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Research paper

Generation by self re-fusion of bovine^3 × 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 BHV1 hyperimmunized calf with 3 types of non-secreting fusion partners. Seven were generated through fusion with the murine × murine (murine^2) hybridoma SP2/0, 3 through fusion with bovine–murine^2 HH previously generated using cells from the same calf, and 68 through fusion with bovine^2-murine^2 HH previously 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 major neutralization targets for BHV1. 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. A cocktail of the 3 BomAb was administered intravenously (i.v.) to a 6-month-old calf and its serum neutralization activity decreased with a half-life consistent with non-immune clearance, suggesting that BomAb may be useful for passive immune treatment of disease in cattle. Rabbits were passively protected by i.v. injection with each of the anti-gB and anti-gD BomAb when challenged i.v. with

Abbreviations: AS, antiserum; b, bovine origin; BHV1, bovine herpesvirus 1; BomAb, bovine monoclonal antibody; Chr, chromosome; dpc, days post challenge; dpi, days post inoculation; gB, BHV1 glycoprotein B; gC, BHV1 glycoprotein C; gD, BHV1 glycoprotein D; gp, glycoprotein; HH, heterohybridoma; IFA, indirect fluorescent antibody; LC, lymphocyte; LN, lymph node; m, murine origin; MumAb, murine monoclonal antibody; MW, molecular weight; VN, virus neutralization.

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BHV1 24 h later. Self re-fusion was shown to be advantageous for efficiently producing HH stably secreting host monoclonal antibodies. The BomAb described should prove useful in studies of the host immune response to BHV1, as reagents, and as sources of bovine immunoglobulin sequences.

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1. Introduction

Bovine herpesvirus 1 (BHV1) causes diseases of global economic significance in cattle and is the subject of national control and eradication programs (reviewed in Levings and Roth, 2013a). BHV1 is a member of the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus (Davison, 2010).

Laboratory rabbits have been infected with BHV1 by a variety of routes, resulting in lesions and disease that were often route-specific (Bindrich, 1960; Armstrong et al., 1961; Persechino et al., 1965; Bwangamoi and Kaminjolo, 1973; Kelly, 1977; Lupton et al., 1980; Rock and Reed, 1982; Brown and Field, 1990). Rabbit infection and disease systems (including those in neonatal rabbits) have been suggested as models for bovine systems, such as vaccine efficacy evaluation (Kelly, 1977; Lupton et al., 1980; Rock and Reed, 1982; Valera et al., 2008).

Alphaherpesvirus envelope glycoproteins (gp) function in the virion envelope's attachment to and fusion with the host cell membrane, and are the targets of protective cellular and humoral host immune responses (reviewed in Levings and Roth, 2013b). BHV1 gps B, C, and D (gB, gC, and gD) are the primary inducers and targets of virus neutralizing (VN+) Ab (Collins et al., 1985; van Drunen Littel-van den Hurk and Babiuk, 1986; Turin et al., 1999) and gB, gC, and gD subunit vaccines were each protective (Babiuk et al., 1987). BHV1 gp epitopes have primarily been characterized using murine mAb (MumAb) (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).

Host species mAb may offer advantages over MumAb in identifying epitopes important in natural infection (Trkola et al., 1996), including protective and immunodominant (Perryman et al., 1990) epitopes, useful for vaccine and diagnostic assay design. They may also be useful for passive protection (Sandborn et al., 2012), and as diagnostic or research reagents. Human mAb have been generated using human-human hybridomas, human-mouse heterohybridomas (HH) and in a variety of other ways (reviewed in Wang, 2011; Sullivan et al., 2011), but the methods other than HH have not generally been available for or employed in other species. In addition, some alternative techniques do not generate mAb representative of the immune response, or may result in mAb limited in quantity or restricted in Ig class (Jessup et al., 2000). Transforming lymphocytes (LC) with species-specific viruses (Epstein-Barr virus for humans, bovine leukemia virus for cattle) is one example where an alternative strategy has been useful for non-murine, non-human species, although the transformed bovine LC usually produce bovine IgM mAb.

HH, also called heteromyelomas, inter-species hybridomas, or xenohybridomas have been and continue to be successfully employed as a source of host mAb for a variety of non-murine laboratory animal (Raman et al., 1994; Ukaji et al., 2011) and veterinary (Flynn et al., 1989; Greenlee et al., 1990; Keggan, 2013) species, including cattle (reviewed in Groves and Tucker, 1989; Groves and Morris, 2000).

Fusions between bovine LC (b) and murine myelomas (m) or murine hybridomas (m^2) have produced HH stably secreting bovine Ig of unknown specificity (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 $b \times m^2$ HH secreting non-neutralizing bovine mAb (BomAb) specific for BHV1 gC. Fusions of non-murine LC with murine myeloma cells have often resulted in loss of secretion as a result of rapid chromosome (Chr) loss. The best studied HH, human-murine HH, lose human Chr preferentially (Crocem et al., 1980; Wollweber et al., 2000). 'Re-fusion' or the use of HH as fusion partners yielded better results in a variety of species (Yang et al., 1990; Wang, 2011), including in cattle. It has been used to increase the number of non-murine Chr in product HH, increasing the likelihood of retaining Chr 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). In one report (Tucker et al., 1984) the re-fused lines carried two to three times the number of non-murine (bovine) Chr as the single-fused HH.

Both $b \times m^x$ (Tucker et al., 1984; Anderson et al., 1986, 1987; Groves et al., 1987; Kennedy et al., 1988; Kemp et al., 1990) and $b^2 \times m^x$ aminopterin-sensitive HH (Anderson et al., 1986, 1987; Tucker et al., 1987; Groves et al., 1988) have been used for re-fusion with bovine LC. In some cases the selected HH fusion partners themselves secreted Ig, but in most cases clones not secreting Ig were chosen for use. In each case the fusion partners and LC were derived from different individuals of the same outbred species.

The aims of this study were: (1) to examine the effect on HH generation rates of increasing degrees of re-fusion with LC from the same individual and stimulated with the same agent; (2) to exploit the increased rate of productive HH generation after re-fusion to derive HH producing VN+ BomAb to BHV1 envelope gp; and (3) to conduct basic characterization of the VN+ BomAb *in vitro* and *in vivo*.

2. Materials and methods

2.1. Immunization of the bovine lymphocyte donor

The immunizing virus was a virulent, low bovine cell culture passage preparation (Center for Veterinary Biologics, CVB, Ames IA) of the Cooper strain (also known as Colorado) BHV1.1 virus, free of bacteria, mycoplasma, and bovine viral diarrhea virus (BVDV).

A seropositive (VN titer 32) subadult Holstein breed cow was repeatedly (up to five times per series) injected s.c. with 5 ml (10^5 plaque forming units [pfu]/ml) of live BHV1. The dosage was divided and injected in multiple sites at 2–4 week intervals into the area drained by a targeted superficial lymph node (LN, prescapular or prefemoral). After an initial unadjuvanted dose, multiple doses of live virus emulsified with equal volumes of Incomplete Freund's Adjuvant (Gibco, Grand Island NY) were injected. At 3–4 days before fusion, unadjuvanted virus was injected in the area. The LN were sequentially targeted, and surgically removed under local or general injectable anesthesia (xylazine, Rompun, Bayer, Shawnee Mission KS). All animal work was approved by the Institutional Animal Care and Use Committee (IACUC). The animal care and use program applying to this and the animal use described in other sections included protocol review, facility inspection, training, and other measures to ensure conformity with the provisions of the current versions of "Guide for the Care and Use of Laboratory Animals" (Anon., 2011a), the Animal Welfare Act (Anon., 2008), and the Stockman's Handbook (Ensminger, 1991).

2.2. Fusions

All fusions were performed by a modification of a standard murine hybridoma protocol (Van Deusen and Whetstone, 1981; Van Deusen, 1984). The cells extracted from the LN by sectioning and sieving were washed and then immediately used for fusion or controlled-rate frozen. Cells from fusions were seeded into 96 well plates at 5×10^5 fusion partner cells/ml. Horse serum was used as nutrient serum, and conditioned media from fusion partner cultures appropriate to each fusion was used.

Three sets of fusions were performed (see Fig. 1), each using a different BHV1-stimulated LN from the same animal. The third set of fusions is the focus of this report. In that set, three fusions were performed on the same day with fresh cells from the same LN, each with a different fusion partner in a 2:1 LC:myeloma ratio.

