine chromosome number and/or sex. Probes for bovine chromosomes X and Y were commercially sourced (IdLabs Inc. Biotechnology, Ontario Canada) and slides hybridized according to manufacturer’s protocol. Analyses were performed under a fluorescence microscope (Axioplan 2, Zeiss Thornwood NY) coupled with a CCD camera (Photometrics, Huntington Beach CA), and images were captured with FISH software (FISH view 4.5, Applied Spectral Imaging Inc., Vista CA). Fifteen samples were tested from 8 lines, representing fusion partners and HH of m2, b x m2, b2 x m2, and b3 x m2 types.

2.6. *Indirect fluorescent antibody assay*

Madin Darby bovine kidney (MDBK) microtiter cell cultures were produced according to CVB Supplemental Assay Method 109 (SAM 109, Anon 2011a). At confluency (1-2 days) the growth medium was decanted and 10-100 TCID\(_{50}\) of BHV1 added per ‘infected well’ (4-8 wells remained uninfected as controls) in 200 ul of maintenance media (media defined in SAM 109). When 10-40% of the cells in the infected wells evidenced cytopathic effect (1-2 days post infection, dpi), the plates were washed with phosphate buffered saline (PBS) and fixed with 80% acetone in water. Plates were air dried at room temperature (rt) and stored at -20 C. For testing, plates were warmed (rt) and soaked in PBS, after which 50 ul of undiluted HH culture fluid (for screening, or other dilution for confirmation or monitoring) was incubated in test wells for 1 hr at rt. Plates were then washed 4 times with PBS, incubated for 1 hr at rt with fluorescein-conjugated anti-bovine immunoglobulin (rabbit or goat origin, National Veterinary Services Laboratories, NVSL, Ames IA) diluted in PBS, washed 5 times with PBS and read using a mercury lamp fluorescent microscope (Orthoplan, Leitz, Rockleigh NJ) at 130X. Selected assays were photographed (Orthomat, Leitz, Rockleigh NJ and Ektachrome 160 or 200, Kodak, Rochester NY). Dilutions in PBS of bovine hyperimmune serum to BHV1 (CVB) served as positive
controls. Hybridoma culture medium and PBS served as negative controls. Infected and uninfected cells were observed in all positive control wells.

For specific activity assays, IFA was performed using affinity purified (Protein G Sepharose 4 Fast Flow, Pharmacia LKB, Piscataway NJ) BoMAb and control Ig. 870016 was purified from nude mouse ascites, and polyclonal anti-BHV1, anti-bovine viral diarrhea virus (BVDV), and non-specific (fetal bovine serum, FBS) Ig from hyperimmune bovine serum and FBS, respectively. Protein was quantified using Lowry assays with rabbit and bovine serum albumin as standards.

For competitive BHV1 IFA, the 3 VN+ BoMAb were each labeled with fluorescein isothiocyanate (FITC) using the NVSL TCSOP0202 method after Hebert et al. (1972). Briefly, the Ig fraction was extracted by ammonium sulfate precipitation, and FITC was bound to the Ig (15 ug FITC/1 mg Ig) through reaction at pH 9.5 at rt. Unreacted FITC was removed through column chromatography (Sephadex G-25, Pharmacia LKB, Piscataway NJ). Each of the 3 VN+ unlabelled BoMab was first incubated in the plate, followed by washing and incubating with the 3 labeled BoMAb, followed by washing and reading.

2.7. Virus neutralization assays

For primary or clone screening assays, undiluted culture supernatants were tested by a modification of SAM 109. Modifications included use of only two wells, virus challenge ranging from 50-500 TCID\textsubscript{50}/25 ul, and observation for cytopathic effect (CPE) at 3-4 days. Culture fluids and production lots of the 3 VN+ BoMAb were titrated using constant virus / varying serum VN assays. These were microtiter CPE-VN (SAM 109) or plaque reduction VN (PRVN) in 35mm wells. The PRVN assay followed CVB Supplemental Assay Method 119 (Anon 2011b), except a high cell culture passage BHV1 was used in place of pseudorabies virus, another varicellovirus.

A pre-/post-attachment VN assay was performed on the 3 VN+ BoMAb using a 4-well modification of SAM 109. Three conditions were studied: Ab inoculum, then virus inoculum and 1 hr incubation (‘0-time VN’); Ab-virus incubated for 1 hour, then the inoculum on cells for 1 hr; virus and media only on cells for 1 hr. Maintenance media was
added after the one hour virus incubation on cells, and the cultures examined for CPE four days later.

2.8. Virus specificity

870005, 870009, and 870016 hollow fiber harvests and dilutions of nude mouse ascites with high anti-BHV1 titers were assayed for IFA or VN activity against other bovine respiratory viruses, and against potential extraneous agents of bovine vaccines and master seeds. Bovine parainfluenza 3 virus (VN, IFA), BVDV (VN), bovine respiratory syncytial virus (VN, IFA), bovine adenovirus (IFA), bovine parvovirus (IFA), bovine reovirus (IFA), and rabies (VN) were tested. Cell culture media and dilutions of SP2/0 ascites were used as negative controls, and dilutions of homologous antiserum used as positive controls.

2.9. Commercial ELISA

A commercial ELISA kit for antibodies to BHV1 (Bovine Rhinotracheitis Virus Antibody Test Kit Screening / Verification, IDEXX, Westbrook ME) was used according to kit instructions, except dilutions for serum were not used. Supernatant media from each of the 3 VN+ BoMAb (870005, 870009, 870016), plus dilutions of nude mouse ascites from 870016 were tested. Cell culture media and dilutions of SP2/0 ascites were used as negative controls.

2.10. Production

Multiple production methods were used to meet various needs, including: routine production, scaleup, and freezing; high purity; high titer; and pilot for large-scale production. Conventional production consisted of flask (25, 75, and 150 cm²) suspension cultures. Serum-free cultures also used flasks, but using medium with added B-12 (0.625 ug/ml), biotin (0.25 ug/ml), bovine insulin (10.0 ug/ml), human transferrin (10ug/ml), and sodium selenite (12.5ug/ml) instead of nutrient serum.
Hollow fiber cell culture was conducted by seeding cells \((1.1 – 1.3 \times 10^8)\) into a cartridge (VitaFiber II, Amicon, Danvers MA) with 30,000 molecular weight cut-off (MWCO). Cultures were perfused with 1.0 L batches of growth medium as used for flask cultures. Twice per week, 25 ml was harvested from the extracapillary space and recirculating growth media was changed. The pH of the culture was maintained by gas exchange with the media reservoir (the entire apparatus was placed in a 5% CO2 incubator).

Ascites production using immunosuppressed Balb/c mice was conducted using modifications of a published technique (Raybould et al., 1985), which used 160 mg/kg cyclophosphamide, equivalent to 4.8 mg/30 g mouse. Mice were injected with 5.0 mg/mouse of cyclophosphamide (Mead Johnson and Co., Evansville IN and Sigma-Aldrich, St. Louis MO) in 0.25 ml SQ one day before cell injection of SP2/0 at 1 and \(5 \times 10^5\), 870005 at \(1 \times 10^5\), 870009 at \(1 \times 10^5\) and 870016 at \(1 \times 10^5\). As a control, \(10^5\) and \(10^6\) cells of 870005 and 870009 were injected without drug. In addition, a trial was performed using up to 5 daily doses of cyclophosphamide at 0, 5.0, 10.0, and 25.0 mg/mouse at days 0-4 prior to intraperitoneal inoculation of \(5 \times 10^6\) cells of @BL5C2.870009. All mice were pristine primed as per routine protocol, and all work was performed under an IACUC-approved protocol.

Ascites production in nude mice was done under commercial contract (Charles River Laboratories, Charles River, MA). The 3 VN+ HH were selected for production in 25 mouse batches, one for each of 870005 and 870009, and two for 870016.

2.11. Isotyping and subisotyping

Isotyping and subisotyping was performed on the 3 VN+ BoMAb using radial immunodiffusion (RID), immunoelectrophoresis (IEP), a modification of the IFA, and by determining the heavy and light chain molecular weights using SDS-PAGE.

A commercial RID kit (Vet-RID, Bethyl Laboratories, Montgomery TX) for bovine Ig (sub)isotype quantitation was used following label directions. A 20X concentrate of conventional culture supernatant media was prepared using centrifugal microconcentrators (Centricon concentrators, Amicon, Beverly MA) and tested. In addition to following the
label instructions, wells were filled four times at 24 hour intervals, and the test read 24 hours after each filling (before refilling) to detect contamination or cross-reaction. Growth media for HH (10% horse serum), kit reference standards, and heterologous standards from the kit (e.g., IgA and IgM for the IgG1 plate) were used as controls.

Gel for IEP (Standard Low mr agarose, BioRad, Hercules CA) was poured 2 mm thick on an 86 x 100 mm film (GelBond film, BioRad, Hercules CA). Wells 2mm in diameter and 2mm x 70mm troughs were cut, with wells and troughs alternating at 10mm intervals. Wells were filled with 15 ul of 15X (870005) or 30X (870009, 870016) flask HH culture supernatant media. Larger diameter wells (4, 6, 9 mm) with larger volumes (2.5, 5, and 10X of standard) were also used to detect any other, lower concentration or contaminating Ig. Troughs were filled with 300ul of goat anti-bovine gamma-globulin (NVSL). Culture media (15ul of 30X media) and anti-BHV1 antiserum (10ul) were used as negative and positive controls, respectively. Electrophoresis was performed at 4V/cm x 1.5 hours. Voltage was measured using needle electrodes. Gels were soaked in PBS to remove unbound protein, dried, stained with Coomassie Blue (Coomassie Brilliant Blue R-250, BioRad, Hercules CA), destained with methanol, dried, and analyzed and photographed.

An isotyping BHV1 IFA was performed using a modification of the previously described IFA. Test wells were incubated with BoMab, washed, incubated with @GBIGG.DAS 17.A4, a mouse MAb specific for bovine IgG1 (kindly donated by Richard Goldsby; Goldsby et al., 1987), washed with PBS, incubated with anti-mouse IgG fluorescein conjugate, washed with PBS, and read. Immunological methods to isotype the light chains were not conducted due to a lack of reagents at the time.

2.12. Polyacrylamide gel electrophoresis (PAGE) and Western blots

Three types of SDS-PAGE were performed: hand-cast full sized (16 x 18 cm x 1.5mm) homogeneous 7.5, 10.0, or 12.5% gels; pre-cast mini (10 x 10 cm x 1.0mm) 4-12% gradient (NuPAGE, Invitrogen Carlsbad CA); and pre-cast microvolume PAGE homogeneous (7.5 or 12.5%) or gradient (10-15 or 8-25%) gels (PhastGel system, Pharmacia LKB, Piscataway NJ). Full sized gels were stained using Coomassie Blue and/or silver stain
Minigels were stained with Coomassie Blue (SimplyBlue, Invitrogen, Carlsbad CA). Microvolume gels were stained using the same Coomassie Blue or silver stain as full size, but using the associated staining apparatus which agitates and heats. High, low, a mixture of high and low, or wide range SDS-PAGE molecular weight (MW) standards (High MW and Low MW, BioRad, Hercules CA and Novex Sharp and MagicMark, Invitrogen, Carlsbad CA) were used appropriate to the gel concentration.

Monitoring the purity of hollow-fiber harvests was done using full sized gels. Apparent MW of Ig H and L chains were determined from gels for hollow-fiber harvest monitoring, from mini-PAGE of concentrates of serum-free culture supernatants, and from full size gels of agarose Protein A-bound Ig. The H and L MW were calculated using the method described for antigen specificity.

Immunoprecipitation was done using detergent-solubilized BHV-1 infected cell cultures (produced similarly to those used for immunoaffinity), concentrates of late-culture hollow-fiber culture harvests, and protein A (Protein A – Sephadex, Pharmacia LKB, Piscataway NJ) or protein G (Sepharose Fast Flow, Pharmacia LKB, Piscataway NJ) linked to highly cross-linked agarose. Sham-inoculated cell cultures or PBS, and dilutions of bovine antiserum to BVDV were used as negative controls, and bovine antiserum to BHV1 was used as a positive control. Mixtures were agitated, and the agarose sediment washed, pelleted, reconstituted in SDS-PAGE sample solution and heated prior to gel application.

Western blots were done with full size (12.5%, 10 aligned-lanes) and microvolume (10-15%, 12 lane or preparatory comb) SDS-PAGE. Separated BHV1 proteins were transferred electrophoretically to nitrocellulose using a full size or mini-gel transfer apparatus (Hoefer Inc., Holliston MA) respectively. The nitrocellulose sheets were placed in full size (Deca-Probe, Hoefer Inc., Holliston MA) or mini-gel (Minblotter 28, Immunetics, Boston MA) gasketed-lane incubation manifolds and reagents applied and removed using pipettes or micropipettors. After blocking at rt and 37C, primary Ab was applied and incubated at 37C (90 min), rt (60 min), and then 4C (16h). Primary Ab consisted of the selected BoMAb (870009 and 870016) or sham preparation, bovine antiserum to BHV1, or nonspecific bovine antiserum (to BVDV). Secondary Ab was anti-bovine peroxidase-labeled immunoglobulin, applied for 2 hr at rt. Control lanes were stained with amido black.
2.13. *Immunoaffinity*

BHV1 was expanded on bovine turbinate and MDBK 850 cm$^2$ roller bottles. At 90-100% CPE, the culture media was decanted, clarified by low speed centrifugation, and ultracentrifuged over a 40% sucrose cushion. The virus pellet was resuspended in 1 ml PBS per ultracentrifuge tube (2-3 ml per roller bottle). Polyvinylpyrrolidone-coated colloidal silica (Percoll, Pharmacia LKB, Piscataway NJ), 90% in 0.25 M sucrose, was used for gradient purification of BHV1 using the method of Svennerholm et al. (1980). Density markers (Pharmacia LKB, Piscataway NJ) (1.050 and 1.070 g/ml) were used, and the preparations ultracentrifuged at 116.5 kg for 20 min in a fixed angle rotor (Ti50, Beckman Coulter, Inc., Brea CA). Fractions ~1.050-1.070 were harvested, pooled, and gradient purified as before. Fractions were harvested, titered, and high virus titer fractions pooled. The gradient-purified BHV1 was diluted 1:8.4 in TBS w/ 1% NP40 and 1% DOC (TBSND), sonicated, and ultracentrifuged 100 kg x 1hr in a swinging bucket rotor (SW 28, Beckman Coulter, Inc., Brea CA) to remove any remaining whole virions and capsids. Uninfected cell debris at the same densities were similarly harvested and treated.

@GBIGG.DAS 17 was purified from mouse ascites using Protein A columns and bound to gel columns (Pierce Immunopure kit, Pierce, Rockford IL). These were used to purify bovine Ig from pools of hollow-fiber culture harvests of four bovine HH-870005, 870009, 870016, and a bovine$^3$ x murine heterohybridoma secreting BoMAb to bovine parvovirus, @BL6-1.013100. @BL6-1.013100 was generated by fusing a 1:1 mixture of the fusion partners for the @BL5C2 fusion (@BL5A1.Y2C2 and Y5A11) with fresh lymphocytes from a bovine parvovirus-stimulated lymph node of a different animal, and cloning twice. Fractions were tested by IFA, and their protein concentrations measured by Lowry. Amounts of 2.51 mg (870005); 10.0 mg (870009); 10.0 mg (870016), and 6.26 mg (013100) were bound to gel columns per product instructions.

The BoMAb columns were loaded with the detergent-solubilized BHV1 preparations (using 3 cycles viral antigen, 10 cycles of sham), washed with TBSND, washed with DH$_2$O, then eluted with 0.1M glycine HCl pH 2.7 into a Tris HCl pH 9.0 ‘cushion’ to avoid damage.
to the eluted protein. Fractions with significant A280 readings were pooled, pH adjusted (from 8.2 to 7.5-7.6) and concentrated 250X using centrifugal concentration cartridges (10,000 MWCO). The same fractions of cell culture control preparations were used.

Apparent MW of BHV1 proteins bound on immunoaffinity columns were determined using microvolume SDS-PAGE. 7.5% homogeneous, 10-15% gradient, and 8-25% gradient gels with 8 lanes were used. 10-15% SDS-PAGE results were used for further analysis. MW was calculated from distance migrated (D) and linear regression on the MW markers, using $\ln \ln \frac{M_o}{MW}$ vs. $\ln D - D_o$ where $M_o$ and $D_o$ are the MW and distance migrated of the heaviest MW marker used.

3. Results

3.1. Heterohybridoma primary culture growth and secretion of specific bovine immunoglobulin

The third set of fusions, which compared degrees of “self re-fusion” resulted in growth in 73.8% of the seeded @BL5C2 ($b^3 \times m^2$), compared to <25% for @BL5C0 ($b \times m^2$) and @BL5C1 ($b^2 \times m^2$) fusions (table 1). Of the @BL5C2 cultures tested, 37.8% were found to secrete antibody specific for BHV1 by IFA, compared to <3.8% for @BL5C0 and @BL5C1 fusions. 42.5% of @BL5C2 cultures were successfully frozen compared to 75-100% of the @BL5C0 and @BL5C1. 72.4% of @BL5C2 lines selected were cloned successfully compared to 71.4-100% of @BL5C0 and @BL5C1. All HH lines selected for recloning were recloned successfully, regardless of fusion.

The 3 HH 870005, 870009, and 870016 were passaged continuously for one year and continued to secrete VN + BoMAb. They were also removed from frozen storage after more than 1 year and secreted VN + BoMAb. @BL5C2.870005, 870009, and 870016 were deposited (under the Budapest Treaty) in the American Type Culture Collection (ATCC, Rockville, MD) and designated HB 9907, 9908, and 9909, respectively. The viability and secretion of the ATCC cultures was confirmed shortly after deposit. In addition, the 3 HH
Table 1: Growth, specific antibody secretion rates, and stability of fusions of various degrees of self re-fusion. Stability measured by secretion through culture preservation (freezing) and cloning.

<table>
<thead>
<tr>
<th>Fusion name</th>
<th>@BL5C0</th>
<th>@BL5C1</th>
<th>@BL5C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>bovine(^1) x murine(^2)</td>
<td>bovine(^2) x murine(^2)</td>
<td>bovine(^3) x murine(^2)</td>
</tr>
<tr>
<td>Fusion partner</td>
<td>SP2/0</td>
<td>@BL5-1.Y1B1 &amp; Y3E6</td>
<td>@BL5A1.Y2C2 &amp; Y5A11</td>
</tr>
<tr>
<td>Cultures seeded</td>
<td>768</td>
<td>1056</td>
<td>960</td>
</tr>
<tr>
<td>Cultures with growth (% of seeded)</td>
<td>190 (24.7)</td>
<td>131 (12.4)</td>
<td>708 (73.8)</td>
</tr>
<tr>
<td>Primaries tested (% of cultures with growth)</td>
<td>185 (97.4)</td>
<td>130 (99.2)</td>
<td>423 (59.7)</td>
</tr>
<tr>
<td>Primaries strong IFA(^+) (% of tested)</td>
<td>7 (3.8)</td>
<td>4 (3.1)</td>
<td>160 (37.8)</td>
</tr>
<tr>
<td>Primaries frozen secreting (% of IFA(^+))</td>
<td>7 (100)</td>
<td>3 (75.0)</td>
<td>68 (42.5)</td>
</tr>
<tr>
<td>Primaries cloned (% of attempted)</td>
<td>5 (71.4)</td>
<td>3 (100)</td>
<td>42 (72.4)</td>
</tr>
<tr>
<td>Lines recloned (% of attempted)</td>
<td>5 (100)</td>
<td>3 (100)</td>
<td>25 (100)</td>
</tr>
<tr>
<td>Lines VN(^+) (% of tested)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (0.71)</td>
</tr>
</tbody>
</table>

\(^1\) Indirect fluorescent antibody
\(^2\) Virus neutralization
were selected for resistance to 6-thioguanine and oubain while continuing to secrete VN+ BoMAb.

3.2. Karyology

Karyotypes of 8 lines representing fusion partners and HH of varying degrees of re-fusion demonstrated a range of chromosome numbers. Although there was significant variability and small sample numbers, there was a trend of increasing chromosome number with increasing bovine re-fusion (table 2). An example karyotype is shown in figure 1. Anomalies included deletions, translocations, re-arrangements, and small markers.

Table 2. Chromosome numbers of fusion partners and secreting heterohybridomas of various degrees of re-fusion.

