Enhanced Bovine Herpesvirus Type 1 Neutralization by Multimerized Single-Chain Variable Antibody Fragments Regardless of Differential Glycosylation

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Single-chain variable antibody fragments (scFvs) with a 2-amino-acid linker capable of multimerization as di-, tri-, or tetrabodies that neutralize bovine herpesvirus type 1 (BoHV-1) in vitro were constructed and expressed in Pichia pastoris. In contrast to the monomeric form, multimeric scFvs had a higher virus neutralization potency, as a result of in vitro virus neutralization potency, as a result of increased avidity via multimerization. The scFvs were expressed in Pichia pastoris, a eukaryotic microorganism capable of expressing scFvs in the secreted form and performing posttranslational modifications such as folding, formation of disulfide bridges, and glycosylation.

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The remarkable specificity to a desired epitope of monoclonal antibodies (Abs) (12) or their variable-region fragments (7) makes these proteins suitable for clinical diagnosis, immunodiagnostics, and therapeutics. A single-chain variable fragment (scFv) is an example of a monovalent Ab fragment where the amino-terminal variable heavy-chain and variable light-chain domains are held together through an artificial flexible peptide linker to form a stable antigen (Ag)-binding site against an epitope. For example, certain viral epitopes provide a suitable target for the binding of scFv to neutralize virus (7). Antibody fragments against human or animal pathogens can be produced economically in plants, mammalian cells, or microorganism-based expression systems such as Escherichia coli or Pichia pastoris. Previously, our laboratory constructed neutralizing scFvs with 7- and 18-amino-acid linkers against bovine herpesvirus type 1 (BoHV-1) (14, 15). The short linker size (0 to 2 amino acids) has been suggested to form trimers, tetramers, and/or higher multimers (6, 13), as it influences the configuration of the variable domains. The increased avidity as a result of such scFv multimerizations may potentially enhance virus neutralization abilities.

BoHV-1 is an etiological agent of infectious bovine rhinotracheitis and infectious postnatal vulvovaginitis in cattle (19, 23). Subsequent to primary viral infection, a lifelong latent infection is established in affected cattle, which can be reactivated during stress (weaning, transport, or crowding) or as a result of immunosuppression. BoHV-1 is also involved in bovine respiratory disease complex (BRDC) or shipping fever, a disease caused by a synergistic effect of viral and bacterial infections leading to severe pneumonia, which may be fatal. The economic losses due to BRDC alone cost the U.S. cattle industry up to 3 billion dollars annually (10). The currently used vaccines do not provide adequate protection against BoHV-1 infection due to associated problems such as viral shedding, abortions, immunosuppression, and reversion to virulence (22). Therefore, passive immunization with neutralizing Abs or their Ag-binding fragments potentially provides an adjunct disease prevention approach for the eradication of BoHV-1 from a herd. In addition, these Ag-binding fragments will provide useful reagents for the specific detection of BoHV-1 in an immunoassay, for example, by immunofluorescence (14, 15).

We demonstrate here the development of a functional multimeric scFv with a 2-amino-acid linker against BoHV-1 with improved in vitro virus neutralization potency, as a result of increased avidity via multimerization. The scFvs were expressed in Pichia pastoris, a eukaryotic microorganism capable of expressing scFvs in the secreted form and performing posttranslational modifications such as folding, formation of disulfide bridges, and glycosylation (4). Little is known about the glycosylation of Ab variable regions, although it has been suggested that it may affect antigen recognition negatively or positively (8). The recombinant multimerized scFvs against BoHV-1 described here are heterogeneous proteins due to differential cleavage and glycosylation, but these proteins retain virus recognition and neutralization functions.
MATERIALS AND METHODS

Hybridoma. A mouse × cattle xenogeneic hybridoma, HB9907 (ATCC, Rockville, MD) (17), secreting monoclonal IgG1 antibody against BoHV-1, provided cDNA, the genetic source of rearranged VDHJH and VJ genes. The hybridomas were grown in RPMI 1640 (Gibco Canada) supplemented with 10% horse serum, 5 mM sodium pyruvate, 0.5 M minimal essential medium (MEM) nonessential amino acids, 1 mM L-glutamine, gentamicin (50 μg/ml), and 5 μM 2-mercaptoethanol (14).