Fusion 5C0 was done using the SP2/0-Ag14 (SP2/0) line (Shulman et al., 1978) as fusion partner. The SP2/0 line had been generated by fusing BALB/c spleen cells (from a mouse immunized with SRBC) with the P3X63Ag8 non-secreting murine myeloma, so for this work it was designated m^2 and the 5C0 fusion products $m^2 \times b^1$.

Fusion 5C1 was done using an equal mixture of 5A0.1B1 and 5A0.3E6 as fusion partner. The fusion partners were products of a previous fusion using frozen LN cells from the subject calf and SP2/0 cells in a 3:1 LC:myeloma ratio. Two HH, 5A0.1B1 and .3E6 (of 480 cultures) transiently secreted anti-BHV1 Ig, and so were chosen as fusion partners. They were selected for methotrexate sensitivity and frozen as individual cultures. The fusion partners were designated $m^2 \times b^1$ and the 5C1 fusion products $m^2 \times b^2$.

Fusion 5C2 was done using an equal mixture of 5A1.2C2 and 5A1.5A11 as fusion partner. The fusion partners were products of a previous fusion using fresh LN cells from the subject calf and an equal mixture of 5A0.1B1 and .3E6 ($m^2 \times b^1$, see above) in a 1:1 LC:myeloma ratio. The @BL5A1 fusion resulted in 4 HH (including 5A1.2C2, of 576 cultures) that temporarily secreted Ig to BHV1. 5A1.2C2 (the only initially secreting culture surviving) and 5A1.5A11 (chosen for robust growth in culture) were chosen as fusion partners. They were selected for methotrexate sensitivity and frozen as individual cultures. The fusion partners were designated $m^2 \times b^2$ and the 5C2 fusion products $m^2 \times b^3$.

2.3. Growth culture retention, cloning, and stability

Primary cultures with observed growth were tested using microtiter (MT) indirect fluorescent Ab (IFA) and MT VN assays using duplicate test wells. Primary cultures from 5C0 and 5C1 fusions were preferentially tested relative to 5C2 (97–100% *vs.* ~60%) due to the relative rarity of cultures with growth for those fusions, and limiting culture expansion and testing capacity. Selected primary cultures positive by IFA (IFA+) from all three fusions were retained, expanded, cloned by dilution, recloned, and controlled-rate frozen, again with preference for 5C0 and 5C1 lines.

Three HH lines (re-clones 5C2.αgB-09, αgC-05, and αgD-16, hereafter referred to only as αgB-09, αgC-05, and αgD-16) were chosen for special scrutiny because they secreted VN+ BomAb (hereafter also referred to by the HH short 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.

2.4. Karyology

Cells and slides were prepared according to the initial steps in the CVB protocol ABRMPRO1101 after Worton and Duff (1979). At the NIH-National Cancer Institute (NCI, Frederick MD) Chr were stained with a trypsin-Giemsa staining technique (Seabright, 1971), karyotyped using a microscope coupled with a charge-coupled device camera, and images were captured with karyotyping software (Band View 5.5, Applied Spectral Imaging, Vista CA). In those cases where Chr morphology was too poor for analysis, fluorescence *in situ* hybridization was used to help determine Chr number and/or sex. Probes for bovine Chr X and Y were commercially sourced (IdLabs Biotechnology, London ON) and slides hybridized according to manufacturer's protocol. Fifteen samples were tested from 8 lines, representing fusion partners and HH of m^2, $m^2 \times b$, $m^2 \times b^2$, and $m^2 \times b^3$ types.

2.5. Indirect fluorescent antibody assay

For primary or (re-)clone screening assays, undiluted culture supernatants were tested by a modification of

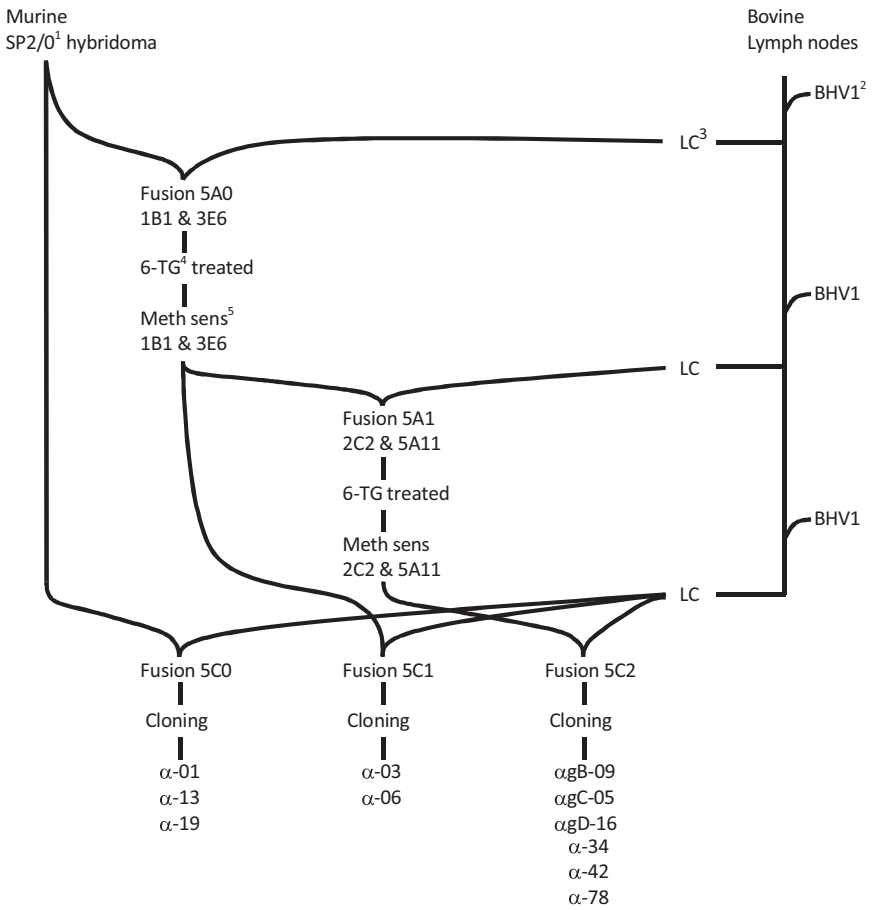

Fig. 1. Flow chart illustrating the re-fusion strategy used to generate heterohybridomas secreting bovine mAb to bovine herpesvirus 1. Only those heterohybridomas and bovine mAb for which results are described in the manuscript are shown. [1]SP2/0-Ag14 line. [2]Bovine herpesvirus 1. [3]Lymphocytes extracted. [4]6-Thioguanine. [5]Methotrexate sensitive.

CVB protocol VIRPRO1014 (Anon., 2011b). Conjugate used was FITC-conjugated anti-bovine Ig (rabbit or goat origin, National Veterinary Services Laboratories [NVSL], Ames IA). Dilutions in PBS of bovine hyperimmune serum (antiserum, AS) to BHV1 (CVB) served as positive controls. Hybridoma culture medium and PBS, as well as uninfected wells served as negative controls. Infected and uninfected cells were observed in all positive control wells.

For specific activity assays of the VN+ BomAb αgD-16, IFA was performed using affinity purified (Protein G Sepharose 4 Fast Flow, Pharmacia LKB, Piscataway NJ) BomAb and control Igs. αgD-16 was purified from nude mouse (NM) ascites fluid (AF), and polyclonal anti-BHV1, anti-BVDV, and non-specific Ig were purified from bovine AS and FBS, respectively. The BHV1 AS (CVB Lot 9) was produced in a single calf by repeated immunization (i.m., i.m., intranasal, i.m. with adjuvant, then i.d. and i.v.) with virulent Cooper strain BHV1. Protein was quantified using Lowry assays with rabbit or bovine serum albumin as standards.

For competitive BHV1 IFA, the 3 VN+ BomAb were each labeled with FITC using the NVSL TCSOP0202 method after Hébert et al. (1972). Each of the 3 VN+ unlabeled BomAb was first incubated in the plate, followed by washing and incubating with one of the 3 labeled BomAb, followed by washing and reading.

2.6. Virus neutralization assays

Titrations of preparations of the 3 VN+ BomAb and BHV1 AS were done using constant virus/varying Ab VN assays. The MT cytopathic effect (CPE) VN method followed CVB Supplemental Assay Method (SAM) 109 (Anon., 2011c). The plaque reduction VN (PRVN) assay followed CVB SAM 119 (Anon., 2011d), except a Cooper strain BHV1 reference virus was used in place of pseudorabies virus, another varicellovirus. VN titers reported were ranges of or nearest 2-folds, or 50% endpoints calculated by linear regression of log of dilution *vs.* log of % neutralization (those points between 10 and 90%). Titers for dilutions of high-titered preparations used in rabbit passive immunization studies were specified based on dilution factor only.