<table>
<thead>
<tr>
<th>Fusion identity</th>
<th>Fusion type</th>
<th>Ig Secretion</th>
<th>Lines tested</th>
<th>Line identities</th>
<th># Samples</th>
<th># Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP2/0 Murine</td>
<td>Murine²</td>
<td>No</td>
<td>1</td>
<td>NA</td>
<td>2</td>
<td>44; 60</td>
</tr>
<tr>
<td>BL5A Bovine</td>
<td>Bovineᵇ x Murine²</td>
<td>No</td>
<td>1</td>
<td>3E6</td>
<td>2</td>
<td>60ᵃᵈ; 71</td>
</tr>
<tr>
<td>BL5C0 Bovine</td>
<td>Bovine x Murine²</td>
<td>Yes</td>
<td>3</td>
<td>8700001 870013 870019</td>
<td>4</td>
<td>64ᵉ; 65ᶠᵉ; 70ᶠʰ; 79</td>
</tr>
<tr>
<td>BL5C1 Bovine</td>
<td>Bovine² x Murine²</td>
<td>Yes</td>
<td>2</td>
<td>870003 870006</td>
<td>3</td>
<td>77ᵗⁱ; 79; 72-8²ᶜ</td>
</tr>
<tr>
<td>BL5C2 Bovine³</td>
<td>Bovine³ x Murine²</td>
<td>Yes</td>
<td>1</td>
<td>870009</td>
<td>4</td>
<td>58ᶠ; 86-90; &gt;90ᶠ; &gt;90ᶠ</td>
</tr>
</tbody>
</table>

ᵃ Mouse chromosome number is 40, SP2/0 number is 58 to 65, modal 61-62 (Sorokina et al., 1986)
ᵇ Cow chromosome number is 60
ᶜ Male
ᵈ Also cells with 120-132
ᵉ Range 62-75
ᶠ XY
ᵍ Also up to 100+
ʰ Range 52-86
ⁱ Range 68-77
Figure 1. An example heterohybridoma karyotype (@BL5C0.870013). 79 chromosomes were observed including XY.

3.3. *IFA and VN*

A variety of IFA patterns were observed with the BoMAb tested (figure 2). Only the homologous unlabeled neutralizing BoMAb competed with each labeled neutralizing BoMAb. 870016 was IFA positive at 500 ng/ml. Polyclonal bovine antiserum to BHV1 was positive at 5,000 ng/ml.
Figure 2. Indirect fluorescent antibody patterns of select bovine MAb and controls. (A) @BL5C2.870005. (B) @BL5C2.870009. (C) @BL5C2.870016. (D) @BL5C0.870001. (E) @BL5C2.870042. (F) @BL5C2.870078. (G) Bovine antiserum to BHV1. (H) SP2/0. (I) @BL5C2.870034. Samples were conventional culture supernatant fluids and $10^{-3}$ of antiserum.
The bovine\(^3\) x murine\(^2\) fusion was the only one to produce HH secreting neutralizing BoMAb. Three were detected: 870005, 870009, and 870016. They were found to neutralize the immunizing BHV1 isolate without complement. Although some (0.75 log\(_{10}\) TCID\(_{50}\), compared to ≥2.25 log\(_{10}\) for traditional VN) activity was demonstrated by 870016 in the 0-time VN, all 3 VN+ BoMAb neutralized <1.0 log\(_{10}\) in 0-time VN, and all reactions by traditional VN were ≥1.0 log\(_{10}\).

The 3 VN+ BoMAb assayed were negative by IFA, VN, or both to all 3 other bovine respiratory viruses and 4 other bovine viruses tested. Of those tested, only higher titered preparations of BoMAb were reactive in the commercial ELISA designed and labeled for serum dilutions. Conventional culture supernatant of 870016 was suspect, and 870016 ascites was positive. Conventional culture supernatants of 870005 and 870009 were negative, as were the cell culture media and SP2/0 ascites included as negative controls.

3.4. Production methods

Conventional culture of the three selected HH was consistently successful. Serum-free cultures were routinely established for high-purity preparations. Hollow fiber cultures achieved VN titers 8-16X those of simultaneous flask comparison cultures (figure 3), with demonstrably greater purity (figure 4). Maximum VN titers were: @BL5C2.870005 – 256; 870009 – 512; 870016 – 512-1024. Titers were higher on both hollow fiber and comparison flask culture on the four-day culture harvests (e.g., days 34 and 41, figure 3).

No ascites was generated in any of the cyclophosphamide-treated syngeneic mice, and some higher doses of cyclophosphamide proved lethal. Ascites was successfully generated commercially using nude mice. Volumes of ascites (25 mouse) lots ranged from 124 to 151 ml. Titers by IFA and VN of the lots were: @BL5C2.870005 - 1600 IFA, 512 VN; 870009 – 1600 IFA, 4096 VN; 870016 – 3200 IFA, 2048-4096 (2689 calculated) VN.
Figure 3. Virus neutralization (VN) titers of twice-weekly harvests from hollow-fiber and flask cultures of bovine MAb @BL5C2.870009.

Figure 4. A comparison of product purity, hollow fiber cultures vs. flask cultures of @BL5C2.870016.

Lanes 1, 8 – molecular weight standards; lanes 2–6 – Week 0, 1, 2, 3, 4 hollow-fiber harvests, respectively; lane 7 – flask culture. 10% full-sized SDS-PAGE gel, 10 lanes, Coomassie blue stain. Image cropped to include entire separation gel, lanes 1-8. Harvests of week 1 and 3 had similar high titers, that of week 4 was ~8-fold less.
3.5. Isotyping and subisotyping

Each of the VN+ BoMAb (@BL5C2.870005, 870009, and 870016) reacted with only anti-bovine IgG1 reagents (not anti-bovine IgG2, IgM, or IgA) in the RID assay. The media control was negative, and kit reference controls were positive with their respective antisera and with the other antisera. Although used for quantitating Ig in the diagnosis of failure of passive transfer (Ameri and Wilkerson 2008), concentrations of the BoMAb could not be measured, because the diameters of the immunoprecipitate ring of all BoMAb preparations (870016 > 870009 > 870005) were below the low standard and below the Y intercept of the standard curve. Although quantities could not be calculated, they were below the 620 mg/dl standard, or less than 31 mg/dl in original culture fluid.

In the IEP assay (figure 5) each of the three BoMAb tested produced a single arc centered slightly to the anode side of the well, consistent with the IgG1 subclass (Butler 1983). The isotype-specific IFA resulted in positive results for each of the neutralizing BoMAb using the anti-bovine IgG1 murine MAb, and the anti-bovine IgG1 murine MAb bound to each of the VN+ BoMAb in immunoaffinity columns. H and L chain molecular weights by SDS-PAGE were calculated to be 48.9 and 26.9 K for 870005 (r = 0.899), 48.6 and 25.6 K for 870009, and 50.4 and 25.3 K for 870016 (figure 6, r = 0.997). All sizes were consistent with IgG1 (heavy) subclass and either lambda or kappa (light) class. One ‘band’ was observed for each H and L chain.

3.6. SDS-PAGE and Western Blot

Mock immunoprecipitation experiments (agarose Protein A and BoMAb without antigen) demonstrated the purity of bound SF preparations and provided additional data on H and L chain MW. When reacted with solubilized BHV-infected cell culture fluids (immunoprecipitation), no proteins were observed in the experimental lanes that were not also observed in at least one negative control lane (figure 7).

Western blots did not result in clear unique reactions for any of the 3 VN+ BoMAb (figure 8). Although faint bands were observed, the same reactions appeared in negative
Figure 5. Immunoelectrophoresis of bovine MAb @BL5C2.870016.
Top gel, wells 1-4, increasing volumes (15, 37.5, 75, 150 ul) of 30X @BL5C2.870016. All troughs contain goat origin anti-bovine IgG (National Veterinary Services Laboratories, NVSL, Ames IA).
Bottom gel wells 1-4, BHV1 antiserum, 1:1, 1:10, 1:1, and 1:10 respectively. Troughs for wells 1-2 and 2-3 contain 1:1 and 1:10 goat origin anti-bovine IgG (NVSL). Trough for wells 3-4 contains 1:10 Commercial anti-bovine IgG (KPL, Gaithersburg MD).
controls. When the sensitivity was increased, reactions with many proteins were observed, but again, negative controls (e.g. irrelevant bovine antiserum) reacted with the same proteins. Similar results were observed using microvolume SDS-PAGE (results not shown).

3.7. Immunoaffinity

Two 10-15% gradient microvolume SDS-PAGE assays on the eluates of each of the VN+ BoMAb (figure 9, top and middle gels) were analyzed ($r = 0.99$). The results were
Figure 7. Immunoprecipitation of BHV1\(^1\) solubilized antigen by bovine MAb and controls. 7.5% full sized SDS-PAGE gel, Coomassie blue and then silver stained. Image cropped to include only lanes 2-19 of original gel, stacking gel interface to sample front.

Lanes
1 – Molecular weight (MW) standards
2 – Empty
3 – BVDV\(^3\) AS \(10^{-2}\) x PB\(^4\)
4 – BVDV AS \(10^{-2}\) x BHV1
5 – BVDV AS \(10^{-3}\) x PB
6 – BVDV AS \(10^{-3}\) x BHV1
7 – BHV1 AS \(10^{-2}\) x PB
8 – BHV1 AS \(10^{-2}\) x BHV1
9 – BHV1 AS \(10^{-3}\) x PB
10 – BHV1 AS\(^2\) \(10^{-3}\) x BHV1
11 – MW standards
12 - @BL5C2.870016 x PB
13 - @BL5C2.870016 x BHV1
14 - @BL5C2.870009 x PB
15 - @BL5C2.870009 x BHV1
16 – Media control x PB
17 – Media control x BHV1
18 – MW standards

\(^1\) Bovine herpesvirus 1
\(^2\) Antiserum
\(^3\) Bovine viral diarrhea virus
\(^4\) Phosphate buffer
Figure 8. Western blot of two bovine MAb and controls with bovine herpesvirus 1 (BHV1) antigens. Lane 1-8 used for immunoreaction, lanes 9-10 stained with amido black. Bovine MAb preparations were 10X hollow fiber culture harvests. Image cropped to include only stacking gel interface to sample front.

Lane 1 - Pre-stained MW standards
Lane 2 - Media control
Lane 3 - @BL5C2.870009
Lane 4 - @BL5C2.870016
Lane 5 – BHV1 bovine antiserum 1:10
Lane 6 – BHV1 antiserum 1:1
Lane 7 - Bovine viral diarrhea virus (BVDV) bovine antiserum 1:1
Lane 8 – BVDV antiserum 1:10
Lane 9 – BHV1 antigen
Lane 10 – Unstained MW standards
Figure 9. SDS-PAGE of eluates from immunoaffinity columns using bovine MAb and bovine herpesvirus 1 (BHV1) detergent solubilized antigens and controls. All samples = 250X concentrates of eluates and preparations used with columns. All gels microvolume gradient SDS-PAGE. Top and middle gels – 10-15%; bottom gel – 8-25%. Images cropped to include only stacking gel interface to near bottom of separation gel. Molecular weight standards – gel 1, lanes 1, 4, 7; gel 2, lane 1; gel 3, lane 8 BHV1 antigen - gel 1, lane 8 (1:7.5); gel 2, lane 8 (1:7.5); gel 3, lanes 1 (1:1), 2 (1:2), 3 (1:4) Cell culture control – gel 2, lane 2 (1:5) @BL5C2.870005 - gel 1, lane 6; gel 2, lane 7; gel 3, lane 7 @BL5C2.870009 - gel 1, lane 5; gel 2, lane 6; gel 3, lane 6 @BL5C2.870016 – gel 1, lane 3; gel 2, lane 4; gel 3, lane 5 Anti-bovine parvovirus bovine MAb - gel 1, lane 2; gel 2, lane 3, 5; gel 3, lane 4
similar and so were averaged (individual values <5% from average). 870005 bound a protein of approximately 118 Kd, consistent with BHV1 gC. 870009 bound proteins of approximately 61, 82, and 132 Kd, consistent with BHV1 gB. @BL5C2.870016 bound proteins of approximately 78 and 147 Kd, consistent with BHV1 gD. The anti-bovine parvovirus BoMAb did not bind any BHV1 protein. Eluates of cell controls contained no proteins or only leached Ig. Results for 8-25 and 7.5% gels were consistent with the 10-15% gels.

4. Discussion

Although techniques other than hybridoma generation can be used to derive host species MAb, some do not generate MAb representative of the immune response, or they may result in MAb limited in quantity or restricted in Ig class (Jessup et al., 2000). Non-murine myeloma fusion partners have been sought with limited success. Protocols using human peripheral blood lymphocytes have been published with the intent of popularizing murine-human HH for analysis and therapy (Jessup et al., 2000).

Hybridomas lose chromosomes rapidly after fusion, and HH especially do so (Wollweber et al., 2000). Random and non-random patterns of loss, retention, and translocation have been observed using a variety of staining techniques including FISH (Wollweber et al. 2000; Nejad et al. 2008). Human-murine HH lose human chromosomes preferentially (Crocem et al., 1980; Wollweber et al., 2000), possibly due to DNA replication rates or spindle formation issues (Nejad et al., 2008). Preferentially retained chromosomes include those coding for Ig (Crocem et al., 1980), those key to survival (Wang et al., 1998), and those associated with Ig production and secretion but not coding for Ig (Raison et al., 1982). Re-fusion has been used to increase the number of non-murine chromosomes in HH. In one report (Tucker et al., 1984) the re-fused lines carried two to three times the number of bovine chromosomes as the single-fused hybridoma. In this study a trend of increasing chromosome numbers with increasing ‘self re-fusion’ was observed, although the data are limited and the ranges overlap. This is consistent both with random loss and with the concept of encoded non-Ig transcripts or proteins enabling retention of same-species
chromosomes. The species origin of the chromosomes or fragments were not determined in the HH tested.

This study demonstrated a higher success rate of generation of HH secreting specific host MAb as a result of repeated self re-fusion, that is, using the modification of re-fusing HH to lymphocytes from the same individual. There was a ≥3-fold increase in primary cultures with growth in selective media, and a ≥10-fold increase in HH secreting specific Ig among those primaries tested, when the b^3 x m^2 fusion was compared to the b^2 x m^2 or b^1 x m^2 fusions using the same stimulated lymphocytes (table 1). It is unknown if higher growth and secretion rates would be observed with even higher degrees of re-fusion. Higher specific HH generation rates have been observed by others using “more bovine” fusion partners for fusions with another cow’s lymphocytes (Tucker et al., 1984; Anderson et al., 1987; Kennedy et al., 1988). The high specific HH generation rate observed here is consistent with a previous study (Anderson et al., 1987), but that study used fusion partners that themselves secreted Ig. In this study, the fusion partners did not secrete Ig, so each of the secreting HH produced would be expected to secrete only specific MAb. When the b^2 x m^2 fusion partners used for @BL5C2 (@BL5A1.Y2C2 and Y5A11) were used in fusions with other bovine lymphocytes (from other individuals, stimulated using other viruses), generation rates were low (similar to @BL5C0 and @BL5C1), further suggesting the “self re-fusion” nature of @BL5C2 was key to the high generation rates. The reason ‘self’ re-fusion appears to work better are unknown, but perhaps the enabling effect leading to greater chromosome retention has an individual-specific nature. The ‘self re-fusion’ method (and the 3 VN+ BoMAb and the 3 HH secreting them as examples) was patented (Levings and Stoll, 1991, since expired).

The stability of @BL5C2 primaries (as measured by ability to maintain secretion through freezing or cloning) was lower than @BL5C0 and @BL5C1 (although the sample numbers for those are low). This may have been due to more rapid and severe chromosome loss, or may be at least partially due to the preferential treatment given to primaries of the latter two fusions. The re-cloning rate of all three fusions was equally high (100%), which may be a reflection of the oft-reported advantage of aggressive re-cloning, or may at least partially be due to the laboratory’s ability to handle the lower number of cultures at that stage of the process. The 3 VN+ HH stably secreted for over a year in continuous culture. No
tests were done to determine the proportion of cells secreting BoMAb at the beginning and end of that period to gain additional information on their stability. The 3 VN+ cultures could also be well-preserved in the frozen state, as all 3 were removed after 1 year for further study and secreted BoMAb.

Monoclonality of hybridomas is always a concern due to the possibility of mixed cultures (addressed by cloning), or fusing two lymphocytes to one myeloma cell. In addition, because a hybridoma can cease secretion while retaining the Ig structural gene, and then resume secretion when stimulated (Raison et al., 1982), re-fusion could result in use of the retained Ig gene, in turn resulting in mixed Ig. The loss of chromosomes after fusion provides an advantage for the latter two problems. In this study, the single bands on PAGE gels suggest monoclonality. Further, the ability to clone cDNA of the H and L chain-encoding mRNA from 870005 (Koti et al., 2010) and 870016 (Levings and Roth, 2012), and the H chain mRNA from 870005 and 870009 (Saini et al., 1997) suggest only one gene for each of H and L are being expressed.

IFA (patterns and competition) and VN (activity presence or absence) results suggested a variety of specificities. The IFA patterns, although limited to observation of only a few characteristics (e.g., membrane, cytoplasm, nucleus; grainy, smooth), may be useful for selection for further study in that some resembled the patterns of hyperimmune serum and some did not (figure 2), suggesting some of the MAb recognize non-immunodominant epitopes. A characterization of specificities of all BoMAb generated was not done, nor a comparison of specificities of the BoMAb generated between the fusions.

The rarity of VN+ BoMAb-secreting HH observed (3/423 @BL5C2 lines tested, or 0.71%) suggests only fusions with large numbers of growing or IFA+ HH are likely to yield such results, or any particular specificity/activity. In this study one VN+ BoMAb was generated for each of the three major neutralization targets, gB, gC, and gD. None of them required complement for VN activity. The characteristics might be used to infer epitope specificity based on previous studies with murine MAb (van Drunen Littel-van den Hurk et al., 1984; Collins et al., 1984; Marshall et al., 1986; Okazaki et al., 1986; Hughes et al., 1988). However, the murine MAb used in the studies may have different effector functions in vitro, and the murine immune response may have targeted different epitopes than the
natural host. None of the 3 VN+ BoMAb had significant neutralization in the 0-time VN trial, suggesting they may not be able to neutralize after attachment. The small neutralization activity observed in the assay as designed may have been due to neutralization of the virus in suspension, before it could attach to cells. Further assay designs could add information regarding this question. The 3 VN+ BoMAb were specific for BHV1, of all bovine viruses tested.

Production from HH cannot employ one of the most commonly used methods of murine hybridoma production, ascites production in syngeneic mice. However, many other techniques are available, and scale-up is achievable. In this study conventional culture, serum-free culture, nude mouse ascites production, and hollow-fiber culture were successfully demonstrated. Each of the methods has its uses, with hollow-fiber being perhaps the most amenable to industrial scale-up among those studied here (Rodrigues et al., 2009). It does not have the animal use and suffering issues of ascites production, and has been used successfully for production of a number of hybridomas (Gramer et al., 2003), including for those used for therapeutic manufacture (Valdés et al., 2001).

One or two hollow fiber cultures, lasting 4-9 weeks, were conducted for each of the HH secreting VN+ BoMAb. VN titers 8-16X those of simultaneous flask comparison cultures were demonstrated (figure 3), although they were 2-8X lower than that of nude ascites. Much greater purity than flask culture was observed (figure 4), in spite of using nutrient medium with serum. The 30,000 average MWKO appeared to allow some BSA to diffuse into the cellular space for harvest. The mechanical, overgrowth/cell death, and acidity issues observed in other studies (Jackson et al., 1996; Valdés et al., 2001) were experienced in our trials. The pilot method used here did not have extensive monitoring or controls (e.g., glucose use, O₂ monitoring, pH adjustment). pH monitoring and adjustment was done through observation and correction of the nutrient media, and harvesting was done on a fixed schedule. A scale-up of the technology including serum-free media and increased controls could be expected to improve purity and yields.

The 3 VN+ BoMAb were all IgG1. The generation of IgG1 is consistent with most other BoMAb reports (Groves and Morris, 2000) and generating IgG in this study is likely due to the repeated immunizations in the area drained by the target lymph node. IgG1 is the
majority IgG sub-isotype in bovine serum (Butler, 1983), so it is not remarkable that IgG1 BoMAb resulted from the parenteral immunization. The other BoMAb generated were not (sub-)isotyped, so a characterization of isotypes within or between the fusions could not be done. The isotypes of the light chains were not determined as part of this study. H and L chain weights by SDS-PAGE (figure 6) were consistent with IgG1 and either light chain class. Later sequencing studies on these BoMAb confirmed the IgG1 isotype of the H chain of 870005 and 870009 (Saini et al., 1997), and 870016 (Levings and Roth, 2012), and established the lambda isotype of the L chains of 870005 (Koti et al., 2010) and 870016 (Levings and Roth, 2012). Lambda is the predominant class of light chains in cattle (91%, Arun et al., 1996).

The BHV1 antigen specificity of the 3 VN+ BoMAb was determined using commonly employed methods. The MW for gB (a.k.a. gI and gp6/11a/16) have been reported as 130/68-74/54 (Misra et al., 1981), 130/74/55 (Marshall et al., 1986; Babiuk et al., 1987), 120/69/55 (Collins et al., 1984), and 117/70/51 (Okazaki et al., 1986). 870009 bound proteins of 132, 82, and 61 Kd, consistent with BHV1 heterodimer gB. The MW for gC (a.k.a. gIII and gp3/gp9) have been reported as 180/77-82 (Misra et al., 1981), 180/97 (Marshall et al., 1986), 180/91 (Babiuk et al., 1987), 82 (Collins et al., 1984), and 157/87 (Okazaki et al., 1986). 870005 bound a protein of 118 Kd, consistent with BHV1 homodimer gC. The MW for gD (a.k.a. gIV and gp5/11b) have been reported as 138/68-74 (Misra et al., 1981), 150/77 (Marshall et al., 1986), and 71 (Okazaki et al., 1986; Babiuk et al., 1987; Tikoo et al., 1990). 870016 bound proteins of 147 and 78 Kd, consistent with the BHV1 homodimer gD.