cDNA synthesis, PCR, and cloning. First-strand cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen) from 5 μg of total RNA (TRIzol; Invitrogen) isolated from HB9907 hybridomas. The VJ recombinations were PCR amplified from cDNA using a sense primer corresponding to framework 1 (FR-1) with a built-in SfiI restriction site (underlined) (sVJ-s [5'-GTGGCCAGCCGCGCCAGGTGTCGTCGACTCAG-3']) and an antisense primer corresponding to FR-4 with the designed flexible linker (VJL-as [5'-AGAACCCTAGGACGTCAGT GTGGTCGGCCGT-3']) (Mobix, McMaster University, Hamilton, Ontario, Canada). Platinum Taq high-fidelity polymerase (Invitrogen) was used to amplify the targeted cDNA using PCR conditions that included hot-start denaturation at 95°C for 30 s followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. Similarly, VDHJH recombinations were amplified by using sense (LVDJ-s [5'-ACACTGAGCTTCTAGGTTCAGTGCAGTGGACGG-3']) and antisense (VDJS-as [5'-CTGGCCGGCGCTTTGGCCACTAGTGAAAGGAGCG- GTGCCAGC-3']) primers (Mobix, McMaster University, Hamilton, Ontario, Canada). The purified recombinant protein secreted by P. pastoris KM71H (Mut+ Arg+; Invitrogen) cells was prepared according to the manufacturer’s instructions for EasySelect. For transformation, 8 μl of P. pastoris was mixed with 5 μl Sacl-linearized recombinant plasmid (~1 μg/μl) and incubated for 3 min at 0°C in a cuvette (0.2-cm gap; Bio-Rad). Electroporation conditions were a voltage of 1.5 kV, a field strength of 7.5 kV/cm, a capacity of 25 μF, a resistance (pulse controller) of 400 Ω, and a time constant of 8.1 ms (Genepulser; Bio-Rad). After the cells were pulsed, 1 ml 1 M sorbitol was added, followed by incubation at 30°C for 1.5 h. The transformants were plated onto 2% yeast extract-peptone-dextrose (YPD) agar supplemented with 1 M sorbitol and 100 μg zeocin/ml and incubated for 4 days at 30°C. Single colonies were grown at 30°C in buffered minimal glycerol complex (BMGY) medium containing 0.5% methanol. Methanol (0.5%, vol/vol) was added every 24 h to maintain induction until supernatant collection at 96 h postinduction.

scFv purification. The His-tagged recombinant protein secreted by P. pastoris KM71H was purified on a nickel-charged affinity column (ProBond; Invitrogen) under native conditions, as described previously (14). The purified recombinant protein was concentrated by using a centrifugal 10,000 molecular-weight cutoff (MWCO) filter device (Millipore, Bedford, MA), and protein concentrations were determined by the use of a Bio-Rad protein assay kit.

Western immunoblotting. Purified scFv was fractionated in 12% SDS-PAGE gels (15a) and stained with Coomassie blue (0.15%, wt/vol) (Brilliant Blue R-250; Fisher). The recombinant protein was electrophoretically transferred onto a nitrocellulose membrane (Protran; Schleicher & Schuell Inc.) and immunodetected by using a mouse anti-His antibody conjugated with alkaline phosphatase (AP) (1:2,000; Invitrogen) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)–nitroblue tetrazolium (NBT) chromogen (Promega). The negative methodological control included the pPICZα (without insert)–transformed P. pastoris KM71H (P4 clone) supernatant that did not express the recombinant protein, according to the manufacturer’s protocol.

FIG 1 PCR amplification of VJ-JH (a), VD-VJ-JH (b), and overlap VD-JH-2L-VJ-JH (c) from cDNA of HB9907 xenogeneic hybridomas secreting IgG1 against BoHV-1. (a) Lane 1, molecular weight (in thousands) ladder; lane 2, HB9907 hybridoma VJ-JH amplicon; lane 3, HB9908 VJ-JH positive control; lane 4, negative control. (b) Lane 1, molecular weight ladder; lane 2, HB9907 VD-VJ-JH amplicon; lane 3, HB9908 VD-VJ-JH positive control; lane 4, negative control. (c) Lane 1, molecular weight ladder; lane 2, VJ-JH-2-codon-linker-VD-VJ-JH amplicon; lane 3, negative control.
Mass spectrometry. Samples of purified and desalted recombinant protein were run on a Waters/Micromass Ultima quadrupole time of flight (Q-TOF) mass spectrometer operated in the positive-ion mode (McMaster Regional Centre of Mass Spectrometry, Hamilton, Ontario, Canada).