For primary or (re-)clone screening (+/−) assays, undiluted culture supernatants were tested using an equal-volume modification of SAM 109. Modifications included use of only two wells, virus challenge ranging from 50 to 500 $TCID_{50}$/25 μl, and observation for CPE at 3–4 days. An equal-volume modification of SAM 119 was

used to monitor BomAb activity (% neutralized) in the immunized calf. A 3-condition modification of SAM 109 using 4 wells was performed to examine post-adsorption neutralization of the 3 VN+ BomAb. The conditions studied were: (1) Ab inoculum, then virus inoculum and 1 h incubation on cells ('0-time VN'); (2) Ab-virus incubated for 1 h, then the inoculum on cells for 1 h ('traditional VN'); (3) virus and media (only) on cells for 1 h (control).

2.7. Virus specificity

αgB-09, αgC-05, and αgD-16 culture supernatants and concentrates thereof, serum free (SF) culture supernatants, hollow fiber (HF) culture harvests, and dilutions of NM AF, all with anti-BHV1 VN titers ≥8 were assayed for IFA or VN activity against other bovine respiratory viruses, and against potential extraneous agents of bovine vaccines and their master seed viruses (Anon., 1991) and cells. Bovine parainfluenza 3 (PI3) virus (VN, IFA), BVDV (VN), bovine respiratory syncytial virus (VN, IFA), bovine adenovirus (IFA), bovine parvovirus (BPV, IFA), bovine reovirus (IFA), and rabies virus (VN) were tested. Cell culture media and dilutions of SP2/0 AF were used as negative controls, and dilutions of homologous AS (bovine AS for IFA) used as positive controls.

2.8. Commercial ELISA

A commercial ELISA kit for bovine Ab to BHV1 (Bovine Rhinotracheitis Virus Antibody Test Kit Screening/Verification, IDEXX, Westbrook ME) was used according to kit instructions, with one modification – the dilutions prescribed for bovine serum were not used. Culture supernatants from each of the 3 VN+ BomAb (αgB-09, αgC-05, αgD-16), plus dilutions of NM AF from αgD-16 were tested. Cell culture media and dilutions of SP2/0 AF were used as negative controls.

2.9. 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 (Rodrigues et al., 2010). Conventional production consisted of flask (25, 75, and 150 cm^2) suspension cultures and was routinely successful. SF cultures were routinely established (also with flasks), using a modified medium (Iscove and Melchers, 1978), with added bovine insulin and human transferrin (Kawamoto et al., 1983) instead of nutrient serum.

HF culture was conducted by seeding cells ($1.1–1.3 \times 10^8$) into the extra-capillary space of a cartridge (Vitafiber II, Amicon, Danvers MA) with 30,000 molecular weight cut-off (MWCO) fibers. Cultures were perfused with 1.0-liter batches of the growth medium used for flask cultures. Twice per week, 25 ml was harvested from the extra-capillary space and recirculating growth media was changed. The pH of the culture was maintained by filtered gas exchange with the media reservoir (the entire apparatus was placed in a 5% CO_2, 37 °C incubator). Extensive monitoring or control of critical culture parameters (*e.g.*, glucose use, O_2 concentration, pH) was not performed. The mechanical, overgrowth/cell death, and acidity issues observed in other studies (Jackson et al., 1996; Valdés et al., 2001) were experienced in these trials.

Conventional AF production in syngeneic mice is not possible for HH, so a trial using immunosuppressed Balb/c mice was conducted using modifications of a published technique (Raybould et al., 1985). 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 PBS s.c. one day before i.p. injection of αgB-09, αgC-05, and αgD-16, and SP2/0 at 1 and/or 5×10^5 cells/mouse. As a control, 10^5 and 10^6 cells of αgB-09 and αgC-05 were injected without drug treatment. 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 i.p. inoculation of 5×10^6 cells of αgB-09. All mice were pristine primed prior to hybridoma injection as per routine hybridoma protocol, and all work was performed under an IACUC-approved protocol. NM AF from each of the 3 VN+ HH (25 mouse batches) was produced commercially (Charles River Laboratories, Charles River MA).

2.10. 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 H and L chain molecular weights (MW) 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 20× concentrate of conventional culture supernatants was prepared using centrifugal concentrators (Centricon concentrators, Amicon) and tested. Wells were refilled 4 times and observed for contaminating Ig. Kit reference controls were used as positive controls. Growth media for HH (10% horse serum) and heterologous kit reference controls (*e.g.*, IgA and IgM for the IgG1 plate) were used as negative controls.

The IEP (Williams and Grabar, 1955) procedure applied to bovine Ig (Butler, 1983) was performed, using standard low negative relative migration (−mr) agarose (2 mm thick), 86 mm × 100 mm (GelBond) film, tris-tricine buffer, and a horizontal electrophoresis apparatus (Bio-Rad, Hercules CA). Wells 2 mm in diameter and 2 mm × 70 mm troughs were cut, with wells and troughs alternating at 10 mm intervals. Wells were filled with 15 μl of 15× (αgC-05) or 30× (αgB-09, αgD-16) flask HH culture supernatants. Larger diameter wells (4, 6, 9 mm) with larger volumes (2.5, 5, and 10× of standard) were also used to detect any other, lower concentration or contaminating Ig. Troughs were filled with 300 μl of goat anti-bovine Ig (NVSL). Culture media (15 μl, 30×) and bovine BHV1 AS (10 μl) were used as negative and positive controls, respectively. Electrophoresis was performed at 4 V/cm × 1.5 h. Gels were soaked in PBS to remove soluble protein, dried, stained with Coomassie Blue, destained with methanol, dried, then analyzed and photographed.

An isotyping BHV1 IFA was performed using a modification of the previously described IFA, using @GBIGG.DAS

17.A4 (hereafter referred to as DAS17) a MumAb specific for bovine IgG1 (hybridoma kindly donated by Richard Goldsby, Amherst College, Amherst MA; Goldsby et al., 1987). Test wells were incubated with BomAb, washed, incubated with the DAS17, washed, incubated with anti-mouse IgG FITC conjugate, washed, and read. Immunological methods to isotype the L chains were not conducted due to a lack of reagents at the time.

2.11. SDS-PAGE and Western blots

Three types of SDS-PAGE were performed: hand-cast full size (16 cm × 18 cm × 1.5 mm) homogeneous 7.5, 10.0, or 12.5% gels; pre-cast mini (10 cm × 10 cm × 1.0 mm) 4–12% gradient gels (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). Gels were stained using Coomassie Blue and/or silver stain. High, low, a mixture of high and low, or wide range SDS-PAGE MW standards (Bio-Rad and Invitrogen) were used appropriate to the gel concentration.

Monitoring the purity of HF harvests was done using full sized gels. Apparent MW of Ig H and L chains were determined using a mini-gel of an ammonium sulfate precipitate of SF culture supernatant (αgC-05), and a full size gel of agarose Protein A-bound Ig (αgB-09 and αgD-16, same gel). The H and L MW were calculated using the method described in Section 2.12.

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, Holliston MA) respectively. The nitrocellulose sheets were processed in full size (Deca-Probe, Hoefer) or mini-gel (Minblotter 28, Immunetics, Boston MA) gasketed-lane incubation manifolds. After blocking at rt and 37 °C, primary Ab was applied and incubated at 37 °C (90 min), rt (60 min), and then 4 °C (16 h). Primary Ab consisted of the selected BomAb (αgB-09 and αgD-16) or sham preparation, bovine BHV1 AS, or nonspecific (BVDV) bovine AS as controls. Secondary Ab was anti-bovine peroxidase-labeled Ig, applied for 2 h at rt. Control lanes were stained with amido black.

2.12. Immunoaffinity chromatography

Viral envelope Ag was prepared using a method similar to Marshall et al. (1986). BHV1 was produced using bovine cell lines in roller bottles. At 90–100% CPE, the culture media was clarified and the virus pelleted through a 40% sucrose cushion. The virus was resuspended in PBS and gradient purified (2 cycles) using polyvinylpyrrolidone-coated colloidal silica (Percoll, Pharmacia LKB), 90% in 0.25 M sucrose, after the method of Svennerholm et al. (1980). Fractions ~1.050–1.070 g/ml (using markers, Pharmacia LKB) were harvested, titered, and high virus titer fractions pooled. The gradient-purified BHV1 was diluted 1:8.4 in Tris-buffered saline with 1% tergitol-type nonyl phenoxypolyethoxylethanol (NP-40) and 1% deoxycholate (DOC) (TBSND), and sonicated. Remaining whole virions and capsids were removed by ultracentrifugation. Uninfected cell debris at the same densities were similarly harvested and treated.