The BHV1 preparation used for immunoaffinity was detergent solubilized, but not exposed to reducing conditions. This resulted in the binding of glycoprotein homo- and hetero-dimers. When subjected to the conditions of SDS-PAGE, some or all of the complexes disassociated, consistent with the reports cited above. The heterodimer gB and the homodimer gD migrated as the dimer and each of its monomers, whereas the homodimer gC migrated as the monomers only. The apparent MW found in this study are consistently higher than, or at the upper end of the reported range, but are consistent with the glycoproteins cited.
Negative Western blot results (figure 8) may suggest 870009 and 870016 are directed to conformational epitopes, and negative immunoprecipitation (figure 7) results may suggest they bind with low affinity. The extensive concentration employed for the affinity chromatography preparations might also indicate low affinity or avidity under the binding or washing conditions.

The major BHV1 glycoprotein complexes gB, gC, and gD are involved in virus attachment and entry. In the primary immune response, they are the primary inducers and targets of neutralizing Ab (Collins et al., 1985, van Drunen Littel-van den Hurk and Babiuk, 1986; Turin et al., 1999). gB, gC, and gD subunit vaccines were all protective, with gD eliciting the highest VN titers and best protection (Babiuk et al., 1987). The emphasis in molecular vaccine design has been on delivery of major gps, and on use of major or minor gps as negative markers (Baranowski et al., 1996; Babiuk et al., 1996; Turin et al., 1999).

Having an immortal and expandable source of bovine Ig specific for immunogenic, protective proteins of a major bovine pathogen provides opportunities for further work in a variety of areas. The bovine Ig can be used as a pure species-specific or isotype-specific reference material for use as controls (e.g., in failure of passive protection assays) or in the generation of further reference materials (e.g., anti-species or isotype antisera, MAb, or conjugates) (Srikumaran et al., 1987). The immortalized cell lines can serve as a source of bovine genetic material, and mRNAs coding for the Ig can be used as examples in species, breed, or allotype sequence studies (Kacskovics and Butler, 1996).

The specific BoMAb might be used as agent-specific reference materials in serologic assays. These would include use as controls in assays that depend on species or isotype, e.g., IFA or ELISA using anti-species Ig conjugates. They would also have advantages over conventional controls in assays that do not depend on species or isotype, e.g., VN due to their purity. As bovine Ig they can be introduced into the host for studies of the bovine immune response, e.g., in passive immunity studies (Booman et al., 1992, Thomas et al., 1998). The VN+ BoMAb derived in this study may be useful in studies of the bovine secretory immune response as they are IgG1, the primary subclass in bovine milk and a significant component of Ig in nasal secretions (Butler 1983). As snapshots of the host immune response to an infectious disease, they may be useful in determining host-relevant epitopes in a way that
whole antiserum (where any epitope-specific antibody population is diluted by the many others) or non-host monoclonal antibody (generated by an animal with no evolutionary background for defense against the agent) cannot (Binns et al., 1993). Determining host-relevant epitopes may assist in vaccine development, particularly of peptide or engineered vaccines (Tikoo 1990). Host MAb with ‘private’ idiotypes may also be exploited for development of anti-idiotypic vaccines (Hariharan et al., 1991, Ladjemi et al., 2011). The sequences derived from the HH may be useful for engineering other antibodies with the variable region or complementarity determining regions of the isolated BoMAb. The generation of oubain and methotrexate resistant HH secreting BoMAb as demonstrated here would allow the generation of ‘tetradomas’ secreting hybrid antibodies with two specificities, two isotypes, and/or two species origins, using conventional hybridoma technology (Moldenhauer 2011).

Many of these uses have already been realized, from the cultures generated and expanded, frozen, and/or deposited at ATCC. In this study 870005, 870009, and 870016 reacted in a commercial RID for bovine IgG1, and 870016 reacted in a commercial ELISA for bovine Ab to IBR. Studies of passive protection (Levings et al., 2012a), epitope competition (Levings et al., 2012b), anti-idiotype generation (Levings et al., 2012c), variable region sequence (Saini et al., 1997; Levings and Roth, 2012), and structure (Koti et al., 2010) have also been done using the BoMAb generated in this study.

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CHAPTER 4. PASSIVE IMMUNIZATION WITH NEUTRALIZING BOVINE MONOCLONAL ANTIBODIES IN A RABBIT MODEL OF BOVINE HERPESVIRUS 1 INFECTION

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Abstract

Rabbits were passively immunized by intravenous injection with three neutralizing bovine monoclonal antibodies (MAb) to bovine herpesvirus 1 (BHV1). The bovine MAb were administered individually at a variety of doses, and polyclonal antiserum to BHV1, fetal bovine serum, non-secreting hybridoma supernatant, and cell culture media were used as control preparations. Rabbits were challenged 24 hours after passive immunization, at 6-8 days of age, with a virus dose shown to be ~90% lethal in age/dose trials. Protection from lethal challenge was conferred by each antibody preparation at high virus neutralizing titers, and protection diminished with diminishing antibody titers. Overall, the results suggest young rabbit infection may serve as a useful infection and immunity model for generalized BHV1 infections of young animals, and that single bovine MAb are protective in this model.

1. Introduction

Bovine herpesvirus 1 (BHV1) causes infectious bovine rhinotracheitis (IBR, McKercher et al., 1955), conjunctivitis (McKercher et al., 1959), infectious pustular vulvovaginitis (Kendrick et al., 1959), infectious pustular balanoposthitis (Huck et al.,

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1971), and abortion (Ormsbee, 1963) in adult cattle, as well as encephalitis (French, 1962a; 1962b) and generalized disease (Van Kruiningen and Bartholomew, 1964) in calves. Subtypes (1.1, 1.2a, and 1.2b – and formerly including 1.3a and 1.3b, now a separate species) were identified by genetic and antigenic analysis (Metzler et al., 1985; Wyler et al., 1989) and were associated with geographic range and prevalence of clinical manifestations (Edwards et al., 1990; van Oirschot et al., 1995; D’Arce et al., 2002). Modified live and killed virus vaccines are produced and marketed for prevention of disease in the U.S. (Anon, 2009) and elsewhere, primarily for the respiratory form which can potentiate secondary pneumonic infection, a disease known as bovine respiratory disease or “shipping fever” (Yates, 1982). The three major envelope glycoproteins of BHV1 are designated gB, gC, and gD. Immunization with each of the glycoproteins has been used to protect calves from lethal combined challenge with BHV1 and *Mannheimia haemolytica* (Babiuk et al., 1987). Definition of the protective immune response to BHV1 at the epitope level through the use of monoclonal antibodies (MAb), particularly those of host origin, could assist in improved vaccine design.

### 1.1. Models of BHV1 Infection

Natural infections with BHV1 have been described in a variety of species. In some cases the investigations were undertaken to determine possible wildlife reservoirs for the virus or closely related viruses, a particular concern in BHV1 eradication programs. Some reports based on serology may in fact be due to infection with closely related herpesviruses (reviewed in Thiry et al., 2006). Even isolated viruses could be mis-identified as BHV1 if the sole characterization method was reaction with BHV1 antiserum. Viruses characterized as “closely related” to BHV1 on initial isolation have since been identified as separate viral species, e.g., cervid herpesvirus 2 (Ek-Kommenon et al., 1986). There have been reports of BHV1 isolation or PCR detection from cattle, goats (Mohanty et al., 1972; Tolari et al., 1990), sheep (Whetstone and Evermann, 1988), mouflon, fallow deer, red deer, roe deer (PCR, Kalman and Egyed, 2005), pronghorn antelope (Hoff et al., 1973), water buffalo (St. George and Philpott, 1972; Ibrahim et al., 1983), wildebeest (Karstad et al., 1983), swine
(Onstad and Saxegaard 1967; Saxegaard and Onstad 1967; Derbyshire and Caplan 1976), mink, and ferrets (Porter et al., 1975). Serological evidence of infection has been cited for cape buffalo (Rweyemamu, 1970), black-faced impala (Karesh et al., 1997), free-ranging (Taylor et al., 1997) and ranched (Sausker and Dyer, 2002) bison, hippopotamus (Kaminjolo et al., 1970), and Asian elephants (Metzler et al., 1990; Bhat et al., 1997).

Experimental infections have been established in cattle (Mckercher et al., 1963; Chow et al., 1964; Snowdon, 1965; Edington et al., 1972; Anon, 2010), goats and sheep (McKercher et al., 1959; Lehmkuhl and Cutlip, 1985; Wafula et al., 1985), mule deer (Chow and Davis, 1964; Nettleton et al., 1988a), red deer (Reid et al., 1986; Nettleton et al., 1988a), reindeer (Nettleton et al., 1988a; 1988b), swine (Woods et al., 1968; Nelson et al., 1972), ferrets (Smith 1978), striped skunks (Lupton et al., 1980a), laboratory rabbits (see below), and eastern cottontail rabbits (Lupton and Reed, 1979). Mice, rats, guinea pigs and the chick embryo are refractory to BHV1 infection (Kendrick et al., 1958; Armstrong et al., 1961; Barenfus et al., 1963; Persechino et al., 1965).

1.2. BHV1 rabbit models

Bindrich (1960) produced vulvovaginitis and conjunctivitis in rabbits with BHV1 but could not serially transfer infection. Armstrong et al. (1961) produced a mild erythematous lesion in rabbits inoculated intradermally (ID) with BHV1, but the virus could not be passaged in this species, and keratitis was not induced when BHV1 was applied to the scarified cornea of the eye. Persechino et al. (1965) produced dermatitis with ID infection, corneal keratitis with intraocular infection, and when the virus was inoculated intracerebrally, meningoencephalitis with flaccid paralysis of the hind limbs. Bwangomoi and Kaminjolo (1973) likewise produced skin lesions in rabbits inoculated ID with BHV1, and they produced epididymitis upon intratesticular inoculation.

Kelly (1977) inoculated rabbits with various doses of a high-titer (>10^8 plaque forming units [pfu] per rabbit) of a high passage (≥50) BHV1.1 (Los Angeles strain). He inoculated adults by the intraperitoneal (IP), intravenous (IV), and intraconjunctival routes. Clinical signs were only observed after intraconjunctival infection, and included hyperemia.
and edema of the conjunctiva, scleral congestion, and swelling of the lids. Microscopic inflammatory lesions in adrenal glands and liver were observed. Pregnant does were inoculated, with no evidence of infection in the concepta. He also inoculated neonates (1-19 days of age) IP and intra-cerebrally. Seven of 13 IP-inoculated neonates (≤7 days old) died, and he noted that 10 day old rabbits were difficult to infect. In the neonates inoculated IP, a severe-fatal generalized infection with multifocal necrosis (most severe in liver and adrenal glands) was observed. Because conjunctivitis was a recognized lesion of BHV1 infection in cattle (Dawson et al., 1962; Mohanty and Lillie, 1970), he suggested that the adult rabbit might be a good model of herpesvirus ocular infection. Because the lesions in the neonates resembled those of aborted bovine fetuses (Kennedy and Richards 1964; Molello et al., 1966), and ultrastructurally resembled cultures of infected bovine kidney cells, he suggested that the neonate rabbit might be useful as an experimental host for studies on the pathogenesis of BHV1 infection.

Lupton et al. (1980b) reported on the infection of rabbits by multiple routes using $10^8$ pfu/ml of moderate (10) passage BHV1.1 (Cooper strain). Injection (IV) of adults (1.0 ml) resulted in a transient (less than 24 hours duration) fever (to 40.6 C) at 24-48 hours post inoculation. No gross lesions were observed. Virus was isolated from adrenal glands and spleen. Foci of fluorescent antibody (FA) staining were observed in the adrenal glands, with staining of scattered individual cells in the spleen and liver. Intraconjunctival (0.2 ml in each sac) exposure in adults resulted in mucopurulent conjunctivitis. Virus was isolated from ocular and nasal secretions, and from the optic and trigeminal nerves. Intradermal (0.1 ml) injection resulted in dermatitis characterized by 24 hours by a hemorrhagic center surrounded by an edematous wheal. Virus was isolated from the serous exudate from the dermal lesion. Fluorescent antibody staining of the tissues immediately surrounding the epithelial ulcer was observed. Intratracheal (1.0 ml) exposure resulted in a transient fever (40.4 and 40.6 C). No other clinical signs were observed. Virus was isolated from nasal and ocular secretions, trachea, lungs and nasal mucosa. Intravaginal (1.0 ml) exposure resulted in mucopurulent vulvovaginitis, by gross and histopathology. Virus was isolated from vaginal swabs, and vulvar and vaginal tissues. Fluorescent antibody staining was observed in the vulvar epithelium.
Infection of dexamethasone-treated adults by the intravenous and conjunctival routes resulted in a systemic infection, with severe conjunctivitis, labored respiration, anorexia and severe depression. Rabbits died or were moribund at days 4 and 7 post-inoculation. Gross lesions included enlarged adrenal glands, hemorrhages on the kidney, multiple foci on the liver, and severe conjunctivitis. Virus was isolated from nasal and ocular swabs, adrenal glands, liver, spleen, and explants of trigeminal and optic nerve fibers. Focal to diffuse zones of FA staining in the adrenal glands and liver, and scattered FA staining in the spleen was observed.

Pregnant rabbits were inoculated IV (1.0 ml) at 17 and 22 days of gestation. In three of four rabbits, fever (40.4 to 40.7 C) for approximately 24 hr was observed, followed by ‘incomplete abortion’ (expulsion of live fetuses or fetid vaginal fluids). The remaining rabbit did not evidence fever, but complete abortion was observed. No other clinical signs were observed. When sacrificed, injected does contained live and dead or decomposing fetuses. Virus was isolated from adrenal glands, placentae, and uteruses. No FA staining was observed on uteruses and placentae. The adrenal glands of aborted rabbits were 2-3 times normal size, with pale corticxes. Virus was not isolated from pools of fetal tissues from aborted or retained fetuses. No lesions were observed in fetal tissues. The cause of BHV1-induced abortion in rabbits appeared to differ significantly from what was reported in cattle. In cattle, virus infection of the placenta was accompanied by viremia and multifocal coagulative necrosis of liver and spleen in the fetus. Abortion occurred 15- to 30 days following BHV1 infections of the dam and several days after fetal death. In rabbits, virus infection and histopathologic lesions were not found in rabbit fetuses, and live fetuses were expelled as early as 18 hours post-inoculation. The authors suggested that the abortions may have been related to the extensive coagulative necrosis observed in the does’ adrenal cortex.

Neonates (4 days of age) were injected IP with 1.0 ml. All died or were moribund 48 hours post inoculation. No gross lesions were observed. Virus was isolated from lungs, kidney, spleen, brain, and adrenal glands. Large foci of FA staining were observed in the liver and adrenal glands. Fluorescent antibody staining of small foci or individual cells was observed in the spleen, brain, and lungs.
The authors observed that the diverse clinical manifestations of BHV1 infection in rabbits were similar to those reported in cattle and included neonatal systemic infection, conjunctivitis, vulvovaginitis, dermatitis, respiratory tract infection, and abortion. Virus isolation and FA staining was associated with lesions from all diseases. The virus isolations from the optic and trigeminal nerves indicated the potential for latent infections in the rabbit. They suggested the rabbit may be well-suited as a laboratory model of the study of BHV1 disease pathogenesis, viral latency, pathogenicity of virus strains, or evaluation of vaccine safety and efficacy.

Rock and Reed (1982) established persistent infection in rabbits after conjunctival inoculation of BHV1.1 (Cooper strain), and reactivated the virus (inoculated eye only) with dexamethasone treatment. They detected BHV1 RNA in the trigeminal ganglion of the latently infected rabbits (Rock et al., 1986), which allowed mapping of the latency-related RNA to a specific region of the BHV1 genome and comparison to HHV1 (Rock et al. 1987). The similarities with pathogenesis of latency in cattle suggested the rabbit would make a useful model of persistent infection. Brown and Field (1990a) inoculated anaesthetized rabbits intranasally using trephine openings, resulting in consistent infections and immune responses. They were able to reactivate the virus using dexamethasone (Brown and Field 1990b).

Chowdhury et al. (1997) infected rabbits with BHV1.1 (Cooper strain) using the trephine method, producing respiratory signs but no infection of lungs or brain. Valera et al. (2005) infected rabbits intranasally with BHV1.1 (Los Angeles strain) using local anaesthesia and instillation of the virus. Clinical signs included nasal discharge, labored breathing, and in some rabbits, conjunctivitis. Intranuclear inclusion bodies, positive immunohistochemistry, and positive PCR reactions in lung tissue were observed. The kidney of one rabbit was also positive by PCR. Virus was recovered from nasal swabs up to six days after inoculation. The authors suggested the model could be used for virulence studies, and to test vaccines and antiviral drugs.
1.3. Passive Immunization for other Alphaherpesviruses in laboratory animal models

Passive immunization has been studied in mouse models for HHV1 or HHV2. Age of mice (1 day to 5 weeks), polyclonal Ab routes (IP, SQ) and doses, virus routes (IN, IM) and doses, and intervals between Ab and virus administration (Ab 24 hr before to 24 hr after virus) varied. However, survival consistently correlated with Ab titer, and inversely correlated with virus dose and time from virus challenge (Luyet et al., 1975; Baron et al., 1976; Oakes and Rosemond-Hornbeak, 1978; Erlich and Mills, 1986).

Dix et al. (1981) administered (IP) mouse MAbs directed against HHV1 gC or gD, as well as hyperimmune rabbit HHV1 and negative serum to 31 day old mice. HHV1 or HHV2 was injected in the footpad 2 hrs before or 24 hrs after immunization. Low-titered MAb preparations protected against HHV1 challenge when administered 2 hrs before challenge, and high-titered ones were protective when administered 24 hrs after challenge. Rector et al. (1982) inoculated 4 week old mice intraorbitally (after scarification) with HHV1 and 4 to 24 hours later IP administered mouse MAb specific for gA/B, gC, gD, or gE. All MAb were protective, although some did not neutralize virus and one was not active in neutralization, complement lysis of infected cells, or ADCC assays. The authors concluded that “the therapeutic effectiveness of a specific monoclonal antibody does not correlate with its immunological reactivity in vitro.” Balachandran et al. (1982) inoculated 4-6 week old mice IP with non-neutralizing mouse MAb against HHV2 gA/B, gC, gD, gE, and gF, then challenged them with HHV2 in the footpad immediately or 3 hours later. Protection correlated with ADCC titers. Bystricka et al. (1997) injected 6-8 week old mice IV with 8 MAbs to three topographically distinct sites on gC and 8 MAbs to two topographically distinct sites on gB. Two hours later HHV1 or HHV2 was injected IP. Protection was observed with many of the MAb, but did not correlate with topographic site or VN titer.

Wathen et al. (1985) injected 22 day old mice IP with each of 4 neutralizing murine MAb, two against each of SHV1 gB and gD, followed by SQ challenge with SHV1. Both anti-gD MAb (directed against two epitopes) protected mice from death, and one anti-gB MAb protected. The murine MAb that did not protect was an IgM Ab requiring complement for neutralization and was directed against the same epitope as the IgG anti-gB MAb that did
protect. Marchioli et al (1988) tested 12 neutralizing MAb directed against SHV1 gB, gC, or gD and swine antiserum by IP inoculation followed by footpad challenge with SHV1. MAb to gC and gD protected, those against gB did not. *In vitro* neutralization titers did not correlate with *in vivo* protection.

Wilks and Coggins (1977) injected 3-4 week old Syrian hamsters IP with 0.1 ml of rabbit antiserum (neutralizing titer 128) to equine herpesvirus 1 (EHV1), 3 days prior to IP EHV1 (Kentucky B strain, hamster adapted) challenge, 3 hours after challenge, or 24 hours after challenge. Treatment 3 days prior increased survival, treatment 3 hours only increased survival time, and treatment 24 hours after had no protective effect.

Stokes et al. (1989) injected 6-8 week old Syrian hamsters intracardiac with 0.1 ml of murine MAb to six EHV1 glycoproteins including gB, gC, and gD 1 hour prior IP or IN challenge with EHV1 (Kentucky D strain, hamster and cell culture passaged). Each of the MAb to gB, gC, and gD conferred protection from challenge - the other MAb did not. The route of virus inoculation influenced the outcome, with MAb treatment reducing tissue virus titers after IP inoculation, but increasing lung titers after IN inoculation in some instances. In the same study, injection with 0.2 ml of hamster antiserum (neutralizing titer 128) 24 hours prior to IP or IN challenge with EHV1 provided protection, injection 24 hours after challenge reduced tissue virus titers, and injection 24 hours prior with a 1:10 dilution had no effect.

This study examined the efficacy of three neutralizing bovine MAb in protection of neonatal rabbits from lethal BHV1 challenge. It is the first use of passive immunization in the model.