Virus neutralization assay. A plaque reduction assay was performed with recombinant scFv to determine its BoHV-1 virus neutralization ability (14). Recombinant scFv3-18L (15) and bovine serum positive for neutralizing antibodies against BoHV-1 (titer, 1:48; Animal Health Laboratory, Ontario Veterinary College) were used as positive controls. The negative controls included fetal bovine serum (FBS), bovine serum albumin (BSA), phosphate-buffered saline (PBS), and Dulbecco’s minimal essential medium (DMEM). To determine if the recombinant protein caused a significant plaque reduction (≥50%), a one-sample t-test (two-tailed P value) was performed (GraphPad Prism, version 4.03; GraphPad Software).

Immunodetection of the BoHV-1 epitope. BoHV-1 (Cooper strain)-infected MDBK cells were grown in DMEM (4% FBS and 1 mM l-glutamine) until approximately a 90 to 100% cytopathic effect was achieved. The clarified supernatant was spun at 115,460 g for 1 h at 4°C (Optima L-80XP ultracentrifuge; Beckman Coulter) on 30% sucrose in a TNE-buffer layer (10 mM Tris, 100 mM NaCl, 1 mM EDTA [pH 7.4]), and the viral pellet was resuspended in Tris-EDTA (TE) buffer (pH 8.0).

Purified BoHV-1 was separated into envelope and capsid protein fractions (6a) and subjected to immunodetection by recombinant scFv in a Western blot. Briefly, BoHV-1 (250 μg) was resuspended in 250 μl viral lysis buffer (10 mM Tris buffer [pH 7.5] [Fisher Scientific], 1% lgepal-630 [Sigma]) and incubated at 20°C for 1 h, followed by centrifugation at 13,148 × g for 90 min at 4°C. The pellet comprising the capsid fraction was washed with viral lysis buffer and resuspended in loading buffer (100 mM Tris Cl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). The envelope protein fraction in the supernatant was precipitated with trichloroacetic acid (TCA) (100%, wt/vol) for 10 min at 4°C, and the pellet was washed three times with 200 μl cold acetone, dried, and resuspended in loading buffer. The samples were boiled for 10 min prior to fractionation on 12% SDS-PAGE gels and transferred onto a nitrocellulose membrane (Protran; Schleicher & Schuell Inc.). The nitrocellulose membrane was washed (0.05% Tween 20 in PBS) and blocked (2.5% Tween 20 in PBS), and the viral epitope was immunodetected by using recombinant scFv (5 μg/ml), followed by mouse anti-His-AP antibody (1:2,000; Invitrogen) and NBT-BCIP chromogen (Promega).

BoHV-1 gB and gE enzyme-linked immunosorbent assays (ELISAs). gB and gE enzyme immunoassays (Herdcheck IBRgB; Idexx, Switzerland) for the detection of BoHV-1-specific antibodies were used to determine whether recombinant scFv recognized the gB or gE immunodominant epitopes of BoHV-1. Briefly, 50 μl of phosphate wash buffer (Idexx, Switzerland) was added to each well coated with BoHV-1 antigen, followed by incubation of BoHV-1 gB- or BoHV-1 gE-specific monoclonal antibody conjugated to horseradish peroxidase (HRP) for 1 h at 4°C. After the plates were washed, TMB substrate solution (3,3′,5,5′-tetramethylbenzi-
dine and H₂O₂) (Idexx, Switzerland) was added, and the reaction was stopped with 1 M HCl after 10 to 15 min. The absorbance was measured at 650 nm (Titertek Multiscan). The blocking of the gB and gE dominant epitopes was calculated as a percentage of the absorbance by the sample relative to the average absorbance value of the negative control (NCav):

\[
\left(\frac{\text{NCav}}{\text{OD}_{\text{sample}}} \times \frac{1}{\text{NCav}}\right) 
\]

100%. For the detection of the gB and the gE dominant epitopes, blocking percentages of 45% and 30%, respectively, were considered negative.