BomAb was prepared by immunoaffinity chromatography (IAC). DAS17 Ig was isolated from AF by 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 then covalently bound to a commercial aldehyde-activated 6% beaded agarose (AminoLink Coupling Gel, Pierce) using product instructions. Columns of the DAS17-agarose were used to purify bovine Ig from pools of HF culture harvests of four bovine HH – αgB-09, αgC-05, αgD-16, and an $m^2 \times b^3$ HH secreting IgG1 BomAb to BPV, 6-1.α-0131 (hereafter referred to only as α-0131). The BPV VN+ α-0131 was generated by fusing each of the fusion partners for the 5C2 fusion (5A1.2C2 and .5A11) with fresh LC from a BPV-stimulated LN of a different animal, cloning, and then re-cloning. α-0131 resulted from the 5A1.2C2 fusion. IAC fractions were tested by IFA, and their protein concentrations measured by Lowry. Amounts of 10.0 mg (αgB-09); 2.51 mg (αgC-05); 10.0 mg (αgD-16), and 6.26 mg (α-0131) were bound to aldehyde-activated agarose gel columns, per product instructions.

The BomAb columns were loaded with the BHV1 envelope Ag preparations (using 3 cycles viral Ag, 10 cycles of sham), washed with TBSND, washed with DH_2O, then eluted with 0.1 M glycine HCl pH 2.7 into a Tris–HCl pH 9.0 'cushion'. Fractions with significant Å280 readings were pooled, the pH adjusted (from 8.2 to 7.5–7.6) and concentrated 250× using centrifugal concentrators (10,000 MWCO, Amicon). The same fractions of cell culture control preparations were collected and treated identically.

Apparent MW of BHV1 proteins purified by IAC were determined using microvolume SDS-PAGE. 7.5% homogeneous, 10–15% gradient, and 8–25% gradient gels with 8 lanes were used (10–15% gels for calculations). MW was calculated from distance migrated (D) and linear regression on the MW markers, using $\ln \ln M_o/\mathrm{MW}$ *vs.* $\ln D - D_o$ where M_o and D_o are the MW and distance migrated of the heaviest MW marker used. Pearson's correlation coefficients (r) were calculated for the linear regressions used. The MW of envelope proteins were compared to those previously reported, including for: gB (gI, gp6/11a/16), gC (gIII, gp3/gp9), and gD (gIV, gp5/11b) (Misra et al., 1981; Marshall et al., 1986; Babiuk et al., 1987; Collins et al., 1984; Okazaki et al., 1986; Tikoo et al., 1990).

2.13. Bovine monoclonal antibody processing in cattle

A seronegative Holstein steer, ~6 months old and ~450 lb (~200 kg) was injected i.v. (jugular vein) with a cocktail of equal volumes (20 ml each) of the 3 VN+ BomAb NM AF described. The NM AF had titers of 383 (αgB-09), 1997 (αgC-05), and 2480 (αgD-16), and the cocktail had a titer of 5520, slightly higher than the sum of the BomAb titers (4860).

The experiment was conducted using an IACUC-approved protocol. Serum samples were taken from the jugular vein at −1 to 15, 20, 27, 34, and 42 days post-inoculation (dpi). Serum was collected twice on day 0, once

immediately before injection of the Ab, and again ~30 min after injection of the Ab, but from the side opposite injection. Equal-volume PRVN assays were performed using a fixed virus amount (47.0–56.0 pfu) and negative sera from cattle of the herd as controls. The % VN of the 2 wells for each bleeding were averaged, and linear regression performed on the averages.

Nasal swabs were taken on days −1 to 15 post-immunization. Each nostril was swabbed with one cotton-tipped swab, and both swabs placed in a tube with 2 ml Eagles' minimum essential medium (EMEM) and stored at −70 °C. PRVN assays were conducted on the swab fluids.

2.14. Passive immunization of neonatal rabbits

The VN+ BomAb dose needed to produce a potentially measurable protective effect in cattle was calculated. Using a calf weight of 200 kg, the published plasma volume for 6 months old grazing Herefords (47.7 ml/kg, Payne and Garner, 1967), and the plasma IgG/total body IgG pool for humans (0.52, Wells, 1978), 1 ml of i.v. inoculum would be initially diluted ~1:10,000, and after distribution diluted ~1:20,000. Therefore 1000 ml of any single Ab preparation of VN titer 2560 would need to be injected to achieve a circulating VN titer after distribution of 128, the mid-point of the range (64–256) of circulating polyclonal Ab titers reported to protect via passive protection (Mechor et al., 1987). Due to the large calculated dose and the uncertain protective effect of any single BomAb, a preliminary trial using the most popular laboratory animal model for BHV1 infection (rabbits) was performed.

Mid-term bred female BHV1-seronegative (VN) New Zealand White rabbits (167, in 16 lots) were commercially sourced (Small Stock Industries, Pea Ridge AR) and whelped in nesting boxes. The inoculated rabbits in the age *vs.* virus-dose mortality trials were 4–22 days old at challenge. The inoculated rabbits in the passive immunization trials were 6–8 days old at challenge.

HH culture supernatants (regular or SF) were used undiluted or concentrated 10–30× at 4 °C using 30,000 MWCO ultrafiltration vacuum or centrifugal devices (Amicon). Similarly concentrated hybridoma culture media or non-secreting hybridoma (SP2/0) culture supernatant were used as sham controls and data combined by series as '0 titer'. In addition, bovine BHV1 AS (see Section 2.5; titer 2760) and serial 2-fold dilutions in EMEM (specified as titer 1380 through 22) were administered, using matching dilutions of FBS for sham preparations. Ab 'doses' reported are VN titers of 1 ml of inoculum (*e.g.*, 1 ml of 1280 titer = 1280, 0.5 ml of 2560 titer = 1280).

Viruses were 3 or 7 (only the $10^{7.7}$ preparation) cell culture passages from a Cooper strain seed virus 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. Third-passage viruses caused typical BHV1 respiratory disease when administered intranasally to cattle (Anon., 2010). Virus titers for age-dose mortality trials were $10^{7.0–8.8}$ pfu/ml, and for passive immunization studies were $10^{7.3–7.5}$ pfu/ml. EMEM was used as a sham virus preparation.

All experiments were conducted using an IACUC-approved protocol. Trials using i.v. injection of adults and temperature measurement were conducted, but the febrile responses reported in the literature were not observed. A trial using i.m. challenge of 14–15 days old rabbits was also conducted, but no clinical signs or deaths were observed. *Per os* and intraocular inoculation of limited numbers of neonates also failed to produce clinical signs or death. Intracardiac (i.c.) challenge of young rabbits reproducibly induced virus-specific disease and death, 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, but rather biased for more even size distribution between treatments. All rabbits were observed daily for 14 days post-challenge (dpc) for clinical signs or death. No clinical signs other than depression were observed prior to death.

Multiple deaths were observed the day of and day after each of sham Ab injection (prior to virus injection) and sham virus injection, with one death in a sham group 2 days after inoculation. Many deaths occurred on day 2 dpc (virus) in sham Ab or no Ab groups, with peak death numbers/group on days 2 and 3. It therefore appeared that injury and virus-specific mortality time-courses overlapped. For purposes of interpretation, deaths observed on the day of or day after either Ab or virus i.c. injection were recorded as injury (non-specific) death, and deaths on 2–14 dpc were scored as virus-specific. Due to varying litter size and injury losses, 0–10 rabbits per litter were scorable as virus-specific deaths or as alive at the end of the observation period. Only rabbits from litters with 2 or more scored rabbits were used for analysis. Reported '*n*' values for groups are for scorable rabbits used for analysis. Although a rigorous statistical analysis could not be performed, for illustration groups of ≥9 (BomAb – due to control group size of 9) or ≥10 (AS) were graphed and analyzed by chi-square with Yates correction (for two groups).

For the age-dose mortality study, three age-groups were tested: 4–7 days old ($n = 29$ rabbits, 5 virus titers $10^{7.0–8.0}$ and sham); 13–14 days old ($n = 23$, 3 virus titers $10^{7.7–8.3}$ and sham); 20–21 days 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. Data was pooled for each age × virus dose, across litters and lots.