2. Materials and Methods

2.1. Animals

New Zealand White rabbits were purchased from a commercial source (Small Stock Industries, Pea Ridge, AR). Mid-term bred female rabbits were purchased and housed in individual cages with cardboard nesting boxes or cardboard-in-plexiglass nesting boxes, and provided commercial, compressed nesting material. Boxes were checked daily and live
births and deaths recorded. Experimental lots ranged from 2 to 24 dams, with 24 being typical. From the 182 pregnant dams eligible for this study, 119 litters with 301 young comprised the population of subjects. Losses were due to death of dams, pregnancy wastage, death of young rabbits before inoculation, and injury death due to inoculation. The inoculated rabbits in the age-dose mortality trails were 4-22 days old at challenge. The inoculated rabbits in the passive immunization experiments were 6-8 days old at challenge. The dams were seronegative to BHV1 by VN.

2.2. Antibody

Preparations of three neutralizing bovine MAb, @BL5C2.870005, .870009, and .870016 (Levings et al., 2012), were used and are hereafter referred to as 870005, 870009, and 870016. Heterohybridoma supernatant fluids (regular or serum-free, as described in Levings et al., 2012) were used unconcentrated or concentrated between 10- and 30-fold using 30,000 molecular weight cutoff ultrafiltration vacuum or centrifugation devices (Amicon, Inc., Beverly, MA) at 4C. One concentrate (870005, titering 2560) was also diluted 1:2 in basal media (calculated titer 1280) and administered. Virus neutralization titers of the MAb preparations ranged from <2 to 2560 (<2 to 2560 for 870005; 3 to 320 for 870009; 18.3 to 1280 for 870016). Preparations of cell culture media concentrated similarly to the heterohybridoma supernatant fluids were used as sham controls. In addition, bovine hyperimmune antiserum to BHV1 (titering 2760) and serial 2-fold dilutions in basal media (calculated titer 1380 through 22) were administered, using matching dilutions of FBS for sham preparations. Antibody ‘doses’ are reported as VN titer of 1ml of inoculum (e.g., 1ml of 1280 titer = 1280, 0.5 ml of 2560 titer = 1280).

2.3. Virus

The viruses used were BHV1, Cooper strain (BHV1.1). Seed virus was received from Dr. T.L. Chow of Colorado State University as fifth cell culture passage of virus isolated from the nasal secretions of an infected calf. Viruses were 3 (all but the 10^7.7
preparation), or 7 cell culture passages from the seed virus cited. Third-passage viruses were challenge viruses used to release batches of USDA-licensed vaccines (Anon, 2010). Virus titers for age-dose mortality trials were $10^{7.0-8.8}$ pfu/ml. Virus titers for passive immunization studies were $10^{7.3-7.5}$ pfu/ml. Eagles’ minimum essential medium (EMEM) was used as a sham virus preparation.

2.4. Virus neutralization assays

Plaque reduction virus neutralization (PRVN) assays were performed as described in the Center for Veterinary Biologics Supplemental Assay Method 119 (Anon 2011), except a high cell culture passage BHV1 was used in place of pseudorabies virus (SHV1), another varicellovirus. Briefly, equal volumes (0.1ml) of test Ab solution and a preparation of virus containing 40-70 pfu/0.1ml) are incubated, placed on cells in 35mm wells, incubated and spread, and then overlaid with semi-solid agar. The cells are fixed and stained and plaques counted. VN titers reported were 50% endpoints calculated by linear regression of log of dilution vs. log of % neutralization (those points between 10 and 90%), or rarely when test dilution data justified, “nearest two-fold.” Calculated titers for dilutions of high-titered preparations were also used as previously described.

2.5. Experimental design

All experiments were conducted under an approved animal use protocol. Trials using intravenous injection of adults and temperature measurement were conducted, but the febrile responses reported in the literature were not observed. A trial using intramuscular challenge of 14-15 day old rabbits was also conducted, but neither clinical signs nor death were observed. Per os and intraocular inoculation of limited numbers of neonates also failed to produce clinical signs. Intracardiac (IC) challenge of young rabbits reproducibly induced virus-specific disease, and was used for all trials reported.

A split litter design was used for all studies. Allocation to treatment was not blinded, and was not totally random -- in some litters allocation was biased by rabbit size, with smallest (and largest) rabbits being assigned to the sham-challenge group. All rabbits were observed
daily for 14 days post-challenge for clinical signs or death. No clinical signs other than
depression were observed prior to death, so only deaths were scored. Death observed on the
day after either IC inoculation was recorded as injury (non-specific) death. Therefore, only
deaths on days 2-14 were counted. Numbers of rabbits per litter surviving all inoculations
ranged from 1-10. Only groups of 2 or more were used for analysis. Analysis was done by
virus dose and antibody identity x dose groups, across litters and lots. Sham inoculation data
was pooled by type (sham virus, sham Ab - media control; and sham Ab – FBS dilutions).
All results reported were obtained using this protocol.

For the age-dose mortality study, three age-groups were tested: 4-7 day old (n=29
rabbits, 5 virus titers 10^{7.0-8.0} and sham); 13-14 day old (n=23, 3 virus titers 10^{7.7-8.3}
and sham); 20-21 day old (n=12, 2 virus titers 10^{8.0-8.3} and sham). At least 20% of each litter was
assigned to the sham challenge group.

For the passive immunization studies, the split litter design initially included group a
= true antibody, true virus; group b = sham antibody, true virus, group c = true antibody,
sham virus; group d = sham antibody, sham virus. 1.0 ml of antibody or matching sham
preparation was given IC on 0 days post inoculation (dpi). Circulating Ab titers were not
assessed due to the small size of the rabbits and attendant risk of sample bleeding. 1.0 ml of
virus or sham preparation was given IC on 1 dpi. In a few cases other volumes were given to
produce alternate doses, or rarely, due to injection difficulties. Preliminary experiments on
20 litters split into groups a through d established no mortality in groups c (n=17) and d
(n=16) other than injury deaths at day 1 post-inoculation, so subsequent litters were split into
groups a and b only. For passive immunity results, results were pooled for two virus doses
(10^{7.3} and 10^{7.5}), for SP2/0 and EMEM media controls, for all dilutions of FBS, and for all
concentrations of media.

3. Results

Virus-specific deaths were observed 2-13 days (only 2 occurred >9 days) post virus
inoculation. In the preliminary age-dose titration studies, survival appeared to increase with
subject age at a given virus dose. For example, at dose 10^{8.0}, survival was 12.5% (n=8) for 4-
7 day old, 60% (n=5) for 12-14 day old, and 100% (n=3) for 20-21 day old rabbits. Survival appeared to decrease with increasing virus dose at a given rabbit age, for rabbits ≤14 days old (no deaths observed in 20-21 day old rabbits at two doses tested, $10^{8.0}$ and $10^{8.3}$). For example, for 13-14 day old rabbits, survival was 100% (n=3) for $10^{7.7}$, 60% (n=5) for $10^{8.0}$, and 16.7% (n=6) for $10^{8.3}$. An age-dose combination was selected for the passive immunization studies to target 60-90% mortality in unprotected rabbits – 6-8 days of age, and $10^{7.3-7.5}$ pfu.

All Ab treatments improved the survival of 6-8 day old virus-challenged rabbits tested in the passive immunity study (see figures 1 and 2). Survival appeared to increase with the titer of treatment antibody. For example: using 870009, survival was 25.0% (n=4), 64.3% (n=14), and 80% (n=5) for preparations of titer 2-4, 184, and 320, respectively; and using 870016 survival was 33.3% (n=3) and 100% (n=4) for preparations of titer 18.3 and 606.4 respectively. With one exception (@BL5C2.870005, 1:2560), all Ab preparations titering ≥320 resulted in ≥80% survival, compared to 41.9% (n=31) for all FBS dilutions, 35.1% (n=37) for all media control preparations, and 10.3% (n=29) for $10^{7.5}$ challenge control (no sham or Ab passive immunization) groups. A clear order of Ab titer-survival was not observed, as illustrated in the antiserum dilution series (see figure 2).

For some Ab treatments with incomplete protection (e.g., 870009 titer 184, antiserum titers 22 and 86), the onset of mortality and the date of e.g., 80% survival was delayed relative to the ‘0 titer’ group (see figures 1 and 2).

4. Discussion

This is the first report of passive immunization to BHV1 using the laboratory rabbit model. Intracardiac immunization and challenge of young rabbits were used as a model of BHV1 infection. A relationship between virus-specific mortality, age, and challenge titer was observed. An inverse age vs. mortality relationship was observed, consistent with previous reports of BHV1 infection in rabbits (Kelly, 1977). A direct relationship between virus titer and mortality was also observed, consistent with reports on HHV1/2 infection of mice (Baron et al., 1976). Testing additional virus titers in the 4-5 and 6-8 day old groups
Figure 1. Protection by three bovine monoclonal antibodies (MAb) of 6-8 day old rabbits from intracardiac challenge with bovine herpesvirus 1\(^1\). (A) @BL5C2.870005; (B) @BL5C2.870009; (C) @BL5C2.870016.

\(^1\) 10\(^{7.3-7.5}\) pfu bovine herpesvirus 1.

Antibody titer is virus neutralization titer of MAb concentrates. Results from media concentrated to match the MAb are pooled and represented as “0” titer.

Group n is number of rabbits in each group. No group <5 was selected for presentation except for @BL5C2.870005, for which no MAb group ≥5 was available.
Figure 2. Protection by dilutions of bovine hyperimmune antiserum (AS) of 6-8 day old rabbits from intracardiac challenge with bovine herpesvirus 1\(^1\).

\(^1\) \(10^{7.3-7.5}\) pfu bovine herpesvirus 1.

Antibody titer is virus neutralization titer of AS dilutions. Results from fetal bovine serum diluted to match the AS are pooled and represented as “0” titer.

Group n is number of rabbits in each group. No group <5 was selected for presentation.

...may have provided clearer demonstration of both the age and titer effects. This study did not examine the effect of changing the intervals between immunization and challenge, the ability to passage the virus in rabbits, or the pathology associated with the fatal infection of the neonate rabbits, as was done in some previous BHV1-rabbit or HHV1/2-mouse infection studies. Using the survival data obtained in preliminary experiments, a dose and age of rabbits was chosen to result in reproducible - but not overwhelming - disease, to enable demonstration of protection.
An inverse Ab VN titer to mortality relationship was observed for all but one (@BL5C2.870005, VN titer 2560) Ab preparation used, although a clear order of Ab titer-survival was not observed. The Ab-survival relationship was consistent with some studies of HHV1/2 polyclonal antibody in HHV1/2 infection of mice (Baron et al., 1976; Erlich and Mills, 1986), but not with some murine MAb studies (Bystricka et al., 1987). Non-neutralizing BoMAb (to gB, gC, gD, or other viral proteins) were not tested in this study. The increased mortality of the high-titered @BL5C2.870005 group compared to lower titered groups using the same bovine MAb may be due to toxicity of the preparation. The time to death was shorter in the immunized group than in the sham-immunized virus-challenged controls, and injury deaths due to Ab appeared to be higher with this bovine MAb (e.g., 71.4% [n=21] for the concentrated preparations titering 52 and 237), suggesting toxicity may have been an issue.

This IV-IV model of protection from generalized BHV1 disease in young rabbits after challenge may be suitable for the study of generalized BHV1 disease in young cattle, or even generalized alphaherpesvirus diseases in the young of other species, including swine and humans. The neonatal mortality without clinical signs (other than depression) is consistent with previous reports (Kelly, 1977; Lupton et al., 1980b). The rabbit passive immunization to BHV1 model might best be compared to the colostral antibody protection of bovine neonates from bovine BHV1 generalized disease (Mechor et al., 1987). It is also analogous to the murine MAb protection of porcine neonates from SHV1 generalized disease (Marchioli et al., 1988). In both cases, however, the route of immunization and challenge differed from the current study. The potential of the BoMAb described here or any MAb to BHV1 for passive immune prevention or therapy of cattle for generalized BHV1 disease has not been tested.

The specific protection observed in this study support the use of various rabbit models to study the immunity of other BHV1 disease syndromes (respiratory disease, conjunctivitis, vulvovaginitis, latency, and encephalitis), if and when the mechanisms of disease can be demonstrated to be similar. Care should be taken in extrapolating from disease models in non-host species, particularly when the disease syndrome produced is dissimilar to the target syndrome in the host (e.g., BHV1 abortion). The usual target for
vaccination is the IBR syndrome, for which this neonate generalized disease model may not be appropriate.

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References


CHAPTER 5. VIRUS, STRAIN, AND EPITOPE SPECIFICITIES OF NEUTRALIZING BOVINE MONOCLONAL ANTIBODIES TO BOVINE HERPESVIRUS 1 GLYCOPROTEINS gB, gC, and gD, WITH SEQUENCE AND MOLECULAR MODELS

A paper to be submitted to *Veterinary Immunology and Immunopathology*

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Abstract

Three bovine monoclonal antibodies (BoMAb) raised to bovine herpesvirus (BHV) 1.1 were tested for reactivity to two isolates of BHV1.1, one of BHV1.2, and two of BHV5 in virus neutralization and indirect fluorescent antibody assays. They were also tested with other herpesviruses infecting cattle and other mammalian alphaherpesviruses, and found negative. Their BHV1.1 epitope specificity was defined by competitive ELISA using peroxidase-labeled murine monoclonal antibodies (MuMAb) that had been previously characterized. The anti-gB BoMAb reacted strongly with BHV1.1 and BHV1.2, and poorly or not at all with BHV5. It competed with a MuMAb specific for a BHV1.1 gB epitope previously shown to only partially cross-react between BHV1 and BHV5. BHV5 gB has nearly the same amino acid sequence as BHV1.1 in the epitope region, but molecular modeling suggests the lack of cross-reactivity is due to masking of the epitope in BHV5 by an adjacent region, which has significant sequence differences between BHV1.1 and BHV5. The anti-gC BoMAb reacted strongly with one isolate of BHV1.1 and BHV1.2, less well with a heterologous isolate of BHV1.1, and poorly or not at all with BHV5. It did not compete with any of the anti-gC MuMAb tested. The anti-gD BoMAb reacted strongly with all BHV1.1, 1.2, and BHV5 isolates tested. It competed with two MuMAb specific for gD

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epitope(s) previously shown to not cross-react between BHV1.1 and BHV5. Sequence analysis and molecular modeling suggest the cross-reactivity of the anti-gD BoMAb is due to it reacting with an epitope-adjacent region or regions conserved between BHV1.1 and BHV5, but not with other alphaherpesviruses. The results suggest the usefulness of combining *in vitro* biological with sequence or structure modeling data to investigate important epitopes of infectious agents.

1. Introduction

The infectious bovine rhinotracheitis (IBR) virus was first isolated in the United States (US) in 1956 (Madin et al., 1956). Infectious pustular vulvovaginitis (IPV) virus was first isolated in Canada and the US in 1958 (Greig et al., 1958, Kendrick et al., 1958). It was indistinguishable from IBR (Gillespie, 1959). Bovine encephalitis virus was first isolated in Australia, and found to be closely related to the IBR virus (French, 1962). It was subsequently identified in other countries including the US (Eugster et al., 1974). Based on molecular similarities and differences the IBR, IPV, and the encephalitis viruses were designated BHV1.1, BHV1.2, and BHV1.3, respectively (Engels et al., 1986; Metzler, 1986). Bovine encephalitis herpesvirus (BEHV) was also suggested for the encephalitic virus (Studdert, 1989). The currently accepted name is BHV5 (Davison et al., 2005). Vaccination or prior infection with BHV1.1 protected calves from histologic lesions of encephalitis resulting from BHV5 infection (Cascio et al., 1999).

Virus-specific proteins including 11 glycoproteins were identified from purified IBR virus (Misra et al., 1981). Monoclonal antibodies (MAb) were used to identify their characteristics, including their inclusion in complexes, molecular weight, glycosylation, virion location, and the biological activity of the glycoprotein through MAb activity, e.g., neutralization with or without C, lysis of infected cells, neutralization before and after attachment (van Drunen Littel-van den Hurk et al., 1984; Collins et al., 1984; Marshall et al., 1986; Okazaki et al., 1986; Hughes et al., 1988). Glycoprotein complexes were variously identified by their positions in PAGE gels, by their molecular weights, by apparent homology with the glycoproteins of other herpesviruses (including human herpesvirus 1, HHV1), and
finally in accordance with the homologous HHV1 glycoprotein. Examples are: gB (named GVP 6/11a/16, 130K/74K/55K, gII, then gB); gC (named GVP 3/9, 180K/91K, gIII, then gC); and gD (named GVP 11b, 150K/77K, gIV, then gD). MAb for the same glycoprotein were identified that possessed key (e.g., neutralization) characteristics and that did not (Collins et al., 1984). BHV1.1, BHV1.2, and BHV5 were differentiated using PAGE gel patterns, cross-neutralization, and MAb (Metzler et al., 1986; Friedli and Metzler 1987; Rijsewijk et al., 1999). The numbers of neutralizing MAb found, the numbers of epitopes associated with neutralization, and the strength of neutralization, led to conclusions regarding major vs. less important neutralization targets (Collins et al., 1984; Marshall et al., 1986).

Panels of MAb were assembled defining overlapping and unique epitopes (van Drunen Littel-van den Hurk and Babiuk, 1985; Hughes et al., 1988; Ayers et al., 1989). Epitopes were determined to be linear or conformational using western blots and ELISA with denatured protein (van Drunen Littel-van den Hurk et al., 1984; van Drunen Littel-van den Hurk and Babiuk, 1985, Hughes et al., 1988). Proteolytic peptide mapping (Marshall et al., 1986), glycosylation effects (van Drunen Littel-van den Hurk et al., 1990a), the association of fusion domains with epitopes (van Drunen Littel-van den Hurk et al., 1992), and glycoprotein modification through passage in non-host cell lines (Shen et al., 1991) were studied. The host serologic response to epitopes was examined using competition with MAb (Ayers et al, 1989; van Drunen Littel-van den Hurk et al., 1990b).

Restriction endonuclease (RE) analysis was used to differentiate BHV1.1, BHV1.2, and BHV5 (and subgroups of 1.2 and 5) (Engels et al, 1981; Misra et al., 1983; Metzler et al, 1985; Brake and Studdert 1985), although a continuum of RE patterns between the BHV1.1 and BHV1.2 has also been suggested (Christensen et al., 1996). The genome maps of BHV1.1 and BHV1.2 (Mayfield et al., 1983) and BHV5 (Engels et al., 1986) were determined and percent identity of BHV1.1 to BHV1.2 (95%) and BHV1.1 to BHV5 (~85%) calculated. Cross-hybridization also provided percent identity estimation -- 95% between BHV1.1 and 1.2 (Seal et al., 1985); >80% between BHV1.1 and BHV5 (Bulach and Studdert, 1990), as well as localization of areas with greater and lesser identity (Bulach and Studdert, 1990; Whetstone et al., 1993; Delhon et al., 2003). Identity was found to be conserved within BHV1.1 and BHV5 groups of isolates. The relationships of other ruminant
herpesviruses to BHV1 was also clarified, and phylogenies constructed (Bulach and Studdert, 1990).

Cloning of RE fragments, with sequencing of partial and complete individual genes, first allowed gene assignment including those for major glycoproteins based on homology with HHV1 and other herpesviruses, e.g., BHV1.1 gB (Whitbeck et al., 1988), gC (Fitzpatrick et al., 1989), and gD (Tikoo et al., 1990). It also allowed sequence comparison between BHVs (Abdelmagid et al., 1995) and amino acid (aa) identity calculations for individual glycoproteins, e.g., gB, 91.9% between BHV1.1 and BHV5 (Ros and Belak 2002); gC, 98.5% between BHV1.1 and BHV1.2, 86.8% between BHV1.1 and BHV5 (Hecht et al., 1995); and gD, 79.9% between BHV1.1 and BHV5 (Gabev et al., 2010). Phylogenies of ruminant herpesviruses based on individual genes also have been constructed (Ros and Belak 1999; 2002; Esteves et al., 2008; Momtaz, 2009).

The primary aa sequence allowed analysis of likely disulfide bridges, glycosylation sites, signal and transmembrane regions, and hydrophobic and –philic areas of the glycoproteins, primarily: gB (Whitbeck et al., 1988); gC (Fitzpatrick, 1989); gD (Tikoo et al., 1990). Secondary structures could also be interpreted from the sequences of gC, (Fitzpatrick et al., 1989; 1990) and gD, (Tikoo et al., 1993). Whole genome sequences, protein sequences, mutation, expression, and recombination allowed studies on molecular processing and examination of biological differences between herpesviruses and between BHV. These included studies of major and minor glycoproteins, receptor use, neurovirulence, and substituting whole glycoproteins between BHV1 and BHV5 (Tikoo et al., 1993; Kopp et al., 1994; Seal and Whetstone, 1994; Baranowski et al., 1996; Schwzyzer and Ackermann, 1996; Spear et al., 2000; Delhon et al., 2003; Al-Mubarak et al., 2004; Gabev et al., 2010). Expressed peptides and synthetic peptides designed from sequence information allowed further refinement of epitope location (Abdelmagid et al, 1995; Chowdhury, 1995; 1997; Rijsewijk et al., 1999), including those for anti-idiotypes (Harihanan, 1991).

Five glycoproteins are involved in HHV1 attachment and entry, as well as fusion of infected cells: gB, gC, gD, gH, and gL (reviewed in Spear et al, 2000; Rey, 2006). It is believed similar mechanisms apply to all alphaherpesviruses except those lacking gD, e.g., Varicella-Zoster virus (HHV3). gC first binds to the cell membrane proteoglycans, although
binding by other glycoproteins e.g., gB and gD, can contribute to binding, and gC is not required for attachment (Engels and Ackermann, 1996). This is followed by the homodimer gD binding to one of a few cellular receptors that vary by cell type and species, although they are usually homologous (Connolly et al., 2001). The binding results in a conformational change in gD that triggers virus envelope – cell membrane fusion by gB or gH/L. gB is a homotrimer with fusion domains similar to the vesicular stomatitis virus fusion glycoprotein (44), and homologues within the herpesviruses are highly conserved. A furin protease site is present on almost all gB homologues (including BHV1 gB), but not on HHV1, HHV2, or HHV4. gH/L is a heterodimer – gH contains domains consistent with fusion, and gL is needed to maintain the correct structure of gH.

Crystal structures of HHV gB (Heldwein et al., 2006) and gD (Pilling et al., 1999; Carfi et al., 2001; Krummenacher et al., 2005) have been determined, which in turn allowed homologous models to be constructed for BHV1.1 (gB, Ros and Belak, 2002 and gD, Tikoo et al., 1990; Goltz et al., 2006), BHV1.2 (gD, Leung-Tack et al., 1994), and BHV5 (gD, Abdelmagid et al., 1995) glycoproteins. Other herpesvirus glycoproteins (e.g., gC) have been partially modeled from crystal structures of other molecules. The determination of the structure of HHV1 functional domains and their relationship to epitopes (Heldwein et al., 2006) may also contribute to understanding BHV1/5 functional regions and epitopes.

This study examined the reactivity of three bovine MAb (BoMAb) with isolates of the closely related BHV1.1, BHV1.2, and BHV5 viruses, their reactivity with other herpesviruses, as well as their competition with well characterized murine MAb (MuMAb) to BHV1.1. Sequences and structure models of glycoproteins of the three viruses were then generated or accessed to help explain the observations.

2. Materials and Methods

2.1. Viruses

Two BHV1.1 isolates were used: Cooper (CO, York et al., 1957; Madin et al., 1956), 10^7.7 tissue culture infective dose 50 (TCID_{50})/ml, from a preparation used by the Center for
Veterinary Biologics (CVB) to evaluate IBR vaccines in cattle; and Los Angeles (LA, York et al., 1957, Madin et al., 1956), 10^{7.6} TCID_{50}/ml, from a preparation obtained from the American Type Culture Collection (ATCC). One BHV1.2 isolate was used: K-22, 10^{7.8} TCID_{50}/ml, from a preparation kindly provided by C.A. Whetstone. Two BHV5 isolates were used: EC-1 (1968 Texas isolate, Eugster et al., 1974), 10^{8.0} TCID_{50}/ml, and EC-2 (1989 Texas isolate, d’Offay et al., 1993), 10^{7.0} TCID_{50}/ml, each from preparations kindly provided by C.A. Whetstone. BHV2 (bovine mammilitis virus), BHV4 (DN599 isolate, Movar virus), Alcelaphine herpesviruses 1 (AlHV1, WC-11 strain, malignant catarrhal fever virus), and AlHV2 (840412 isolate, hartebeest malignant catarrhal fever virus) were provided by the National Veterinary Services Laboratories (NVSL). The suid herpesvirus 1 (SHV1, pseudorabies virus), equine herpesvirus 1 (EHV1, equine abortion virus), feline herpesvirus 1 (FHV1, feline viral rhinotracheitis virus), and canine herpesvirus 1 (CHV1) preparations used were from preparations used by CVB for cell culture titrations and neutralization assays. Human herpesvirus 1 (HHV1) was from a preparation obtained from the ATCC.

2.2. Antibodies

Nude mouse ascites of three BoMAb (Levings et al., 2012) were used for bovine alpha-herpesvirus VN and IFA assays: anti-gB @BL5C2.870009; anti-gC @BL5C2.870005; and anti-gD @BL5C2.870016, hereafter referred to as 870009, 870005, and 870016, respectively. VN titers of ascites were 512-4096 and IFA titers were 1600-3200. Three bovine polyclonal antisera were used: anti-BHV1 (BHV1 AS, produced using Cooper isolate); anti-bovine viral diarrhea virus (BVD AS, produced using 4 isolates), and anti-parainfluenza 3 virus (PI3 AS, produced using an RF-like isolate). The BVD and PI3 antisera had been demonstrated to not react in VN or IFA assays with BHV1.1. A negative control ascites was produced in BALB/c mice using the SP2/0 cell line (SP2/0). All antibody preparations were stored frozen. Prior to use, they were thawed, low-speed centrifuged to clarify, heat treated (56 C x 35 min) to inactivate complement (C), and stored at 4 C. Other preparations of BoMAb (e.g., hollow-fiber cultures, as described in Levings et al., 2012) were used for some of the herpesvirus specificity assays.
The MuMAb conjugated and used in cELISA assays have been described previously: D9, F2, G2, C11, G1 (Collins et al., 1984); 4407, 4807, 5106, 5606, 1507, 1808, 2905, 3002, 6003, 1102, 1106, 3402, 4906, 5006 (Marshall et al., 1986); 1E11, 1F8, 1F10, 3F3, 5G2 (van Drunen Littel-van den Hurk et al., 1985); 10C2, 3C1, 3E7, 9D6 (Hughes et al., 1988); and 3D9S (van Drunen Littel-van den Hurk and Babiuk, 1986).

2.3. Cell cultures and media

The Madin Darby bovine kidney (MDBK) cell line, minimum essential media with supplements (MEM), growth media, and maintenance media as described (Anon, 2011a) were used for BHV1.1, BHV1.2, and BHV5 virus expansion as well as virus neutralization (VN) and indirect fluorescent antibody (IFA) assays. All FBS used was found free of BVDV virus and antibody, then gamma-irradiated.

2.4. Virus Neutralization (VN) assays

Constant antibody-varying virus (alpha) VN assays were performed using BHV1.1, BHV1.2 and BHV5. Viruses were diluted $10^{-2}$ to $10^{-9}$ in MEM with 2% guinea pig complement (GPC). 150 ul of each of the antibody preparations (neat) and MEM as a control were mixed with 150 ul of each dilution of each of the viruses (final concentration of 1% GPC) in 96 well plates for neutralization. The plates were incubated for 2 hours (agitated at 1 hour) in a 37 C, humid, 5% CO$_2$ incubator. Cell culture inoculation and incubation followed standard CVB assay methods (Anon 2011a). Cytopathology was read using light microscopy (40X, 100X) on days 3, 4, 5, and 6. Endpoint virus titers at day 6 were calculated using the Reed-Muench method (Reed and Muench, 1938).

Constant virus-varying antibody (beta) VN assays were performed using EHV1 and standard CVB protocols using homologous antiserum and MEM 10% horse serum as positive and negative controls respectively. FHV1 and CHV1 beta VN assays were performed by CVB using standard CVB protocols (similar to Anon 2011a and 2011b), using homologous antiserum as a positive control, and SP2/0 ascites and FBS as negative controls.
2.5. **IFA assays**

25 ul of each of the five BHV1.1, 1.2, and BHV5 viruses diluted in MEM were inoculated into each of 8 wells of 96 well plates containing one-day-old MDBK cells from which the growth media had been decanted. All viruses were diluted to contain $100\pm26$ TCID$_{50}$ per inoculum. Heterologous viruses were diluted to contain additional doses to ensure adequate infection and detection. MEM was inoculated into 8 wells as a negative control. Plate incubation, fixation, storage and preparation were as described (Levings et al., 2012). Each antibody preparation was diluted 1:100 in PBS and 50 ul placed in each well. 50 ul of PBS was added to equivalent wells as a negative control. The plates were incubated 2 hours at RT. Further assay steps including observation and photography were as described (Levings et al., 2012).

IFA assays using other viruses were performed using similar methods, using cell cultures of the virus’ host species or other permissive cultures. Homologous antisera (or direct conjugate, for HHV1) was used as a positive control, and heterologous (anti-bovine parvovirus, as described in Levings et al., 2012) BoMAb preparations (for BHV2, BHV4, SHV1) or SP2/0 ascites (for AlHV1 and AlHV2) were used as negative controls. AlHV1 and AlHV2 assays were performed by NVSL personnel using a 1:20 dilution of ascites and infected bovine turbinate (AlHV1) and lamb kidney (AlHV2) cells.

2.6. **Competition ELISA**

Competition ELISA tests between horseradish peroxidase (HRPO)-conjugated MuMAb and nude mouse ascites containing the three BoMAb were performed as described previously (Ayers et al., 1989). Briefly, ascites fluid dilutions were added to microtiter plates coated with 200 ng per well of purified BHV1. After a 30 min incubation at 37 C, HRPO-conjugated MuMAb was added without washing, and the plates were incubed for another 30 min at 37 C. Plates were then washed, substrate added, stopped, and read at 492 nm.
Competition was calculated as percent reduction relative to the conjugate without competing Ab

2.7. Sequence analysis

Amino acid (aa) sequences were accessed on UniProtKB (http://www.uniprot.org/; UniProt Consortium, 2010; Jain et al., 2009): BHV1.1 Cooper isolate gB (P12640) and gD (Q76PF1); BHV1.2 K-22 isolate gB (Q9Q0A6 partial sequence) and isolate ST gD (Q08100); BHV5 N565 isolate gB (Q9QAP7) and TX89 (equivalent to EC-2) gD (Q65535); and HHV1 strain KOS gB (P06437) and gD (P57083). Additional alphaherpesvirus gD sequences were also accessed: SHV1 (Q8B3S6); EHV1 (P24872); FHV1 (Q89634); and CHV1 (O41524). Sequence alignments were done using ClustalW2 (Larkin et al., 2007) within UniProtKB and lalign (http://www.ch.embnet.org/software/LALIGN_form.html; Huang and Miller, 1991).

2.8. Molecular modeling

The model for BHV1.1 gB was constructed from the accessed aa sequence using SWISS-MODEL (http://swissmodel.expasy.org/; Arnold et al., 2006; Kiefer et al., 2009; Peitsch, 1995) in automatic mode (Arnold et al., 2006; Schwede et al., 2003; Guex and Peitsch, 1997). The program used the HHV1 gB crystal structure 2GUM as a template. The BHV1.1 gB model was compared with the already constructed BHV5 gB model (Q9QAP7, which also used 2GUM as a template), accessed from the SWISS-MODEL Repository release 10.2.1 (Kiefer et al., 2009; Kopp and Schwede, 2004), and to the HHV1 gB crystal structure accessed at the Research Collaboratory for Structural Bioinformatics protein data bank (RCSB PDB) (http://www.pdb.org; Berman et al., 2000).

The models for BHV1.1 gD, BHV1.2 gD, and BHV5 gD were constructed from the accessed aa sequences using SWISS-MODEL in automatic mode. The program used the HHV1 gD crystal structure 2c36 as a template. The gD models were compared to each other, compared to models for the same molecules already constructed using the HHV1 gD crystal
structure 1jma as a template (accessed from the SWISS-MODEL Repository), and compared to the HHV1 gD crystal structures 2c36 and 1jma accessed from the RCSB PDB. RasMol version 2.7.4.2 (http://rasmol.org/; Sayle and Milner-White, 1995; Bernstein, 2000) was used to visualize and analyze PDB files.

3. Results

3.1. Virus Neutralization (VN) Assays

Ascites containing each of the 3 BoMAb were reacted with the 5 bovine alphaherpesvirus isolates. Results are shown in figure 1. The anti-gB BoMAb 870009 and anti-gC 870005 were specific for BHV1 isolates, although 870005 neutralized the LA BHV1.1 isolate less than the homologous CO BHV1.1 isolate, and neutralized the BHV1.2 isolate more than either BHV1.1 isolate. The anti-gD BoMAb 870016 and the anti-BHV1 bovine polyclonal antiserum neutralized all isolates completely. The 3 BoMAb tested were negative by VN with EHV1, FHV1, and CHV1.

3.2. Indirect Fluorescent Antibody Assays

Ascites containing each of the 3 BoMAb were reacted with fixed cell cultures infected with the five bovine alphaherpesvirus isolates. Results are shown in table 1 and figure 2. The anti-gB BoMAb 870009 was specific for BHV1 isolates. The anti-gC 870005 reacted more weakly with the isolates EC-1 and EC-2 than with the homologous virus CO. The anti-gD bovine MAb 870016 and anti-BHV1 polyclonal antiserum reacted strongly with all isolates. Equivalent results were observed in plates fixed 1 day and 2 days after virus inoculation and in wells inoculated with 100 or alternate TCID\textsubscript{50}. When the three ascites were retested at higher concentrations (1:30 and 1:50 rather than 1:100), the reactivity was increased slightly where it was weak, but the differences were still evident. A weak reaction was observed using 870009 and 870005 with BHV4-infected cell cultures, but not using 870016. The three BoMAb were negative by IFA with BHV2, SHV1, and HHV1.
Figure 1: BHV1-specific and non-specific neutralization\(^1\) by three BHV1.1-generated bovine MAb\(^2\) and controls.

\(^1\) Reported in log\(_{10}\) virus neutralized per 0.025 ml inoculum, compared to the geometric mean of the two media titers. Because the lowest dilution examined was 10\(^{-2}\), if none of the wells at any dilution evidenced virus infection, the highest possible titer of the preparation was 10\(^{1.50}\). As a result, all neutralized titers over 4.00 are greater than or equal to the value shown.

\(^2\) For explanation of virus, bovine MAb, and antiserum abbreviations please refer to notes for table 1.

3.3. Competition ELISA (cELISA)

Ascites containing the 3 BoMAb were competed against conjugated MuMAb directed against the epitopes of gB, gC, and gD. Results are shown in table 2 and figures 3-4. The gB-specific BoMAb 870009 competed with the MuMAb conjugate 1F10. The gC-specific BoMAb 870005 did not compete with any of the 9 anti-gC MuMAb conjugates tested. The gD-specific bovine MAb, 870016, competed with two MuMAb conjugates, 3402 and 9D6.
Table 1: Bovine herpesvirus (BHV) 1-specific and non-specific reactions in indirect fluorescent antibody assays by three BHV1.1-elicited bovine MAb.

<table>
<thead>
<tr>
<th></th>
<th>BHV1.1</th>
<th>BHV1.2</th>
<th>BHV5</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>CO$^1$</td>
<td>LA$^2$</td>
<td>K-22$^3$</td>
<td>EC-1$^4$</td>
</tr>
<tr>
<td>870009$^7$</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>870005$^5$</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>870016$^9$</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>BHV1 AS$^{10}$</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SP2/0$^{11}$</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BVD AS$^{12}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PI3 AS$^{13}$</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 BHV1.1 Cooper isolate  
2 BHV1.1 Los Angeles isolate  
3 BHV1.2 K-22 isolate  
4 BHV5 EC-1 isolate  
5 BHV5 EC-2 isolate  
6 uninfected cell control  
7 anti-BHV1.1 glycoprotein B (gB) @BL5C2.870009  
8 anti-BHV1.1 glycoprotein C (gC) @BL5C2.870005  
9 anti-BHV1.1 glycoprotein D (gD) @BL5C2.870016  
10 polyclonal bovine antiserum to BHV1.1  
11 ascites to (non-secreting) murine hybridoma SP2/0  
12 polyclonal bovine antiserum to bovine viral diarrhea virus  
13 polyclonal bovine antiserum to parainfluenza 3 virus

3.4. Sequence analysis of BHV1.1, BHV1.2, and BHV5 gB and gD

The alignment of BHV1.1 and BHV5 gB showed high identity - 92.3% in 948 aa overlap (1-932 BHV1.1, 1-947 BHV5), with a score of 0. The ClustalW alignment (figure 5, table 3) shows identity is also high (91.8%) in the BHV1.1 1F10 epitope region (BHV1.1 gB aa 380-440). The amino-adjacent region has high identity, but the carboxy-adjacent region has lower identity, particularly a 50 aa region starting 50 aa carboxy-adjacent of the epitope and continuing toward the carboxyl terminus (78.0% identity). Only a partial (219 aa) BHV1.2 gB sequence was available (aa 192-410 of BHV1.1), which covers the amino-adjacent area discussed, and some of the epitope. It had 100% sequence identity with BHV1.1 over those aa.
Figure 2: BHV1\textsuperscript{1}-specific and non-specific reactions in indirect fluorescent antibody assays by three BHV1.1-generated bovine MAb\textsuperscript{2}

\textsuperscript{1} For explanation of virus, bovine MAb, and antiserum abbreviations please refer to notes for table 1

A, B, C, D = anti-gB BoMAb 870009 with isolates CO, LA, K-22, and EC-1, respectively

E, F, G, H = anti-gC BoMAb 870005 with isolates CO, LA, K-22, and EC-1, respectively

I, J, K, L = anti-gD BoMAb 870016 with isolates CO, LA, K-22, and EC-1, respectively

M = BHV1 AS with isolate CO; N = SP2/0 with isolate CO; O = BHV1 AS with uninfected cells; P = BHV1 antiserum with isolate EC-1
Table 2: Epitope identification of three anti-BHV1.1 bovine MAb using competitive ELISA with panels of characterized murine MAb.

<table>
<thead>
<tr>
<th></th>
<th>gB</th>
<th></th>
<th>gC</th>
<th></th>
<th>gD</th>
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<tr>
<td></td>
<td>Epitope (^1)</td>
<td>MAb(^2)</td>
<td>870009(^3)</td>
<td></td>
<td>Epitope</td>
<td>MAb</td>
</tr>
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<td></td>
<td>Competed</td>
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<td></td>
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<td></td>
<td>G1</td>
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<tr>
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<td></td>
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<td>V</td>
<td>1F10</td>
<td>+</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(^1\) Epitope nomenclature varies with the source of MAb and work done with each. System used here follows Collins, et al. 1993.

\(^2\) Order of MAb follows established epitope maps (Ayers et al., 1989) for each of the glycoproteins and define overlapping and unique epitopes.

\(^3\) For explanation of bovine MAb identities please refer to notes for table 1.
Figure 3: Epitope identification of an anti-BHV1.1 \(^1\) gB bovine MAb (870009) using competitive ELISA with selected murine MAb. Shown are data for the competing (1F10) and a representative non-competing (3F3) murine MAb.

\(^1\) For explanation of virus and bovine MAb abbreviations please refer to notes for table 1.

Figure 4: Epitope identification of an anti-BHV1.1 \(^1\) gD bovine MAb (870016) using competitive ELISA with selected murine MAb. Shown are data for the two competing (3402 and 9D6) and a representative non-competing (3D9S) murine MAb.

\(^1\) For explanation of virus and bovine MAb abbreviations please refer to notes for table 1.
Figure 5. Alignment of amino acid sequences of BHV1.1\(^1\) and BHV5 gB demonstrating areas of high and lower identity.

Dark shading indicates identity, moderate shading indicates lack of identity but presence of amino acid. Symbols under alignment: * = identity; : = conservation (e.g., charge); . = semi-conservation (shape).

\(^1\) For explanation of virus and glycoprotein abbreviations please refer to notes for table 1.
Table 3. BHV5$^1$ amino acid (aa) sequence identities with BHV1.1 (%) of select gB and gD epitopes and adjacent regions demonstrating areas of high and lower identities

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Epitope or Adjacent region</th>
<th>Alignment Positions$^2$</th>
<th>% Identity with BHV1.1</th>
<th>Glycoprotein aa Number of aa</th>
<th>Glycoprotein aa Number of aa</th>
</tr>
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<tbody>
<tr>
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<td>73.3$^4$</td>
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<td>9D6 52-126</td>
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<td>92.0</td>
<td>53-127</td>
<td>75</td>
<td>52-126</td>
</tr>
<tr>
<td></td>
<td>Amino-adjacent</td>
<td>33$^7$</td>
<td>93.9</td>
<td>20-52</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Carboxy-adjacent</td>
<td>80</td>
<td>90.0</td>
<td>128-207</td>
<td>80</td>
</tr>
<tr>
<td>9D6 164-216</td>
<td>53</td>
<td>90.6</td>
<td>165-217</td>
<td>53</td>
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<td></td>
<td>Amino-adjacent</td>
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<td>85-164</td>
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</tr>
<tr>
<td></td>
<td>Carboxy-adjacent</td>
<td>80</td>
<td>82.5$^8$</td>
<td>218-297</td>
<td>80</td>
</tr>
</tbody>
</table>

$^1$ For explanation of virus and glycoprotein abbreviations please refer to notes for table 1
$^2$ From ClustalW alignments, see figures 5 and 6
$^3$ The 50 positions farthest from the epitope of this 100 have 58.0% identity and include an 8 aa insertion in BHV5 relative to BHV1.1.
$^4$ All 4 mismatches are located in a 9 aa area (55.6% identity)
$^5$ The 34 aa adjacent to the epitope are 100% identical
$^6$ The 17 aa adjacent to the epitope are 100% identical
$^7$ This region includes the amino terminus of the mature protein and does not include the 18 aa signal sequence
$^8$ The 65 aa adjacent to the epitope are 90.8% identical
The lalign alignment of BHV1.1 and BHV1.2 gD showed high identity - 89.1% in 417 aa overlap (aa 1-417 for both viruses), with a score of 1.3e-283. The lalign alignment of BHV1.1 and BHV5 gD showed less identity - 81.4% in 415 aa overlap (BHV1.1 aa 6-417, BHV5 aa 7-417), with a score of 2.7e-228. The ClustalW alignment of BHV1.1 and BHV5 (figure 6, table 3) shows lower identity in the 3402 epitope region. The amino-adjacent and carboxy-adjacent regions have high identity.