For the passive immunization studies, the split litter design initially included: group a = true Ab, true virus; group b = sham Ab, true virus; group c = true Ab, sham virus; and group d = sham Ab, sham virus. One ml of Ab or matching sham preparation was given i.c. on 0 dpi. Circulating Ab titers were not assessed due to the small rabbit size and attendant risk of sample bleeding. 1.0 ml of virus or sham preparation was given i.c. on 1 dpi = 0 dpc. Preliminary experiments on 16 litters split into groups a through d established no mortality in groups c ($n = 19$) and d ($n = 14$) other than injury deaths at days 0–1 dpi or dpc, so subsequent litters were split into groups a and b only. For passive immunity analysis, results were pooled for the two virus doses ($10^{7.3}$ and $10^{7.5}$), for all concentrations of SP2/0 supernatant and culture media Ab controls for each BomAb, and for all dilutions of FBS controls for bovine AS.

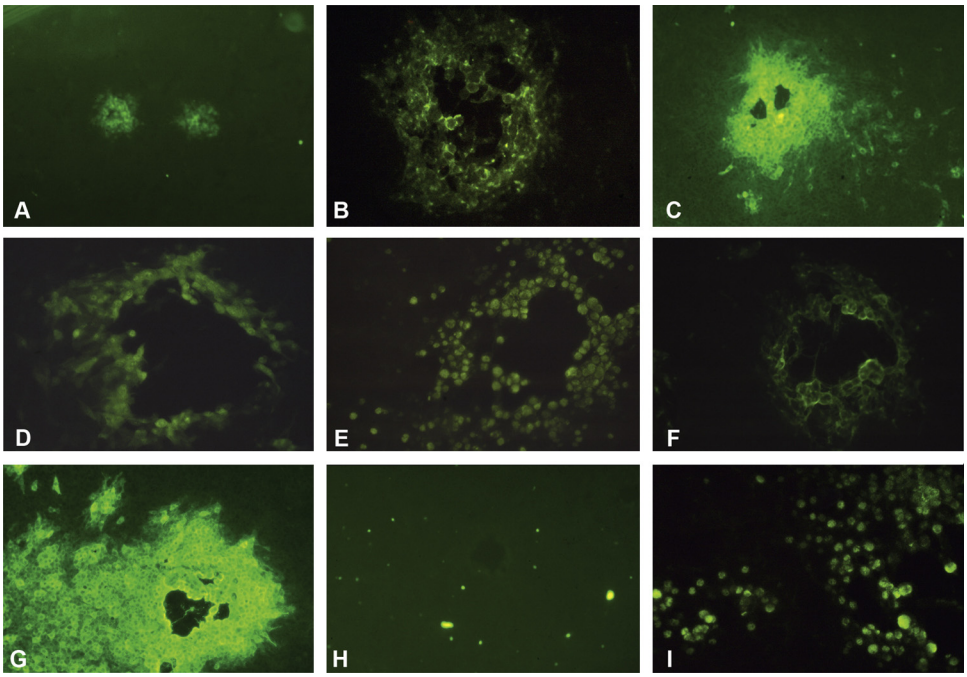

Fig. 2. Indirect fluorescent antibody patterns of select bovine mAb and controls with bovine herpesvirus 1 (BHV1) infected cell cultures. (A) 5C2.αgB-09. (B) 5C2.αgC-05. (C) 5C2.αgD-16. (D) 5C0.α-01. (E) 5C2.α-42. (F) 5C2.α-78. (G) Bovine antiserum to BHV1. (H) SP2/0. (I) 5C2.α-34. Samples were conventional culture supernatants and 10^{-3} of antiserum.

Analysis for one BomAb (αgC-05) could not be completed due to low (<5) numbers in all five Ab titer groups. Toxicity of preparations likely contributed to the low numbers, as injury deaths due to Ab administration were high (*e.g.*, 76% [$n = 16/21$] for the concentrated preparations titering 52 and 237), and the time to scored deaths (*e.g.*, 20%, 50% mortality) was also shorter in αgC-05-immunized groups than in the sham-immunized virus-challenged controls.

3. Results

3.1. Heterohybridoma growth and secretion of specific bovine immunoglobulin

In the "self-re-fusion" comparison (Fig. 1), growth was observed in 3 times as many seeded 5C2 ($b^3 \times m^2$) cultures as for 5C0 ($b \times m^2$) and 5C1 ($b^2 \times m^2$) fusions (Table 1). Of the 5C2 cultures tested, 10 times as many were found to secrete Ab specific for BHV1 by IFA, when compared to 5C0 and 5C1 fusions (Table 1). Stability of secretion was similar between fusions, as evidenced by successful cloning and re-cloning of lines.

The 3 HH αgB-09, αgC-05, and αgD-16 continued to secrete VN + BomAb after one year of continuous passage, and after being stored frozen for more than one year. They were deposited (under the Budapest Treaty) in the American Type Culture Collection (ATCC, Rockville MD) and designated HB 9908, 9907, and 9909, respectively. The viability and secretion of the ATCC cultures was confirmed shortly after deposit. In addition, the 3 HH continued to secrete VN+ BomAb after being selected for resistance to 6-thioguanine and oubain.

3.2. Karyology

Karyotypes of 8 lines representing fusion partners and HH of varying degrees of re-fusion demonstrated a range of Chr numbers. Although there was significant variability and sample numbers were small, there was a trend of increasing Chr number with increasing bovine re-fusion (Table 2). The species origin of the Chr could not be determined. Anomalies included deletions, translocations, re-arrangements, and small markers.

3.3. Indirect fluorescent antibody, virus neutralization, virus specificity, and commercial ELISA assays

A variety of IFA patterns were observed with the BomAb tested (Fig. 2). Affinity purified αgD-16 was IFA positive at 500 ng/ml, whereas affinity purified Ig from polyclonal bovine BHV1 AS was positive at 5000 ng/ml. Only the homologous unlabeled VN+ BomAb competed with each FITC-labeled VN+ BomAb in IFA.

The $m^2 \times b^3$ fusion 5C2 was the only one to produce HH secreting VN+ BomAb. Three were detected: αgB-09, αgC-05, and αgD-16. They were found to neutralize the immunizing BHV1 isolate without C. Although some (0.75 $\log_{10}$ $TCID_{50}$, compared to ≥2.25 $\log_{10}$ for traditional VN) activity was demonstrated by αgD-16 in the 0-time VN, all 3 VN+ BomAb neutralized <1.0 $\log_{10}$ in 0-time VN, and neutralized ≥1.0 $\log_{10}$ by traditional VN, suggesting they may be unable to neutralize after virus attachment.

Table 1
Growth, specific antibody secretion rates, and stability of fusions with increasing degrees of self re-fusion. Stability measured by secretion through culture cloning.

Fusion name	5C0	5C1	5C2
Type	Bovine[1] × murine[2]	Bovine[2] × murine[2]	Bovine[3] × murine[2]
Fusion partner	SP2/0	5A0.1B1 & 3E6	5A1.2C2 & 5A11
Cultures seeded	768	1056	960
Cultures with growth (% of seeded)	190 (24.7)	131 (12.4)	708 (73.8)
Primaries tested (% of cultures with growth)	185 (97.4)	130 (99.2)	423 (59.7)
Primaries strong IFA+[a] (% of tested)	7 (3.8)	4 (3.1)	160 (37.8)
Primaries cloned (% of attempted)	5 (71.4)	3 (100)	42 (72.4)
Lines recloned (% of attempted)	5 (100)	3 (100)	25 (100)
Lines VN+[b] (% of tested)	0 (0)	0 (0)	3 (0.71)

[a] Indirect fluorescent antibody.
[b] Virus neutralization.

Table 2
Chromosome numbers of fusion partners and secreting heterohybridomas with increasing degrees of murine[a] × bovine[b] re-fusion.

Fusion identity	Fusion type	Ig secretion	Lines tested	Line identities	# Samples	# Chromosomes
SP2/0[c]	Murine[2]	No	1	NA	2	44; 60
5A0	Bovine × murine[2]	No	1	3E6	2	60[d,e]; 71
5C0	Bovine × murine[2]	Yes	3	α-01, α-13, α-19	4	64[f]; 65[g,h]; 70[g,i]; 79
5C1	Bovine[2] × murine[2]	Yes	2	α-03, α-06	3	77[g,j]; 79; 72–82[d]
5C2	Bovine[3] × murine[2]	Yes	1	αgB-09	4	58[g]; 86–90; >90[g]; >90[g]

[a] Mouse chromosome number is 40.
[b] Cow chromosome number is 60.
[c] SP2/0 chromosome number is 58–65, modal 61–62 (Sorokina et al., 1986).
[d] Male.
[e] Also cells with 120–132.
[f] Range 62–75.
[g] XY.
[h] Also up to 100+.
[i] Range 52–86.
[j] Range 68–77.