Figure 6. Alignment of amino acid sequences of BHV1.1\(^1\) and BHV5 gD demonstrating areas of high and lower identity. Dark shading indicates identity, moderate shading indicates lack of identity but presence of amino acid. Symbols under alignment: * = identity; : = conservation (e.g., charge); . = semi-conservation (shape).

\(^1\) For explanation of virus and glycoprotein abbreviations please refer to notes for table 1.

The BHV1.1 gD 3D9 epitope has been reported to be either aa 52-126 (which would include the 3402 epitope) or aa 164-216. The ClustalW alignment of BHV1.1 and BHV5 (figure 6, table 3) shows the 52-126 region has high identity. The epitope’s amino-adjacent and carboxy-adjacent regions also have high identities. The ClustalW alignment (figure 6, table 3) shows the 164-216 region has high identity. The amino-adjacent region has
somewhat high identity, and the carboxy-adjacent region lower identity, but the area immediately carboxy-adjacent to the epitope has high identity. By comparison, the region bracketed by BHV1.1 aa 281 and 332 (52 aa) and BHV5 aa 282 and 332 (51 aa) has <40% identity between BHV1.1 and BHV5.

3.5. Molecular modeling

The similarity of BHV aa sequences to HHV1 was examined to indicate the reliability of modeling BHV glycoproteins on HHV1 glycoprotein crystal structures. The lalign alignment of BHV1.1 and HHV1 gB showed 49.0% identity in 904 aa overlap (BHV1.1 aa 29-928, HHV1 aa 25-896), with a score of 8e-275. The region within that alignment used in the modeling (BHV1.1 aa 122-755, HHV1 aa 110-723) had 48.4% identity. The lalign alignment of BHV5 and HHV1 gB showed 49.5% identity in 882 aa overlap (BHV5 aa 66-943, HHV1 aa 47-896), with a score of 8.1e-275. The region within that alignment used in the modeling (BHV5 aa 129-770, HHV1 aa 111-724) had 47.0% identity. The ClustalW alignment of BHV1.1, BHV5, and HHV1 gB is shown in figure 7. It shows areas of identity between the three viruses of 1-10 aa, and none of the areas of low identity are those of the epitope or adjacent areas.

The lalign alignment for BHV1.1 and HHV1 gD showed 28.7% identity in 251 aa overlap (BHV1.1 aa 46-292, HHV1 aa 63-306), with a score of 4.6e-21. The region overlapping that alignment used for modeling (BHV1.1 aa 32-272; HHV1 aa 24-282) had 23.7% identity. The lalign alignment for BHV1.2 and HHV1 gD showed 29.1% identity in the same 251 aa overlap (as BHV1.1), with a score of 1.8e-21. The region overlapping that alignment used for modeling (BHV1.2 aa 32-272, HHV1 aa 24-282) had 24.1% identity. The lalign alignment for BHV5 and HHV1 gD showed 26.9% identity in 286 aa overlap (BHV5 aa 47-330, HHV1 aa 63-341), with a score of 5.6e-23. The region overlapping that alignment (BHV5 aa 33-373, HHV1 24-282) had 24.5% identity. The ClustalW alignment of the BHV1.1, BHV1.2, BHV5, and HHV1 is shown in figure 8. It shows areas of identity between the four viruses of 1-4 aa, and none of the areas of low identity are those of the epitopes or adjacent areas.
Figure 7. Alignment of amino acid sequences of BHV1.1\(^1\), BHV5, and HHV1 gB demonstrating areas of high and lower identity and similarity. Dark shading indicates identity, moderate shading indicates one virus’ amino acid is not identical. Symbols under alignment: * = identity; : = conservation (e.g., charge); . = semi-conservation (shape).

\(^1\) For explanation of virus and glycoprotein abbreviations please refer to notes for table 1. HHV1 = human herpesvirus 1.
Figure 8. Alignment of amino acid sequences of BHV1.1, BHV1.2, BHV5, and HHV1 gD demonstrating areas of high and lower identity and similarity. Dark shading indicates identity, moderate shading indicates one virus’ amino acid is not identical. Symbols under alignment: * = identity; : = conservation (e.g., charge); . = semi-conservation (shape).

For explanation of virus and glycoprotein abbreviations please refer to notes for table 1. HHV1 = human herpesvirus 1.

The model generated for BHV1.1 gB based on HHV1 gB crystal structure 2GUM is shown in comparison with the model accessed for BHV5 gB (also based on crystal structure 2GUM) in figure 9, in ‘ribbon mode’ for ease of domain and region visualization. The locations of the first and last aa of the mapped 1F10 epitope, as well as a representative aa of the carboxy-adjacent region are labeled. The carboxy-adjacent region appears to be shifted toward the viral envelope in BHV5 compared to BHV1.1, partially covering the 1F10 epitope region.
Figure 9. Ribbon models of BHV1.1\textsuperscript{1} gB (a) and BHV5 gB (b) based on the HHV1 gB crystal structure 2GUM. The amino acids corresponding to the carboxyl region of domain II of HHV1 are represented in green. The virus envelope would be to the bottom of each model, and the other two protomers of the gB trimer would be to the left.

\textsuperscript{1} For an explanation of virus and glycoprotein abbreviations please refer to notes for table 1. HHV1 = human herpesvirus 1.
The models generated for BHV1.1, BHV1.2, and BHV5 gD based on HHV1 gD crystal structure 2c36 are shown in figure 10, in ‘spacefill’ mode to illustrate surface vs. internal aa. Select aa of the 3402 and 9D6 epitope regions are labeled on the BHV1.1 model to illustrate the aa of those epitopes exposed to the surface of the molecule, and the adjacency of exposed areas of regions BHV1.1 aa 92-106 (or 52-126) and 164-216. The BHV1.1 gD model indicates that aa 81-102, 124-161, and 180-183 are exposed, and on adjacent loops.

The ClustalW sequence alignment of the gD aa corresponding to the noted exposed and adjacent areas of BHV1.1, BHV1.2, BHV5, and several alphaherpesviruses not reacting with 870016 is shown in figure 11. BHV1.2 and BHV5 amino acid sequence identity with BHV1.1 is >75% in those regions. The amino acid sequence identity for the other viruses shown is <52%.

4. Discussion

4.1. gB

The anti-gB BoMAb 870009 reacted strongly in VN (without C) and IFA with BHV1.1 and BHV1.2, but weakly with BHV5. It competed with one MuMAb in the panel, 1F10, which had previously been shown not to neutralize BHV5.

1F10 had been previously shown to react with BHV1.1 in radio-immunoprecipitation (RIP) and western blot, to ‘weakly’ neutralize virus equally with and without C, to have weak avidity, to not react with denatured antigen in ELISA, and to not react in antibody-C lysis of infected cells (van Drunen Littel-van den Hurk et al., 1984; 1985). In studies comparing BHV1.1 and BHV5 reactivity, 1F10 strongly neutralized BHV1.1, and while positive by RIP for BHV5, did not neutralize the virus (Collins et al., 1993). 1F10 reactivity was not affected or was even slightly increased when BHV1 gB was de-glycosylated (van Drunen Littel-van den Hurk et al., 1990b), and it was unable to block cell fusion by baculovirus-expressed gB (van Drunen Littel-van den Hurk et al., 1992). The 1F10 epitope elicited an antibody response when purified gB was used to immunize calves (van Drunen Littel-van den Hurk et al., 1990b), and was one of the most immunodominant epitopes in
Figure 10. Spacefill models of BHV1.1\(^1\) gD, BHV1.2, and BHV5 gD, based on the HHV1 gD crystal structure 2c36. The virus envelope would be to the top of each model.

\(^1\) For an explanation of virus and glycoprotein abbreviations please refer to notes for table 1. HHV1 = human herpesvirus 1.
Figure 11. Alignment of the glycoprotein D (gD) amino acid sequences of various assayed alphaherpesviruses\(^1\) for the areas exposed in bovine herpesvirus (BHV) 1.1 gD, demonstrating the differences in degrees of identity with BHV1.1. Amino acid identity with BHV1.1 is designated by bold and underline. Numbering is according to BHV1.1 sequence.

\(^1\) SHV1 = suid herpesvirus 1, EHV1 = equine herpesvirus 1, FEHV1 = feline herpesvirus 1, CAHV1 = canine herpesvirus 1, HHV1 = human herpesvirus 1

vaccinated, stressed calves (Ayers et al., 1994). It was designated epitope V, and was one of 4 neutralizing epitopes (of 5 defined epitopes) on gB (van Drunen Littel-van den Hurk et al., 1985). The 1F10 epitope was localized in mammalian cell transfection experiments to aa 380-440 of gB (Fitzpatrick et al., 1990). The aa sequence included cysteines, potential N-glycosylation sites, and the predicted secondary structure included alpha helices and beta sheets (Fitzpatrick et al., 1990). Peptides covering the 380-440 region did not elicit an ELISA response in calves, but did include a T-cell epitope as measured by peripheral blood mononuclear cell proliferation (Gao et al., 1999). In another study the 380-440 region was not identified as a B-cell epitope, but the hydrophilicity index shows two strong peaks in this area for both BHV1.1 and BHV5 (Ros and Belak, 2002).
Domain II of HHV1 gB is made up of two segments, aas 142-153 and 364-459 (Heldwein et al., 2006). These areas correspond to aa 154-165 and 375-470 of BHV1.1 (Whitbeck et al., 1988). The HHV1 homolog of the BHV1 1F10 epitope therefore lies entirely in the larger, carboxyl region of the domain. HHV1 neutralizing monoclonal antibody H1838 targets HHV1 aa 390-410 and its epitope is exposed on the surface (Heldwein et al., 2006). Peptides 389-398 and 441-459 did not produce fusion as peptides in some domains did (Galdiero et al., 2008). This is consistent with the inability of MAb 1F10 to block fusion by BHV1 peptides. However, mutations in domain II of the HHV4 homologue of gB prevented fusion (Backovic et al., 2009). The authors suggested that the domain is important in binding to other HHV4 glycoproteins or to cellular receptors.

We modeled BHV1.1 gB from the crystal structure of HHV1 gB (2GUM, Heldwein et al., 2006). The validity of the model is supported by the relatively high aa identity and similarity between BHV1.1 and HHV1 gBs over the regions modeled, the “remarkable conservation of the positions of all 10 Cys residues”, the similarity of glycosylations sites, and the match in hydrophilicity index (Whitbeck et al., 1988). The quality assessment plot from SwissModel showed good anolea scores for the epitope region and the 100 aas amino-adjacent, with greater uncertainty for the 50 aas carboxy-adjacent to the epitope (data not shown). Similar identity and model quality assessment results were observed for the accessed BHV5 model. Although HHV1 gB is not furin cleaved, the HHV1 model was protease cleaved in the same area, and was therefore noted as an appropriate structure for modeling gB including naturally cleaved gB (Heldwein et al., 2006).

The model localized the epitope to the section of the carboxyl region of domain II closer to the viral envelope of BHV1.1, made up of an alpha helix and beta pleated sheets (figure 9). The segment of domain II more distal from the viral envelope in BHV1.1 (made up of aa carboxy-adjacent to the epitope and arranged in alpha helices and unstructured areas) is shifted toward the virion envelope in BHV5, partially covering the epitope (totally covering it on one side). A useful marker for this change is aa 493 in BHV1, corresponding to aa 500 in BHV5. The carboxy-adjacent region includes many differences in aa sequence between BHV1.1 and BHV5, including an insertion. The hydrophilicity plots are similar in the epitope and amino-adjacent regions but different in the carboxy-adjacent region.
(Whitbeck et al., 1988). A comparison could not be made for BHV1.2, as the carboxy-
adjacent region sequence is unavailable.

The partial but not total covering is consistent with the IFA and neutralization
observations of this study. 870009 reacted much more weakly with BHV5 than BHV1.1, but
did react with BHV5 in both VN and IFA assays (assays using antigen in its native
configuration). MuMAb 1F10 reacted in RIP assays (denatured protein), but did not
neutralize BHV5 (native configuration), suggesting that the linear epitope (whose aa
sequence is well conserved) is totally covered in BHV5. Although the difference in
reactivity between the two competing MAb could be due to differing assay sensitivities,
higher concentration or stronger avidity/affinity of the BoMAb, the scenario of overlapping
or adjacent epitopes on BHV1.1 (one covered and one only partially covered in BHV5) for
the two MAb would explain their (<100%) competition when using BHV1 and the results
observed for each of them when using BHV5 in assays using denatured and native
configuration protein. Use of peptides in gB-binding assays would help elucidate the
epitopes if the 870009 epitope is also linear.

4.2. gC

The anti-gC BoMAb 870005 reacted strongly in IFA with BHV1.1 and BHV1.2, but
weakly with BHV5. It neutralized (without C) with a similar pattern, although it neutralized
the heterologous BHV1.1 isolate LA less than the homologous isolate CO, and neutralized
the BHV1.2 isolate K-22 more than the homologous virus (by \geq 0.9 \log_{10}). It may be that the
configuration of BHV1.2 gC makes the 870005 epitope more accessible. Alternatively,
perhaps the limited bovine immunoglobulin variable genes are “better programmed” for
BHV1.2 than BHV1.1, but given the hypermutation diversity-generating mechanism
(reviewed in Butler, 1997) of the bovine humoral response, the latter seems unlikely.

870005 did not compete with any of the MuMAb in the panel. Although some
studies (van Drunen Littel-van den Hurk et al., 1984; Collins et al., 1984; van Drunen Littel-
van den Hurk et al., 1985; Fitzpatrick et al., 1990) have noted only one or a few
neutralization epitopes on BHV1.1 gC (particularly without C), others (Marshall et al., 1986;
1988, Marshall and Letchworth, 1988; Dubuisson et al., 1992) cited many or all epitopes as neutralizable. The complete gC aa sequences of BHV1.1, BHV1.2, and BHV5 are known, but no crystal structure for HHV1 gC has been published.

The available information was insufficient to speculate on the target for BoMAb 870005.

4.3. gD

The anti-gD BoMAb 870016 reacted strongly in VN without C and IFA assays with BHV1.1, BHV1.2, and BHV5. It competed with two MuMAbs in the panel, 3402 and 9D6 (3402 more strongly).

3402 had been previously shown to describe a unique epitope (designated V) on gD, and to neutralize BHV1. All 5 gD epitopes described were neutralization epitopes. The gD epitopes were more sensitive than those of gB or gC to neutralization as measured by MAb activity per ug Ab. 3402 did not require C to neutralize BHV1, but was much stronger with C, and it reacted strongly in antibody-C lysis of infected cells. 3402 competed for antigen binding with convalescent bovine serum after primary infection unlike some other MuMAb (Marshall et al., 1988). The MuMAb, when used alone or in combinations, did not passively protect calves from infection (Marshall and Letchworth, 1988). 3402 prevented viral attachment to cells (enhanced with C), neutralizing before and after attachment, with and without C. It reduced plaque size in infected cell cultures (plaque size was not rescued by PEG) (Dubuisson et al., 1992). Vaccinated-stressed calves responded to the 3402 epitope (Ayers et al., 1994). 3402 did not react with BHV5 (isolate corresponding to EC-1) by RIP, and it was suggested 3402 and 9D6 react with the same epitope (Collins et al., 1993). Gene truncation experiments using an E. coli expression system mapped the epitope to BHV1.1 gD aa 52-126. A peptide (aa 92-106) designed to include a BHV1.1/BHV5 divergent sequence blocked 3402 binding, and elicited a virus neutralization response in rabbits (Abdelmagid et al., 1995). The 92-106 region was predicted from the aa sequence to be hydrophilic (Tikoo et al., 1990).
The 9D6 MAb was demonstrated to react on western blot and with denatured antigen in ELISA. It strongly neutralized without C, and neutralized post-adsorption, although much less than pre-adsorption. It reacted in antibody-C lysis of infected cells. The epitope was designated 1b (Hughes et al., 1988). Calves responded to the 9D6 epitope when immunized with purified gD (van Drunen Littel-van den Hurk et al., 1990b). 9D6 was negative by RIP to BHV5 (isolate corresponding to EC-1), and it was suggested 9D6 and 3402 react with the same epitope (Collins et al., 1993). Gene truncation experiments using a vaccinia expression system mapped the epitope to aa 164-216. The aa sequence included cysteines, no potential N-glycosylation sites, and the predicted secondary structure included alpha helices and beta sheets (Tikoo et al., 1993). Gene truncation experiments using a baculovirus expression system, however, mapped the epitope to aa 52-126 (Abdelmagid et al., 1998). This region would encompass the mapped 3402 epitope.

The competition of 870016 with both MAb (to different degrees, and not 100%), and the uncertainty in the location of the 9D6 epitope relative to the 3402 epitope, suggest three explanations: a) the two MuMab epitopes are the same, the MuMAb have different avidities, and 870016 targets that epitope or a nearby epitope; b) the two epitopes are different but located nearby, so binding one epitope partially blocks the other (870016 could target either epitope or a third, nearby or related epitope); or c) the two epitopes are different but located close to each other, and 870016 binds a conformational epitope that includes part or all of both 3402 and 9D6 epitopes). Either option b) or c) could also apply if 9D6 binds two areas (one in the 52-126 region and one in the 164-216 region). 870016 reacts with both BHV1.1 and BHV5, indicating it does not target the specific epitope of either 3402 or 9D6 (neither of which neutralize BHV5).

We modeled BHV1.1 gD from a crystal structure of HHV1 gB (2c36, Krummenacher et al., 2005) to determine the exposed aa of the MuMAb putative epitopes. The validity of the model is supported by the moderately high aa identity between BHV1.1 and HHV1 gDs over the regions modeled, and the conservation of the positions of the cysteine residues (Tikoo et al., 1990). The quality assessment plot from SwissModel showed good anolea scores for the entire region modeled (data not shown). The identity and model quality assessment results were similar for BHV1.1, BHV1.2, and BHV5 models. It should be noted...
that ‘gaps’ are observed between some modeled areas in the 2c36 and particularly the 1jma models, indicating some error in the models. Neither the complete amino-terminal strand thought to bind cellular receptors, nor the complete carboxy-terminal strand thought to bind to gB or gH/gL was included in the HHV1 crystal structures or the BHV models presented. Both strands are known to adhere to the exterior of the globular structure included in the crystal structures and models. It is therefore possible some of the aa observed to be exposed in the model are actually covered by other gD areas. Although gD is a dimer, the dimer model 2c36 does not indicate close proximity of the protomers in the ‘exposed areas’ described here.

The 3402 (92-106) epitope includes exposed aa 92-102 (figure 10). The 9D6 ’52-126 option’ epitope includes exposed aa 81-102. Both include a 9 aa (aa 92-100) exposed area of low identity (55.6%) between BHV1.1 (and BHV1.2, which has an identical aa sequence) and BHV5. The aa changes make the 9 aa area of BHV5 more polar and less basic than BHV1.1, and appear to change the shape as well. Within the subject ‘loop,’ the exposed aa adjacent to the 9 aa region are 100% conserved between BHV1.1 and BHV5, as are the next 20-30 aa both amino- and carboxy-adjacent, suggesting either structural necessity, lack of immune pressure, or both. The 11 aa area BHV1.1 81-91, which is 100% conserved among BHV1.1, BHV1.2, and BHV5, but only 9.0 – 63.6% conserved with other alphaherpesviruses tested, suggests a possible 870016 epitope that could explain both MuMab competition and the BoMAb isolate and virus reactivities.

If 9D6 targets 164-216, exposed aa of that ‘loop’ are the 4 aa BHV1.1 180-183 (figure 10), with one aa change from BHV1.1 (and BHV1.2) to BHV5 (T->A), making the BHV5 area less polar, but not appearing to change its shape. Other alphaherpesviruses contain additional changes in this area. The adjacent areas are well conserved between BHV1.1 and BHV5 (>90%), again suggesting structural necessity or lack of immune pressure. Another area of the 164-216 region is exposed on the virion-proximal part of gD. Binding to this area would seem unlikely to compete with 3402. If the 4 aa area 180-183 is immunologically different between BHV1.1 and BHV5 it would be consistent with 9D6’s isolate reactivity but not 870016’s, and if it is not, the reverse would be true. In either case if
the small area were part of or all of an epitope, the MAb reacting with it might compete with 3402 due to its nearby location.

Another ‘loop’ exposed and adjacent to the 3402 epitope at the envelope-distal end of the globular gD structure is aa 124-161. Although not identified as part of either the 3402 or 9D6 epitope options, if 870016 reacted with this area (highly conserved between BHV1.1, BHV1.2, and BHV5, but not other alphaherpesviruses – particularly BHV1.1 aa 130-153) it could explain the BoMAb’s competition with the MuMAb as well as its strain/virus reactivity. A T cell epitope has been mapped to the immediately adjacent region (aa 161-172, Tikoo et al., 1995).

Use of peptides in gD-binding assays would help elucidate the epitopes if the 870016 epitope is linear. None of the regions at the distal end of gD are implicated in binding cellular receptors or binding other viral glycoproteins to trigger envelope-membrane fusion (Whitbeck et al., 1999, Connolly et al., 2005), although the 124-161 region is near the carboxy-terminal pro-fusion domain when gD is in its unbound state. It is possible the MAb binding to the globular part of gD prevent the conformational change that results from cellular receptor binding (and results in release of the carboxy-strand to bind gB or gH/gL), but the mechanism of neutralization remains to be fully described.

4.4. Summary

The three neutralizing BoMAb studied had unique profiles in their reactivity with the 5 bovine alphaherpesvirus isolates studied. Two (anti-gB 870009 and anti-gC 870005) had different reactivities between BHV1.1/BHV1.2 and BHV5, and if this pattern were confirmed with additional isolates of each, would be useful in bovine antibody assays differentiating the viruses. The BoMAb (870016) recognizing all bovine alphaherpesviruses but none of the other herpesviruses infecting cattle tested (including BHV2, BHV4, SHV1, AlHV1 and AlHV2) would also be useful in bovine antibody assays, should the pattern be confirmed with additional isolate testing. The similarities and differences between the gB- and gD-specific BoMAb reactivities and those of the MuMAb with which they competed provided an opportunity to examine the epitopes relative to primary and tertiary protein
structure. Explanations for the reactivity of each, and further questions to investigate resulted. The studies demonstrate the potential usefulness of combining in vitro immunological and virological activity data with sequence and modeling data in epitope investigations.

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References


CHAPTER 6. GENERATION OF MURINE MONOCLONAL ANTI-IDIOTYPIC ANTIBODIES SPECIFIC FOR A NEUTRALIZING BOVINE MONOCLONAL ANTIBODY TO BOVINE HERPESVIRUS 1

A paper to be submitted to Veterinary Immunology and Immunopathology

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Abstract

The antigen-binding fragment (Fab) of a neutralizing bovine monoclonal antibody (MAb) specific for bovine herpesvirus 1 glycoprotein D was used to generate murine anti-idiotypic (αId) MAb. Multiple αId from three hybridoma fusions bound in ELISA to the immunizing bovine MAb, but not to controls from the same animal matched in heavy chain sub-isotype and light chain type. One murine αId MAb inhibited virus neutralization by the immunizing bovine MAb, indicating that its specificity is at or near the bovine MAb antigen binding site, and possibly represents an “internal image” of the antigen. Such αId antibodies have proven useful for vaccination, diagnostics, and in studies of cellular receptors.

1. Introduction

Bovine herpesvirus 1 (BHV1) is an important pathogen of cattle, causing infectious bovine rhinotracheitis, conjunctivitis, infectious pustular vullovaginitis, infectious pustular balanoposthitis, and abortion in adult cattle, as well as encephalitis and generalized disease in calves (Gibbs and Rweyemamu 1977). Modified live and killed virus vaccines are produced and marketed for prevention of these disease syndromes in the U.S. (Anon, 2012) and elsewhere, primarily for the respiratory form which can potentiate secondary pneumonic infection, a disease known as bovine respiratory disease complex or “shipping fever” (Yates

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1982). The three major envelope glycoproteins of BHV1 are designated gB, gC, and gD. Immunization with each of the glycoproteins has been used to protect calves from lethal combined challenge with BHV1 and Mannheimia haemolytica (Babiuk et al. 1987).

The idiotype (Id) of an Ab is the collection of antigenic determinants (idiotopes) within the variable region. Jerne (1974) postulated that Id-anti-Id networks contribute to the regulation of the immune response. A common nomenclature for the Ab network is receptor - antigen (Ag) - Ab1 - Ab2 (anti-Id) - Ab3 (anti-anti-Id), and will be used for descriptions in this study. Idiotopes can be described as ‘public’ (shared by multiple individuals of a species, and likely coded by variable genes, or potentially representing identical or similar paratopes) or ‘private’ (unique to an individual, and likely the result of mutation and nucleotide addition in recombination). Anti-Id antibodies may also be described as ‘paratope-induced’ (Ab2β), and ‘(other) epitope-induced‘ (Ab2α). The former may be considered an ‘internal image’ of the antigen epitope – therefore Ab2=Ag and Ab3=Ab1 (meaning Ab2 binds to receptor and Ab3 binds to Ag) for this case. Nisonoff and Lamoyi (1981) proposed that internal image αIds be used for vaccination.

Id vaccination for lymphoma, where the Id is a tumor-specific antigen (Bendandi 2009; Houot and Levy 2009; Sakamaki et al. 2011), and αId vaccination for carcinoma, where the αId represents an internal image of the antigen (Reinart et al. 2000; Ladjemi et al. 2011), have completed human clinical trials and are in further development. In each case vaccination has been demonstrated to elicit immune responses to the target Ag. In addition to vaccination, αIds have been used to study cell receptors and for diagnostics (reviewed by Ameri and Zhou, 2006).

Anti-Ids have been successfully used to elicit (or suppress) immune responses in several viral systems (reviewed in Koprowski and Herlyn 1986; Dalgleish and Kennedy 1988), including to herpesviruses (Kennedy et al. 1984; Gell and Moss 1985; Lathey et al. 1987; Gurish et al. 1988, Zhou and Afshar 1995), and to BHV1 in particular. Whetstone et al. (1988) generated αId rabbit polyclonal antibodies (PAb) and murine MAb (MuMAb) to a neutralizing MuMAb to BHV1 gB. The αId interfered with virus neutralization by the Ab1, and were recognized by rabbit antisera to BHV1. Orten et al. (1988) generated rabbit PAb αId to a neutralizing MuMAb to gD. It inhibited Ab1 binding to virus, and virus inhibited
Ab2-Ab1 binding. Srikumaran et al. (1990) generated bovine PAb $\alpha$Id to a neutralizing MuMAb to gD. The $\alpha$Id inhibited binding of Ab1 to virus, and mice immunized with the $\alpha$Id produced neutralizing Ab (Ab3). The $\alpha$Id also bound to permissive cell cultures (suggesting receptor-binding). Hariharan et al. (1991) generated bovine MAb (BoMAb) $\alpha$Id to a neutralizing MuMAb to gB. The $\alpha$Id inhibited Ab1 binding to gB, and mice immunized with the Ab2 produced Ab3 that reacted with gB and neutralized BHV1. Orten et al. (1991) generated rabbit PAb $\alpha$Id to a neutralizing MuMAb to gD. When mice were immunized with the Ab2, a humoral (neutralizing Ab) response was observed, but not a cellular response, as measured by lymphocyte proliferation and cytokine production. AbdelMagid et al. (1992) generated rabbit PAb to neutralizing MuMAb to gB and gD. Each bound their respective Ab1 and inhibited neutralization by it, and treatment of cells with Ab2 blocked virus infection. Orten et al. (1993) used rabbit PAb to gB and gD to immunize calves, resulting in a slight reduction of clinical signs and one calf producing BHV1-neutralizing antibodies. Varthakavi and Minocha (1996) used an anti-anti-gB to identify a 56 kDa cellular receptor. The Ab2 was blocked from this recognition by Ab1, and virus blocked the Ab2 from binding to the cellular receptor.

Study of the idiotypic network in a non-rodent, non-primate host species such as cattle, for an economically important disease such as that caused by BHV1, can provide important information. The immune system in cattle achieves humoral immunological diversity using different mechanisms than in humans or rodents (Butler 1997). Cattle have few, closely related variable genes, which might increase the occurrence of public idiotopes (in addition to internal image Ab2) that might be used to elicit antigen-specific Ab3. However, the bovine emphasis on untemplated mutation may reduce or eliminate that conservation. Use of the host animal for the disease allows for efficacy assays and ease of transfer to field use of resultant products. A study using bovine Ab1, Ab2, and Ab3 would be ideal, but presents numerous challenges. Bovine Ab2 (Srikumaran et al. 1990, Haraharan et al. 1991) and Ab3 (Orten et al 1993), and bovine protection from disease (Orten 1993) have been studied in previous trials. This study used bovine Ab1 in an attempt generate murine Ab2 for further work.
2. Materials and Methods

2.1. Preparation of Immunogen (Ab1)

Immunogen consisted of Fab of a BoMAb which neutralizes BHV1 through specific reaction with the viral gD. The BoMAb, @BLC52.870016 (hereafter referred to as 870016), is secreted by a bovine\(^3\) x murine\(^2\) heterohybridoma (HH) and has been described elsewhere (Levings et al. 2012a).

The 870016 Ig was purified from commercially-produced (BioProducts for Science, Madison, WI) nude mouse ascites in two steps. In the first step, protein G affinity chromatography (Protein G Sepharose 4 Fast Flow, Pharmacia LKB, Piscataway NJ) was performed on diluted, filtered ascites using the recommended buffers. High 280 nm-absorbance fractions from multiple trials were pooled, concentrated using centrifugal ultrafiltration devices (Centricon microconcentrators, Amicon Division, Danvers MA) with 30K molecular weight cutoff (MWCO), and checked for purity by microvolume SDS-PAGE. The second step consisted of Ab affinity chromatography using a MuMAb specific for bovine IgG1. The hybridoma @GBIGG.DAS17 (Goldsby et al. 1987, MuMAb hereafter referred to as DAS17) was kindly donated by Richard Goldsby (Amherst College, Amherst MA). The DAS17 Ig was isolated from ascites using protein A affinity chromatography using mini-columns from a commercial kit (Immunopure Fab Preparation Kit, Pierce, Rockford IL) and the recommended buffers. The DAS17 was covalently bound (by primary amines via reductive amination) to a commercial aldehyde-activated 6% beaded agarose (AminoLink Coupling Gel, Pierce, Rockford IL) using product instructions. The protein G-isolated 870016 preparation from step 1 was then applied to a column of the DAS17-agarose, incubated, the column washed, and 870016 eluted using 0.1M glycine, pH 2.8. The eluate was pH neutralized, its protein concentration tested using the Lowry technique, and the cleavage efficacy and final product purity checked by microvolume SDS-PAGE.

A commercial kit (Immunopure Fab Preparation Kit, Pierce, Rockford IL) including papain immobilized on 6% cross-linked beaded agarose was used to cleave the purified 870016. The kit instructions were generally followed, except two (not one) digestions were
performed, and the Protein A steps were substituted with Protein G due to its superior binding to bovine Ig (Boyle and Reis 1987). Uncleaved Ab and crystallizable fragment (Fc) were bound to Protein G using the process described for 870016 purification, and the unbound fraction collected. The unbound fraction was tested for purity by microvolume SDS-PAGE, and quantified.

Anti-BHV1 reactivity of the 870016 Fab was tested by indirect fluorescent antibody (IFA) tests, using rabbit origin (RO) anti-bovine polyclonal conjugate (which would be expected to contain labeled Ab to bovine Fab). Dilutions of the Fab preparation were tested, with equivalent concentrations of protein G-prepared whole 870016, antiserum to BHV1 and other bovine serum. The specificity of the anti-BHV1 reactivity was tested using a ‘blocking IFA’. Dilutions of the 870016 Fab preparation were applied to the IFA plate, followed by dilutions of supernatant fluids of HH culture pros of 870016, two other IgG1 BoMAb specific for BHV1 gB and gC (@BL5C2.870009 and @BL5C2.870005, respectively, hereafter referred to as 870005 and 870009), and bovine BHV1 antiserum. This was followed by DAS17 (assumed to be anti-bovine IgG1 Fc), then PAb RO anti-mouse conjugate, and then read. All incubations were 45-60 minutes at rt, with PBS washes between each step.

2.2. Immunization

Twelve Balb/C mice were initially immunized (see table 1), using 3 doses of 870016 Fab (5, 25, and 100 ug per mouse) and two route/adjuvant combinations (s.c. with Freund’s incomplete adjuvant [FIA] and i.p. without adjuvant), or two mice per group. Unadjuvanted injections were 0.2 ml, and adjuvanted injections were 0.4 ml (equal volumes aqueous solution and FIA, emulsified). At intervals after priming the mice were bled, their serum tested for reactivity with 870016, and further immunized. Mice were selected for fusion based on their serum reactivity. For fusions @@16B and @@16C, mice were injected i.v. with 25ug of immunogen 3 days before fusion. All animal work was approved by the institutional animal care and use committee.
2.3. Mouse selection, fusions, and initial culture

Mice were selected for fusion based on their serum’s binding ELISA reactivity with 870016 hollow-fiber (HF) product relative to HF media control. For initial decision-making and the @@16B fusion, 4-fold dilutions starting from 1:10 (various sets of 1:10-1:2560 depending on bleed) were used. For the @@16C fusion, mice were tested in the same assay for reactivity with 870016 HF product vs. HF media, and with purified 870016. For the @@16D fusion, reactivity with 870016 relative to reactivity with 870005, a BoMAb of different antigen specificity but the same heavy chain sub-isotype (IgG1, Levings et al., 2012a) and light chain type (lambda, Koti et al., 2010) as 870016 (Levings et al., 2012a, Levings and Roth, 2012) was used.

All fusions were performed using a standard hybridoma protocol (Van Deusen and Whetstone, 1981; Van Deusen 1984). Briefly, the spleens were sectioned and sieved, and extracted cells were washed and fused with non-secreting hybridoma SP2/0 cells (Shulman et al. 1978) using polyethylene glycol 1540. Ratios of spleen cells to SP2/0 were: 3:1 for @@16B; 4:2:1 for @@16C; and 2:1 for @@16D. Cells were seeded into 96 well plates at 1 x 10^4 SP2/0 per well, in 0.2 ml/well (7 ¾ plates @@16B; 5 plates @@16C; 5 plates @@16D). Nutrient serum for seeding was a 2:1 mix of horse serum and FBS.

2.4. Hybridoma and (re-)clone selection

Culture supernatants of primary wells with robust growth were tested for murine Ig secretion by commercial ELISA. For example, for the @@16D fusion, of 480 wells seeded, 368 had growth, and 342 were tested for murine Ig. Those positive for murine Ig were tested by Ab1-binding ELISA (Ab1-ELISA) for reactivity with whole 870016 as described for each fusion, using equivalent DAS17 (positive) and SP2/0 (negative) preparations as test sample controls. Those with specific reactivity to 870016 were retained and cloned by limiting dilution. Clones were selected similarly to primaries, and were sub-cloned, resulting in‘re-clones.’
For the @@16B fusion, primaries were selected based on Ab1-ELISA using 870016 HF product, then 870016 HF product relative to HF media. Clones and re-clones were selected based on Ab1-ELISA reactivity with purified 870016 (protein-G and immunoaffinity purified, 50 ug/ml) and 870016 HF product relative to HF media, and with Ab1-ELISA using 32X serum free (SF) product of 870016, 870005, and SP2/0.

For the @@16C fusion, primaries were selected based on Ab1-ELISA using 870016 HF product relative to HF media. They were controlled-rate frozen and stored while one suspect culture was examined, then expanded and tested. Primaries were (sub)isotyped. Clones and re-clones were selected based on Ab1-ELISA reactivity using 32X serum free (SF) product of 870016, 870005, and SP2/0.

For the @@16D fusion, primaries were selected based on Ab1-ELISA using 870016 HF product relative to HF media and 870005 HF product. @@16C.043100 was used as an additional control in Ab1-ELISA tests. Primaries were (sub)isotyped. Clones and re-clones were selected based on Ab1-ELISA reactivity using 32X serum free (SF) product of 870016, 870005, and SP2/0. In some cloning selections, @@16D.011 (clone) or .011100 (reclone) was included as an additional control.

2.5. Hybridoma production

Production of BoMAb Ab1 for use in testing has been previously described (Levings et al. 2012a). Production of murine hybridomas followed the standard protocol cited. In some cases supernatants from ‘extinction cultures’ (cultures left in same media beyond normal growth/expansion phase) and cultures adapted to SF media were tested. In some cases culture fluids were concentrated using centrifugal ultrafiltration devices with 30K MWCO prior to testing. Small-volume ascites production was used for testing certain clones, using standard hybridoma protocols and varying only by the numbers of mice used. Additional production, isolation, and purification techniques were applied to select re-clones for certain assays, and are described in the sections on those assays.
2.6. ELISAs

Murine Ig detection and characterization were done using commercial murine isotyping or sub-isotyping ELISA kits (Mouse monoclonal isotyping kit, Mouse monoclonal sub-isotyping kit, HyClone Laboratories, Logan, Utah) according to kit instructions. Murine Ig was detected in culture fluids using the isotyping kit, using 1:1 IgG:IgM conjugate (final dilution of each as recommended in kit). Isotyping and subisotyping of supernatants, concentrates, ascites or purified fractions was done using the corresponding kit.

All Ab1-binding ELISAs (Ab2 binding to immunogen Ab1 870016 or controls coated on plate) used components of the commercial murine isotyping kit (e.g., plate coating buffer, conjugates, chromophore) and 96-well plates, and were performed according to kit instructions (except Ab1 was substituted for capture Ab). Plate coating preparations cited (e.g., 32X SF, 50ug/ml) refer to solutions diluted 1:100 in the kit plate coating solution and applied 0.1 ml/well.

Multiple 870016 preparations were used as the target (bound to plate), including dilutions of HF product, protein G/DAS17-purified product, and 32X SF product. Analogous Ab1 controls of 870005, SP2/0, or media were used for HF and 32X SF products. The purified 870016 product was always used in association with a culture 870016 product with the culture product controls. Control Ab2 included DAS 17, SP2/0, @@16C.2D12, @@16D.011 or .011100 (a reclone of .011), and diluent (media, PBS). Control Ab1s and Ab2s were always used on the same plate. Comparison of sample Ab2 reactions with the Ab1 preparations relative to that of DAS17’s reaction was used to overcome issues of standardization of Ab1 material used to label the plate – i.e., the material was produced equivalently, but other characteristics (e.g., ug/ml of bovine Ig) were not known.

In a modification of the Ab1-ELISA assay, unlabeled Ab2 preparations were tested for their ability to block or compete with binding of conjugated @@16D.011100 (hereafter referred to as 011100) to Ab1. Horseradish-peroxidase (HRPO) -conjugated 011100 was produced as described in Levings et al., 2012b. Briefly, Ig was prepared from ascites by salt (ammonium sulfate) precipitation, anion-exchange (DEAE) fractionation, and protein G chromatography (hereafter referred to as ASDG preparation). A commercial kit
(ImmunoPure Activated Peroxidase Kit, Pierce, Rockford IL) was used to label the Ig with HRPO. In a ‘block and competition’ assay, dilutions of unlabeled 011100 ASDG were incubated in Ab1-ELISA plates (32X SF products of 870016, 870005, SP2/0 were bound to the plate). Without removing the unlabeled Ig, labeled 011100 was then added, followed by an incubation, washing, and substrate development. In the ‘blocking’ assay, ASDG preparations of 011100, 025100, and SP2/0 were incubated in Ab1-ELISA plates, followed by washing, labeled 011100 addition, incubation, washing, and substrate development.

To assay the ability of Ag to block Ab1-Ab2 binding, concentrates and dilutions of BHV1 ($10^6$ plaque forming units/ml) and dilutions of other viruses (bovine parainfluenza virus 3 and suid herpesvirus 1) were used. Virus preparations were incubated in Ab1-ELISA plates (32X SF products of 870016, 870005, SP2/0 were bound to the plate), followed by washing, labeled 011100 addition, incubation, washing, and substrate development. 1-2 hour room temperature (rt) incubations were used.

2.7. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Microvolume SDS-PAGE was performed as described in Levings et al., 2012a. Briefly, microvolume SDS-PAGE (PhastGel system, Pharmacia/LKB, Piscataway NJ) homogeneous (12.5%) or gradient (10-15%) gels were used following product instructions. A mixture of high and low SDS-PAGE molecular weight (MW) standards, and Coomassie Blue or silver stain (BioRad Laboratories, Hercules CA) were used, employing the associated staining apparatus which agitates and heats.

2.8. Fluorescent antibody tests

Indirect fluorescent antibody (IFA) assays were conducted as described previously (Levings et al. 2012a). 870016, 870016 Fab and other bovine Ig preparations were applied to fixed plates of BHV1 infected bovine cell cultures, incubated, washed, and binding detected using PAb RO anti-bovine conjugate.
870016 Fab was tested for its ability to block the binding of whole 870016 or other anti-BHV1 Ig (Ab1) to fixed BHV1-infected bovine cell cultures. Binding of whole bovine Ig was detected by using DAS17 and then PAb RO anti-murine conjugate.

Ab2s were tested for their ability inhibit the binding of whole 870016 or other anti-BHV1 Ig (Ab1) to fixed BHV1-infected bovine cell cultures (IFA inhibition test, or IFIT). Dilutions of Ab1 were incubated with dilutions of Ab2, or Ab1 was diluted in Ab2. The Ab1-Ab2 mixtures were incubated, and then IFA performed as described above.

2.9. Virus neutralization inhibition test (VNIT)

Ab2s were tested for their ability to bind whole 870016 and other anti-BHV1 Ig (Ab1) and prevent the Ab1 from subsequently neutralizing BHV1. Un-neutralized BHV1 was detected by plaque formation in bovine cell cultures. The assay followed the virus neutralization (VN) assay described previously (Levings et al., 2012a), except: Ab1 was incubated with Ab2 prior to the mixture’s incubation with virus; and the Ab1-Ab2 and Ab-virus incubations were 1-2 hours at rt. Tests with 2-3 wells per test condition were conducted, using 870016, 870005, and SP2/0 as Ab1 with one Ab2 preparation, on the same plate. Samples tested included: concentrates of culture fluid (10X of conventional culture, 50X salt-precipitation of conventional culture, 200X of SF culture); concentrates (salt-precipitation) and dilutions of ascites; salt-precipitation and anion-exchange from ascites Ig (ASD); and papain-treated preparations of the ASD Ig.