The 3 VN+ BomAb assayed were negative by the assays performed for all of the seven viruses infecting cattle tested. Of the preparations tested, only the higher titered ones of BomAb αgD-16 were reactive in the commercial ELISA designed for bovine serum Ab.

3.4. Production

HF cultures appeared to grow quickly and at times achieved VN titers 8–16× those of simultaneous flask comparison cultures (Fig. 3), with demonstrably greater purity (Fig. 4). Maximum VN titers are shown in Table 3. The

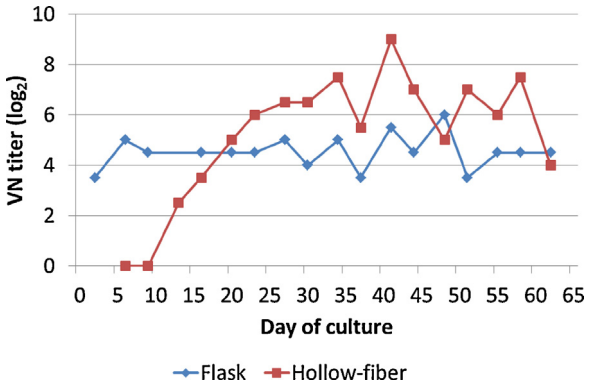

Fig. 3. Virus neutralization (VN) titers of hollow fiber and simultaneous flask cultures of bovine monoclonal antibody 5C2.αgB-09 through time.

30,000 average MWCO appeared to allow some nutrient serum albumin to diffuse into the extra-capillary space for harvest with the BomAb (Fig. 4).

No AF was generated in any of the cyclophosphamide-treated syngeneic mice, and some higher doses of cyclophosphamide proved lethal. NM AF was successfully generated commercially. Volumes of NM AF (25 mouse) lots ranged from 124 to 151 ml. Titers by IFA and VN of the lots ranged from 1600 to 3200 and 512 to 4096, respectively (Table 3).

3.5. Isotyping and subisotyping

Each of the VN+ BomAb (5C2.αgB-09, αgC-05, and αgD-16) reacted with only anti-bovine IgG1 reagents in the

Table 3
Bovine monoclonal antibody[a] virus neutralization titers by production method.

	αgB-09	αgC-05	αgD-16
Flask[b]	32–64	16–32	128
Hollow fiber[c]	512	256	512–1024
Ascites[d]	4096	512	2048–4096

[a] Bovine monoclonal antibodies 5C2.αgB-09, 5C2.αgC-05, and 5C2.αgD-16.
[b] Maximum titer of flask culture conducted in parallel with hollow fiber culture.
[c] Maximum titer.
[d] Nude mouse ascites fluid.

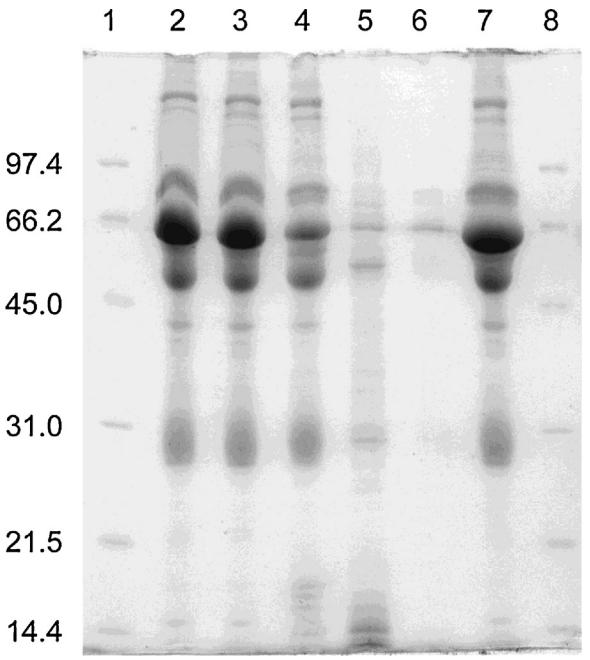

Fig. 4. Bovine monoclonal antibody (5C2.αgD-16) product from hollow fiber cultures *vs.* a simultaneous flask culture, demonstrating increasing purity of hollow figure culture through time. Lanes 1, 8 – molecular weight standards; lanes 2–6 – weeks 0, 1, 2, 3, 4 hollow fiber harvests, respectively; lane 7–flask culture. 10% full-sized SDS-PAGE gel, 10 lanes, Coomassie blue stain. Image cropped to include entire separation gel, lanes 2–9 of original gel. Harvests of week 1 and 3 (lanes 3 and 5) had similar high titers, that of week 4 (lane 6) was ~8-fold less.

RID assay. In the IEP assay each of the three BomAb produced a single arc centered slightly to the anode side of the well (results not shown), consistent with the IgG1 subisotype. The subisotype-specific IFA resulted in positive results for each of the VN+ BomAb using the anti-bovine IgG1 MumAb DAS 17 (results not shown), and the MumAb bound each of the VN+ BomAb in IAC. Calculated H and L chain MW of the single band observed in SDS-PAGE were consistent with IgG1 (heavy) subclass and either lambda or kappa (light) class (see Section 3.6). The results of each of the assays not only confirm the IgG1 subisotype identity, but also support the monoclonality of the 3 VN+ HH.

3.6. SDS-PAGE and Western blots

H and L chain molecular weights by SDS-PAGE were calculated to be 48.6 and 25.6 K for αgB-09 ($r = 0.997$), 48.9 and 26.9 K for αgC-05 ($r = 0.899$), and 50.4 and 25.3 K for αgD-16 ($r = 0.997$). SDS-PAGE of agarose-Protein A bound BomAb demonstrated the purity of bound SF preparations and confirmed H and L chain MW (see Fig. 5 for αgD-16 results).

Full-size Western blots did not result in clear unique reactions for any of the 3 VN+ BomAb (see Fig. 6 for αgB-09 and αgD-16 results), indicating they may react with conformational epitopes. Although faint bands were observed, the same reactions appeared in negative controls. When the sensitivity was increased, reactions with many proteins were observed, but again, negative controls (*e.g.* irrelevant bovine AS) reacted with the same proteins. Similar results were observed using microvolume SDS-PAGE (results not shown).

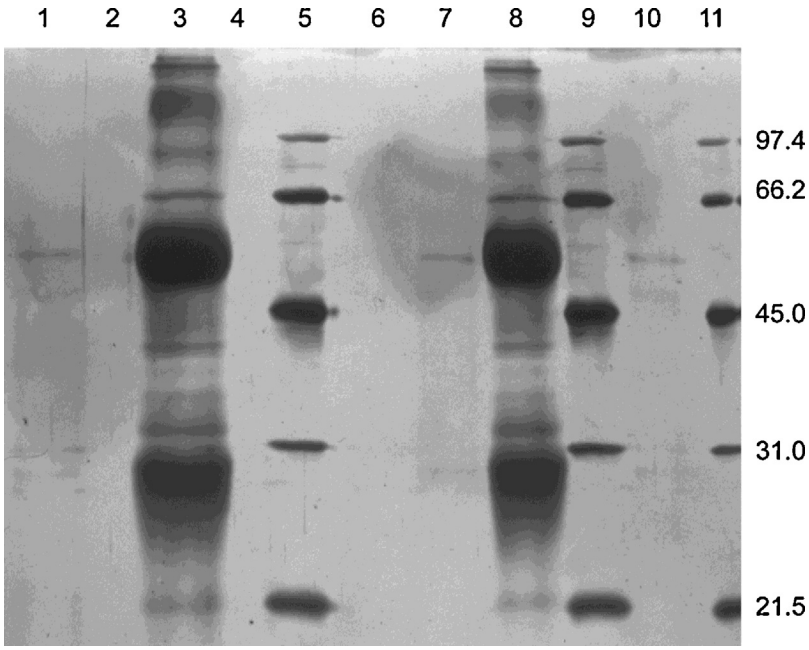

Fig. 5. Molecular weight determination of Heavy and Light chains of 5C2.αgD-16 and controls. Ig was bound to Agarose-Protein A and treated for SDS-PAGE. SDS-PAGE 12.5% full sized gel, silver stain. Lanes 1, 7, and 10, 5C2.αgD-16; lanes 3 and 8, anti-BHV1 bovine antiserum; lanes 5, 9, 11, molecular weight standards; lanes 2, 4, and 6, empty. Image cropped to include only stacking gel interface to below 21.5 standard, lanes 6–16 of original gel.

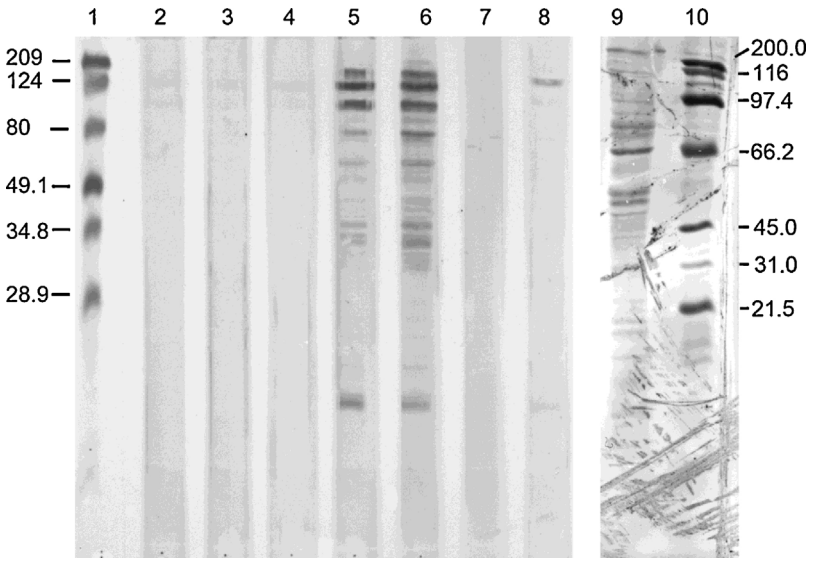

Fig. 6. Western blot of two bovine mAb and controls with bovine herpesvirus 1 (BHV1) antigens. Lanes 1–8 used for immunoreaction, lanes 9–10 stained with amido black. Bovine mAb preparations were 10X hollow fiber culture harvests.
Image cropped to include only stacking gel interface to sample front. Lane 1 – Pre-stained molecular weight standards. Lane 2 – Media control. Lane 3 – 5C2.αgB-09. Lane 4 – 5C2.αgD-16. 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 bovine antiserum 1:10. Lane 9 – BHV1 antigen. Lane 10 – Unstained molecular weight standards.

3.7. Immunoaffinity chromatography

The results of the two 10–15% gradient microvolume SDS-PAGE assays on the VN+ BomAb IAC eluates (Fig. 7) were similar (individual values <5% from average), and so were averaged and analyzed ($r = 0.99$). αgB-09 bound proteins of approximately 61, 82, and 132 Kd, consistent with BHV1 gB. αgC-05 bound a protein of approximately 118 Kd, consistent with BHV1 gC. αgD-16 bound proteins of approximately 78 and 147 Kd, consistent with BHV1 gD. The anti-BPV 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 (results not shown).

3.8. Bovine monoclonal antibody processing in cattle

The percent neutralized of a fixed amount of virus for sera days 0–42 post-Ab dpi for the immunized calf are shown in Fig. 8 (for clarity, negative VN results for days −1 and 0 pre-inoculation are not shown). Linear regression on the average % activity resulted in the equation '% neutralized = 91.6–(2.017 × dpi)' ($r = 0.96$), so Ab $t_{½} = 24.8$ days. Excluding the first 5 days (to account for distribution to the extravascular space), did not change the $t_{1/2}$. The nasal swab on day 5 post immunization (only) was VN+ (titer 2).

3.9. Passive immunization of neonatal rabbits

Deaths scored as virus-specific (134) were observed 2–13 dpc (two >9 dpc). Highest death losses in sham Ab or no Ab, virus-challenged groups were observed on 2 and 3 dpc.

In the age *vs.* virus-dose mortality trials, although subject numbers were small, relationships were observed. Survival appeared to increase with subject age at a given virus dose. For example, at dose $10^{8.0}$, survival was 12.5% ($n = 1/8$) for 4–7 days old, 60% ($n = 3/5$) for 12–14 days old, and 100% ($n = 3/3$) for 20–21 days old rabbits. Survival appeared to decrease with increasing virus dose at a given rabbit age, for rabbits ≤14 days old (no deaths were observed in 20–21 days old rabbits at two doses tested, $10^{8.0}$ and $10^{8.3}$). For example, for 13–14 days old rabbits, survival was 100% ($n = 3/3$) for $10^{7.7}$, 60% ($n = 3/5$) for $10^{8.0}$, and 0 ($n = 0/5$) for $10^{8.3}$ pfu. Using the trial data, an age × virus-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.

In the passive immunity study, preparations of the VN+ BomAb αgB-09 and αgD-16, as well as of the bovine BHV1 AS, improved the survival of 6–8 days old virus-challenged rabbits (see Fig. 9). Survival appeared to increase with the titer of treatment Ab for each of the 3 Ab, although a clear order of Ab titer-survival was not observed when all groups were examined, possibly due to small (5–9) numbers in many groups. All preparations of αgB-09, αgD-16, and BHV1 AS titering ≥320 resulted in ≥80% survival, compared to 41.9% ($n = 13/31$) for all FBS dilutions, 35.1% ($n = 13/37$) for all media control preparations, and 10.7% ($n = 3/28$) for $10^{7.5}$ challenge control (no sham or Ab passive immunization) groups. For some Ab treatments with incomplete protection the onset of mortality and the date of *e.g.*, 20% mortality was delayed relative to the '0 titer' group (see Fig. 9).

Chi-square values for αgB-09 titer 184, αgD-16 titer 1024, and bovine BHV1 AS titer 345 and 1380/2760 relative to 'titer 0' groups were significant (<0.05), and that for BHV1 AS titer 86 was not. However, 'expected' group sizes of <5 were found in all but the BHV1 AS 1380/2760 group. The 3 Ab could not be compared for protective activity

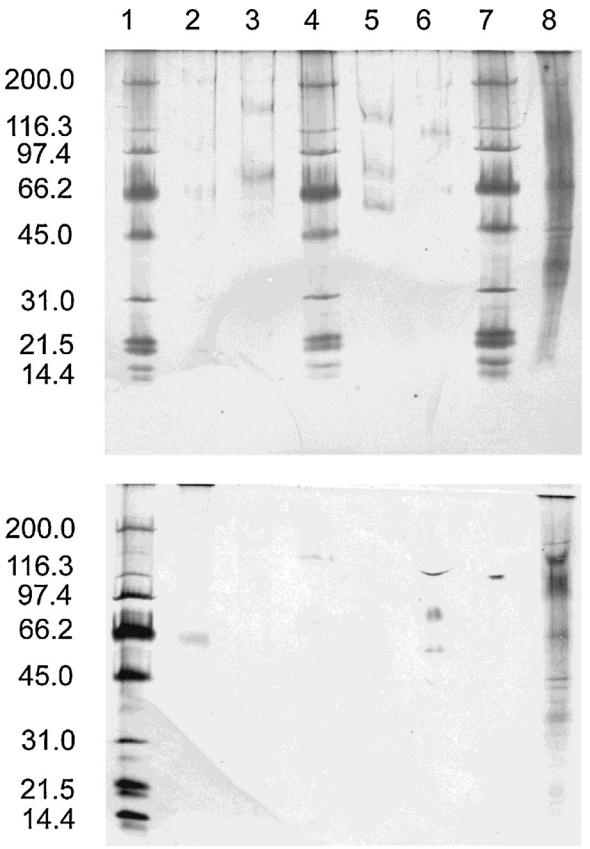

Fig. 7. SDS-PAGE of eluates from immunoaffinity columns using bovine monoclonal antibody and bovine herpesvirus 1 (BHV1) detergent solubilized antigens and controls. All samples = 250× concentrates of eluates and preparations used with columns. Both gels microvolume gradient SDS-PAGE, 10–15%. Images cropped to include only stacking gel interface to near bottom of separation gel. Molecular weight standards – top gel, lanes 1, 4, 7; bottom gel, lane 1. BHV1 antigen (1:7.5) – top gel, lane 8; bottom gel, lane 8. Cell culture control (1:5) – bottom gel, lane 2. 5C2.αgB-09 – top gel, lane 5; bottom gel, lane 6. 5C2.αgC-05 – top gel, lane 6; bottom gel, lane 7. 5C2.αgD-16 – top gel, lane 3; bottom gel, lane 4. Anti-bovine parvovirus bovine mAb – top gel, lane 2; bottom gel, lanes 3, 5.