3. Results

3.1. Immunogen (Ab1) reactivity

The Ab1 Fab inocula were pure, as demonstrated by microvolume SDS-PAGE (data not shown). Ab1 Fab retained immunological reactivity as shown by IFA using PAb anti-bovine Ig conjugate. Endpoints in ug/ml were: Fab ≤5.0; whole 870016 Ig ≤0.5, IBR AS Ig ≤5.0, BVD AS Ig >500, FBS Ig ≤50. Specific reactivity of the Fab was also demonstrated by
its ability to block whole 870016 IFA binding as indicated by anti-bovine IgG1 MuMAb (DAS17) followed by anti-murine conjugate. Endpoint concentrations for 870016 were increased in an Fab concentration-dependent manner (e.g., 100 ug/ml of Fab resulted in a ‘whole Ig’ 870016 endpoint of 100 ug/ml, whereas 10 ug/ml of Fab resulted in a whole Ig 870016 endpoint of ~1.0 ug/ml). No concentration tested (100, 10, and 1 ug/ml) of 870016 Fab affected the endpoint in IFA of 870005, 870009, or IBR AS Ig. Fab was not detected by DAS17 followed by mouse conjugate, consistent with the assumption that DAS17 is specific for bovine IgG1 Fc.

3.2. Hybridoma selection and reactions in immunoassays

As indicated in table 1 by the mice selected for further immunization and fusions, higher priming doses (25 and 100 ug vs. 5 ug) resulted in higher specific immune responses. The Ab2 of interest generated in the fusions are listed in table 2, along with the immunogen and controls.

The @@16B fusion resulted in 5 cultures reacting well with 870016 in Ab1-ELISA. One primary culture (3G9) was selected based on specificity, was cloned and re-cloned, and isotyped (IgM). Figure 1 shows the 870016-specific reactivity of a re-clone (G91100) in Ab1-ELISA. However, culture fluids reacted with media and 870005 to some degree and were negative in the IFA inhibition and VNIT assays. The hybridoma did not produce ascites well, and the ascites produced had questionable binding Ab1-ELISA results. Therefore this hybridoma was not selected for further study.

The @@16C fusion resulted in 13 cultures reacting well with 870016 relative to media control in Ab1-ELISA. Five cultures were selected based on differential 870016/870005 reactivity, and four were (sub)isotyped (3 IgM, 1 IgM with IgG2 and IgG3a reactivity). Clones of each of the five were negative by virus-blocking ELISA, and the four tested by IFIT were negative. Re-clones were negative by VNIT, but one re-clone (043100) of an IFIT-negative clone reacted in IFIT, and so was selected for further study. It was used as a control in Ab1-ELISAs of cultures from the fusion @@16D. In addition, the primary 2D7 (IgG1) was examined due to its reactivity with 870016, 870005 and 870009 relative to
Table 1. Immunization schedule and dosages for anti-idiotypic antibody generation. Immunogen consisted of Fab fragments of bovine monoclonal antibody @BL5C2.870016.

<table>
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<th>Mouse no.</th>
<th>Day of immunization series</th>
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<th>25</th>
<th>40</th>
<th>46</th>
<th>49</th>
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<th>158</th>
<th>159</th>
<th>161</th>
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<td></td>
<td>B</td>
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<td>25ug SQ FIA</td>
<td>B</td>
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<td></td>
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<tr>
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<td>B</td>
<td>25ug SQ FIA</td>
<td>B</td>
<td></td>
<td>B</td>
<td>25ug SQ FIA</td>
<td>B</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>B</td>
<td>B</td>
<td></td>
<td>B</td>
<td>25ug IP</td>
<td>B</td>
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<td></td>
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<td></td>
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</tr>
<tr>
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<td></td>
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<tr>
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<td>B</td>
<td>B</td>
<td>B</td>
<td></td>
<td>B</td>
<td>25ug 1v6</td>
<td>@@16B fusion</td>
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<td></td>
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<tr>
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<td>B</td>
<td>B</td>
<td>B</td>
<td>25ug 1v</td>
<td>B</td>
<td>25ug 1V</td>
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</tr>
<tr>
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<td>B</td>
<td>B</td>
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<td>B</td>
<td>25ug SQ FIA</td>
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</table>

1SQ = subcutaneous
2FIA = Freund’s Incomplete Adjuvant
3B = bled
4IP = intraperitoneal
5IV = intravenous

Table 2. Identities of the antigen, antibodies or controls, and anti-idiotypes or controls generated and tested in the study.

<table>
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<th>Specificity</th>
<th>Full name</th>
<th>Abbreviation Used</th>
<th>Description</th>
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<td></td>
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<tr>
<td>Antigen</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ag</td>
<td></td>
<td>Bovine herpesvirus 1 glycoprotein D</td>
<td>BHV1 gD</td>
<td>A viral envelope glycoprotein essential for BHV1 entry and cell infection</td>
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<td>Antibody 1 (Ab1)</td>
<td>Ab to Ag</td>
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<td>870016</td>
<td>A neutralizing bovine IgG1 MAb to BHV1 gD</td>
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<td>870005</td>
<td>A neutralizing bovine IgG1 MAb to BHV1 gC</td>
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<tr>
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<td>Control</td>
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<td>870009</td>
<td>A neutralizing bovine IgG1 MAb to BHV1 gB</td>
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<tr>
<td></td>
<td>Control</td>
<td>@BL5C0.87001</td>
<td>C0-01 870025</td>
<td>Bovine MAb to unknown Ags of BHV1</td>
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<td>Antibody 2 (Ab2)</td>
<td>Anti-idiotypes</td>
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<td>Murine MAb to @BL5C2.870016 antigen-binding fragments</td>
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<td>Murine MAb to bovine IgG1</td>
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Figure 1. Preferential binding in ELISA of $$@@16B.G93100$$ (Ab2) to $$@BL5C2.870016$$ (Ab1)$^1$.

$^1$Please see table 2 for Ab identities and abbreviations.
Media control for Ab1 MAb is hybridoma nutrient media.
All wells zeroed to lowest value, then PBS control for each preparation subtracted.

media in a pattern similar to the DAS17. It was used as a control for $$@@16C$$ assays. A re-clone of this hybridoma (2D1200) reacted in IFIT with 870005 as well as 870016, similar to DAS17. In both cases, IFA was inhibited only when the Ab2 was reacted with Ab1 before the Ab1 was reacted with Ag. Figure 2 shows the reactivity of one clone of each line selected for re-cloning, as well as the 2D1200 re-clone (its reactivity with SP2/0 was higher than typical in the test shown in the figure).

The $$@@16D$$ fusion resulted in 20 cultures (renamed 01-20) reacting well in Ab1-ELISA with 870016 relative to 870005 and media controls. 19 were (sub)isotyped – 16 were IgM, 1 was mixed IgM/IgG3, 1 was IgG2b and 1 was IgG3). The 7 strongest reactors (all IgM) were cloned, and 6 were re-cloned successfully. Two with the best binding ELISA results (reclones 011100 and 025100) were selected for further study. Figure 3 shows binding ELISA results using 870016 and four control bovine MAb Ab1 preparations, in which both 011100 and 025100 demonstrate minimal reactivity with all Ab1 except the immunogen 870016. The binding of HRPO-labeled 011100 to 870016 in ELISA was
Figure 2. Preferential binding in ELISA of select @@16C clones (Ab2) to @BL5C2.870016 (Ab1)\textsuperscript{1}.

\textsuperscript{1} Please see table 2 for Ab identities and abbreviations. Bovine MAb preparations are 32X serum-free products. Media control is SP2/0 supernatant. Results for @@16C clones are from single wells, those for SP2/0 and DAS17 are averages of 9 wells (9 plates).

Figure 3. Preferential binding in ELISA of @@16D.01110 and .025100 (Ab2) to @BL5C2.870016 (Ab1)\textsuperscript{1}.

\textsuperscript{1} Please see table 2 for Ab identities and abbreviations. Bovine MAb preparations are 32X serum-free products. Media control is SP2/0 culture supernatant. Results from two plates. Results for C0-01 and -25 are from single wells; those for media, 870005 and 870009 are averages of two wells, and those for 870016 are averages of 8 wells.
blocked in a roughly dose-dependent manner by unlabeled 011100 (data not shown).
Unlabeled 025100 did not block labeled 011100 binding (2.78 870016/Media O.D. vs. 1.71 for unlabeled 011100 and 3.62 for the PBS control). However, the sham SP2/0 did inhibit labeled 011100 binding (1.32 870016/Media in the same trial).

Figure 4 shows virus blocking ELISA results with 011100. At 8X Ab1 concentration (3 in figure), 10^6 pfu BHV1 reduced 011100 binding of 870016 by 18.7%, compared to increasing DAS17 binding to 870016 by 34.8%, and reducing DAS17 binding to 870005 by 5.9%. Figure 5 shows typical VNIT results (BoMAb % VN varied by assay) with 011100, demonstrating a dose-dependent inhibition of 870016 neutralization of BHV1. 011100 did not inhibit 870005 VN, DAS17 inhibited VN by both 870016 and 870005 in a

![Figure 4](image)

Figure 4. Antigen^1 inhibition of @16D.011100 (Ab2)^2 binding to @BL5C2.870016 (Ab1) in ELISA.

^1 10^6 plaque forming units/ml of bovine herpesvirus 1.
^2 Please see table 2 for Ab identities and abbreviations.
Concentrations of 011100 and controls are expressed on the x-axis as 2-fold (2^n)
Figure 5. Inhibition by @@16D.011100 (Ab2)\(^1\) of @BL5C2.870016 (Ab1) neutralization of bovine herpesvirus 1. Increasing plaque counts evidence inhibition of the virus neutralization by Ab1.

\(^1\)Please see table 2 for Ab identities and abbreviations.

10-fold dilutions of neat ascites and of 7X Ig (salt precipitation) ascites preparations
Slopes = 011100 x 870016, 8.01; 011100 x 870005, 0.80; DAS17 x 870016, 10.15; DAS17 x 870005, 13.09; SP2/0 x 870016, 1.30 (2 points only); SP2/0 x 870005, 3.67 (2 points only).

dose-dependent manner, and SP2/0 inhibited neither 870016 or 870005. Figure 6 shows the impact of papain treatment on VNIT activity. Papain treatment reduced DAS17’s inhibition of both 870016 and 870005 VN activity. It also reduced 011100’s inhibition of 870016 VN activity, making it essentially undetectable (compared to SP2/0) in the trial shown. The figure also shows the typical minimal VNIT activity of (untreated) 025100 on 870016.

4. Discussion

Generation of αId involves careful selection of immunogen (Ab1) and immunization dose, route, adjuvant, and schedule (a wide variety is represented in Sharpe et al. 1984, Uytdehaag et al. 1985, Whetstone et al. 1988, Gurish et al. 1988, and Bona 2005), as well as mouse screening/hybridoma selection assays. This study involved immunizing mice with BoMAb Fab using a variety of doses, routes, schedules, and one adjuvant present or absent.
Figure 6. Reduction by papain treatment (Tx) of the neutralization-inhibiting activity of @@16D.011100 (Ab2) relative to untreated (Untx). Decreased plaque counts evidence the uninhibited activity of neutralizing Ab1.

1 Please see table 2 for Ab identities and abbreviations
2 Average of 3 wells (3 plates)

The approach eliminated response to the bovine Fc antigens most commonly responded to in a cross-species Ig immunization. However, the mice could respond to bovine constant features of the constant heavy chain domain 1 (CH1), constant domain of the light chain (CL), or allotypic markers on those domains, in addition to the public and private idiotopes of interest on the variable heavy (VH) and variable light (VL) regions.

To better select for αId Ab2, an immunoassay was used which compared the reactivity of the Ab2 with whole immunogen BoMAb Ab1 vs. its reactivity against a BoMAb from the same animal (870005) matched in heavy chain sub-isotype and light chain type. This would eliminate the selection of an Ab2 MuMAb for CH1 or CL. Allotypes have been described on bovine lambda chain and IgG1 (unknown, but potentially on the CH1) Ig (reviewed in Butler 1983). The use of a matched control from the same animal reduces but
does not eliminate the possibility of selecting an allotype-specific Ab2. Finally, because
cattle have relatively few (and use fewer) closely sequence-related VH and VL genes, Ab2 to
common, non-epitope related public idiotopes were less likely to be selected using the
comparative immunoassays. 870005 and 870009 are known to react with different BHV1
glycoproteins than 870016, and the other bovine MAbs from the same animal tested
(@BL5C0.870001 and @BL5C0.870005) do not neutralize BHV1, so by definition they do
not have the same paratope-related private idiotope. It is therefore likely, but not assured,
that the Ab2s selected for study in the three fusions (particularly the @@16D fusion, tested
with four control BoMAb) are directed against private idiotypes of the immunogen bovine
Fab. Other in vitro assays (inhibition of the IFA or VN activity of Ab1) further suggested the
αId nature of the Ab2 derived.

The poor sensitivity of the screening assay (Ab1-ELISA) may have resulted in not all
Ab2 of interest being selected. The Ab1 used for plate binding in ELISAs had to be
enriched. The inclusion of DAS17 as a positive control in all assays (and all assay plates)
was used to demonstrate the presence of Ab1 – typical reactions with 870005 and other
BoMAb were higher than those with 870016. The Ab2 often had to be concentrated for
optimal results, including in some cases small-volume ascites production and concentration.
Confirmatory or characterization immunoassays such as IFIT and VNIT routinely required
concentrated Ab2 preparations.

One MuMAb Ab2, @@16C.2D1200, reacted similarly to DAS17 in the Ab1-ELISA
assay, despite being generated against Fab. It also reacted in IFIT with 870005 as well as
870016, similar to DAS17. The results suggest it may be specific for bovine IgG1 CH1 or
lambda and as such could be useful as a reagent. Of note is that both the MuMAb only
inhibited IFA when allowed to bind the Ab1 before the Ab1 bound to Ag (not after),
demonstrating that the effect was an “anti-Ab1” effect, not a ‘covering up’ of conjugate sites.

Of note in this study is the consistent selection of murine IgM Ab2s. Although the
anti-murine conjugate mix detected the IgG DAS17 control well, it is possible that the higher
valence of IgM resulted in IgM-secreting hybridomas being consistently selected due to
higher avidity. To partially correct for such selection bias, isotyping at the primary selection
stage was used for the @@16C and @@16D fusions to assist selection of IgG Ab2. In
addition, the 16D fusion was conducted using a mouse without the typical booster dose 3 days before fusion, in case that dose was somehow serving as a primary immunization. In both fusions, IgM Ab2 remained the majority of selected primaries, and the IgG Ab2 that were selected were rapidly down-selected due to lack of consistent reactivity.

At least one 870016-specific αId was selected in each of 3 fusions. However, the stability and ascites production for some lines was problematic. Some of the lines could perhaps be further studied with more aggressive recloning and in vitro high concentration production (e.g., hollow-fiber culture).

The two cultures selected for characterization (011100 and 025100) were 870016-specific by Ab1-ELISA, stable, and produced ascites well. 011100 was reactive in the VNIT and 025100 was not, and unlabeled 025100 did not compete with labeled 011100 in Ab1-ELISA, suggesting these are αIds directed against two idiotopes of 870016. In the Ab1-ELISA, SP2/0 also showed significant competition, but it should be noted that purified SP2/0 ascites Ig would consist of a mix of proteins compared to 011100 or 025100, which makes direct comparison difficult.

The ability of 011100 to inhibit VN of 870016 suggests it is specific for a paratope-associated idiotope, and possibly is an ‘internal image’ of the BHV1 gD epitope. The binding site for 011100 was not determined. Because papain treatment significantly reduced 011100 VNIT activity (to 0.1 X untreated preparation levels), as it did DAS17 activity, it may be that some of the VNIT activity is due to agglutination of multiple Ab1 molecules, or steric hindrance of the other binding site of the target Ab1. Neither rules out the possibility of the αId being directed against the paratope.

The ability of BHV1 to inhibit binding of 011100 to 870016 also suggests 011100 is specific for a paratope-associated epitope. The results in that assay are not conclusive because BHV1 did not reduce 011100-870016 binding by a large amount (18.7%). However, that figure is over three times the reduction of BHV1 on DAS17-870005 binding (5.9%) and the reduction is the reverse of BHV1’s large ‘enhancement effect’ for DAS17-870016 binding (34.8%).

In vitro assays alone may be insufficient to characterize the αId generated in this study. Immunization (e.g., of mice) using 011100, 025100, or other Ab2 described here may
provide the amplifying effect needed to characterize them, as well as providing proof-of-concept for their use in vaccination of the host species, cattle.

Anti-Id vaccines have been proposed for difficult situations – hypervariable Ag (e.g., influenza, human immunodeficiency virus), and for Ag difficult to mimic with peptide (e.g., carbohydrate). Several authors have proposed their use for BHV1 diseases because of the problem of latency and safety using attenuated live vaccines. Use of the murine Ab2 resulting from this study (e.g., 011100 or 025100) might elicit BHV1 neutralizing antibodies in cattle, as bovine Ab2 elicited in mice (Srikumaran et al. 1990, Hariharan et al. 1991). The advantages of their use (potential rapid onset of immunity due to cross-linking B cell receptors, ease of production) would have to be weighed against the disadvantages (extremely restricted specificity, potential vaccinate immune response to the murine constant regions) within the context of other products available or in development.

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CHAPTER 7. GENERAL CONCLUSION

In this series of studies, 78 bovine x murine heterohybridomas secreting bovine monoclonal antibodies (MAb) to bovine herpesvirus 1 (BHV1) were generated using a unique self-refusion method. Three bovine MAb of interest were examined for stability, scalability, and activity in a variety of *in vitro* assays, as well as antigen specificity. They were found to target the three major neutralization targets (envelope glycoproteins) of the virus.

The three bovine MAb were each demonstrated to protect against lethal intravenous BHV1 challenge in a neonatal laboratory rabbit disease model when administered at high concentrations intravenously. The model may be useful for infection and immunity studies relative to generalized BHV1 infections of young animals.

The three bovine MAb appeared to be specific for the immunizing virus or a closely related bovine herpesvirus, enhancing their potential as diagnostic reagents, should reactivity with a diversity of isolates of those viral species be demonstrated. Their epitopes were mapped, with unexpected results relative to their biological activity. Comparison of published glycoprotein sequences and molecular modeling allowed epitope-specific explanations for the results.

The antigen-binding fragment (Fab) of one of the neutralizing bovine MAb (specific for BHV1 gD) was used to generate murine MAb specific for the bovine MAb idiotype. One murine anti-idiotype inhibited virus neutralization by the immunizing bovine MAb, indicating that its specificity is at or near the bovine MAb antigen binding site. The murine MAb may therefore also be useful, for vaccination or diagnosis.

The generation and exploration of the *in vitro* and *in vivo* activities of bovine MAb demonstrates the utility and potential of host MAb for serious diseases of cattle. The physical products (the bovine MAb) were demonstrated to be potentially useful in diagnosis and immunization, as well as in the definition of neutralization epitopes of the virus. The concepts explored can be used for other host species and other diseases.

Further work could include increasing the number of self re-fusion cycles and observing for further increases in growth/secretion lines, or using the BL5A1.Y2C@ and
.Y5A11 (murine2 x bovine2) in fusions with lymphocytes from other cattle and comparing growth/secretion rates with the @BL5C2 fusion in this study. It could also include characterizing additional bovine MAb from the fusions described here, or additional similar fusions, including antigen and epitope definition to broaden and confirm the conclusions of this work. The elementary scale-up production methods could be improved, allowing further work with the subject MAb but also application to other heterohybridomas. A more rigorously designed rabbit protection study would confirm or deny the dose-dependent passive immunization seemingly observed in these studies, and could be expanded to a more complete rabbit immunization model (potentially including active immunization of the dams). Oral administration of the bovine MAb to neonate calves at titers similar to colostrum, followed by challenge in some animals would provide information on the potential for passive immunization against generalized neonatal BHV1 disease cattle. Those and other routes and doses could be used to explore protection from bovine respiratory disease (BRD) or other diseases. The bovine MAb studied could be tested against additional murine MAb to further characterize the epitopes (particularly for the anti-BHV1 gC @BL5C2.870005). Reactivity of the bovine MAb with peptides in ELISA or virus neutralization inhibition assays could also help with epitope definition. The anti-idiotypic antibodies generated could be administered to syngeneic mice and with modification, to cattle to examine the potential of anti-idiotypic vaccination for BHV1 and BRD. These and additional bovine MAb or their mRNAs could be sequenced, their complementarity determining regions defined and analyzed, and their structures modeled. Finally, the bovine MAb could be crystalized alone and in complex with their antigens to examine their structures and binding characteristics, including shape of ‘loops’ and any ‘induced fit.’ Expansion of such examinations to new species such as cattle could increase the understanding of immunoglobulin origins and function.
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