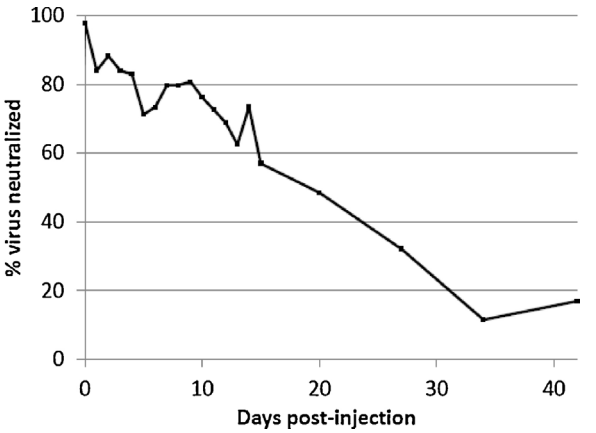

Fig. 8. Virus neutralization activity of serum from a passively immunized calf through time. Bovine monoclonal antibodies were injected IV at day 0. % of virus (47.0–56.0 pfu) neutralized by equal volumes of serum.

(*e.g.*, titer of 50% protection) due to low subject numbers and few titers tested.

4. Discussion

This is the first report of a direct comparison of HH fusions using 'degrees of self-refusion' and using the same immunogen. The increased Chr number and higher numbers of growing and secreting HH observed with increased re-fusion are consistent with previous re-fusion reports. However, the 10-fold increase in secreting HH in the $b^3 \times m^2$ fusion relative to the other fusions, and the low production when the same $b^2 \times m^2$ fusion partners were used with another individual's bovine LC (5 of 1056 cultures using 5A1.2C2 and 2 of 1056 cultures using 5A1.5A11 in the BPV fusions) suggest the 'self-refusion' strategy deserves further study. Inclusion of further degrees of self-refusion could be instructive, although in at least one case additional degrees of refusion were reported to be counter-productive (Greenlee et al., 1990).

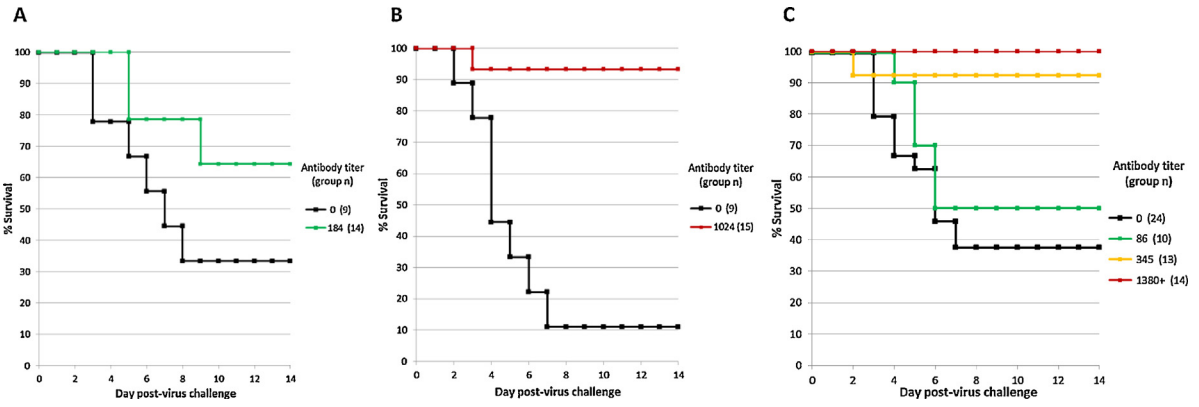

Fig. 9. Protection by two bovine monoclonal antibodies (BomAb) and bovine antiserum (AS) of 6–8 days old rabbits from intracardiac challenge with bovine herpesvirus 1 ($10^{7.3–7.5}$ plaque forming units of bovine herpesvirus 1). (A) 5C2.αgB-09; (B) 5C2.αgD-16; (C) AS. Antibody titer is virus neutralization titer of mAb concentrates or AS dilutions. '1380+' group is combined 2760 and 1380 titer groups. Results from media concentrated similarly to the BomAb or fetal bovine serum diluted equivalently to the AS are pooled for each series and represented as "0" titer. Group n is number of rabbits in each group. No group <9 (BomAb) or <10 (AS) was selected for presentation.

The generation in the $b^3 \times m^2$ fusion of a large number of stably secreting HH with a diversity of specificities for the many BHV1 Ags enabled the isolation of HH producing VN+ BoMAb specific for the major BHV1 VN targets gB, gC, and gD, which should prove useful for further study. 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 such as the RID demonstrated here), or in the generation of further reference materials (*e.g.*, anti-species or isotype AS, 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 3 VN+ BomAb were all IgG1, consistent with most other HH BomAb reports (Groves and Morris, 2000), and not surprising considering the parenteral immunization used (Butler, 1983). IgG1 is the majority IgG sub-isotype in bovine serum, the primary subclass in bovine milk and a significant component of Ig in nasal secretions (Butler, 1983). For this reason the HH generated could be particularly useful for constructing 'bovinized' Mab using MumAb to other cattle pathogens.

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. 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 AS (where any epitope-specific Ab population is diluted by the many others) or non-host mAb (generated by an animal with no evolutionary background for defense against the agent) cannot (Binns et al., 1993). Studies of compilations of BomAb generated using whole virus could determine immunodominant epitopes. Determining host-relevant and strongly immunogenic epitopes may assist in vaccine development, particularly of peptide or engineered vaccines (Tikoo et al., 1990) as well as in the development of anti-idiotypic vaccines (Hariharan et al., 1991; Ladjemi et al., 2011).

The BomAb could also 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, as was demonstrated in this study. As bovine Ig they can be more easily introduced into the host for studies of the bovine immune response to the agent, *e.g.*, in passive immunity studies (Booman et al., 1992; Thomas et al., 1998).

In this study the $t_{1/2}$ value of 24.8 days observed in the immunized calf is similar to the common range of published values for cattle of 19 (Menanteau-Horta et al., 1985) to 23 days (Mechor et al., 2001), indicating the Ab was recognized as 'self' on this primary immunization. Although only observed on one day, the VN+ nasal swab suggests greater transfer of active Ab to nasal secretions than that predicted (2% of circulating Ab, Wells et al., 1977; Marshall and Letchworth, 1988), as the calf serum titer was 4 at the time. This may be due to the BomAbs' IgG1 isotype.

In the age × virus-dose rabbit mortality trials, the inverse age *vs.* mortality relationship observed was consistent with previous reports of BHV1 infection in rabbits (Kelly, 1977), and the direct relationship between virus titer and mortality observed was consistent with reports on human herpesvirus 1 and 2 (HHV1/2) infection of mice (Baron et al., 1976). The BHV1-induced neonatal mortality without clinical signs (other than depression) observed in this study was consistent with previous reports (Kelly, 1977; Lupton et al., 1980).

In the rabbit passive immunization trials (the first reported for this host species and virus), the titer-dependent protection observed was consistent with some studies of HHV1/2 polyclonal Ab in HHV1/2 infection of mice (Baron et al., 1976; Erlich and Mills, 1986), but not with some HHV1/2 MumAb studies (Bystrická et al., 1997). Non-neutralizing BomAb (to gB, gC, gD, or other BHV1 proteins) were not tested for comparison in this study as was done in some HHV1/2 MumAb studies (Rector et al., 1982; Balachandran et al., 1982). Although the rabbit model used in this study may be useful for *e.g.*, generalized neonatal disease, it may not be appropriate for the usual target of vaccination, bovine respiratory disease.

The pilot-scale production demonstrated here has been used for production of many hybridomas (Gramer et al., 2003), including for those used for therapeutic product manufacture (Valdés et al., 2001). The sequences derived from the HH may be useful for engineering other Ab with the V 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 Ab with two specificities, two isotypes, and/or two species origins, using conventional hybridoma technology (Moldenhauer, 2011).

Many of these uses have already been realized for the BomAb described here. Studies of host passive protection (Levings et al., unpublished), epitope competition and anti-idiotype generation (Levings, 2012), as well as variable region sequence (Saini et al., 1997), and structure (Koti et al., 2010) have been done using the VN+ BomAb generated in this study.

Conflict of interest

The authors declare no conflicts of interest.

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