RESULTS

Construction of anti-BoHV-1 scFv with a 2-amino-acid linker.
The variable lambda light- and heavy-chain rearrangements amplified from HB9907 hybridoma cDNA were combined in an overlap PCR (Fig. 1) together with a 2-codon (GGT TCT) linker and cloned into the pPICZ [H9251] vector in the VL-VH configuration with a built-in c-myc epitope and His tag targeted for expression under the influence of the AOX1 promoter. Upon the screening of recombinant clones, the scFv2L5 clone was isolated and analyzed for nucleotide sequence (Fig. 2). The deduced protein sequence and secondary structure, presented as IMGT Collier de Perles in Fig. 3, demonstrated the characteristic strands, loops, and turns typical of variable domains. The supernatant of P. pastoris (KM71H strain) transformed with the scFv2L5 plasmid, expressing the recombinant protein after 96 h of induction with 0.5% methanol, was subjected to immunochemical analysis. The recombinant protein secreted by P. pastoris, designated scFv5-2L, demonstrated the presence of an approximately 34-kDa protein (Fig. 4) in a Western blot, consistent with the expected theoretical molecular mass of 30.3 kDa. These experiments demonstrated the stable expression of scFv with a 2-amino-acid linker in a VL-linker-VH configuration in P. pastoris that could be purified via aHis tag on nickel-charged affinity resin.

Differential glycosylation in recombinant scFvs expressed in P. pastoris.
Mass spectrum (Q-TOF) analysis of scFv5-2L showed multiple peaks at around 27 kDa and weaker signals at around 30.3 kDa (Fig. 5a). A careful analysis of the data, taking into consideration a 5-Da margin for readout accuracy (see Table S1 in the supplemental material), showed differential cleavage that resulted in a loss of the His tag on scFvs. Nevertheless, these scFvs without a His tag copurified on Ni-chelating resin with properly expressed scFvs with a His tag via noncovalent multimerization. This consistent observation provides indirect evidence for scFv5-2L multimerization per se as a result of the 2-amino-acid linker. The addition of 1 to 9 mannose residues and other unidentified small adducts (Fig. 5a) seemed to provide further scFv heterogeneity.

Mass spectrum analyses of monomeric scFv3-18L showed the most prominent signals at around 31.7 kDa (Fig. 5b; see also Table S1 in the supplemental material), where recombinant protein heterogeneity was due to the differential cleavage of the α-signal se-

FIG 3 Two-dimensional IMGT Collier de Perles of the amino acids (shown as one-letter abbreviations) of the V₅-linker-V₃₄ region of recombinant scFv5-2L expressed in Pichia pastoris. Hydrophobic amino acids (I, V, L, F, C, M, A, and W) in β-strands are shown in blue; proline (P) is shown in yellow. The loops are limited by amino acids shown in squares (anchor positions). Arrows indicate the direction of the β-strands. Blue, light green, and dark green loops represent CDR1L, CDR2L, and CDR3L, respectively; red, yellow, and purple loops represent CDR1H, CDR2H, and CDR3H, respectively; and hatched circles correspond to missing positions according to the IMGT numbering system for V and V-like domains (11, 16). The linker composed of glycine and serine amino acids is shown in the green box.

FIG 4 Immunochemical analysis of secreted recombinant scFv5-2L expressed in Pichia pastoris. Western blot using scFv5-2L, scFv3-18L, and scFv5-2L (KM71H strain) transformed with the scFv2L5 plasmid.
Recombinant scFv against BoHV-1 recognizes an epitope on the 32-kDa envelope protein. In a Western blot, recombinant scFv5-2L recognized an approximately 32-kDa protein in the envelope fraction of BoHV-1 (Fig. 8). Such an immunodetection of the 32-kDa protein in the BoHV-1 envelope is specific, as it was absent in the capsid fraction (Fig. 8). The scFvs with 7- and 18- amino-acid linker sizes are known to specifically recognize BoHV-1 antigen in infected but not uninfected MDBK cells in an immunofluorescence assay (14, 15). Furthermore, two immunodominant envelope glycoproteins, gB and gE, of BoHV-1 were not recognized by either multimeric scFv5-2L or monomeric scFv3-18L in a blocking ELISA (Fig. 9). These findings suggest that neutralizing recombinant scFvs against BoHV-1 likely recognize a non-gE or -gB immunodominant epitope on the viral envelope.

**DISCUSSION**

With the objective to enhance the BoHV-1 neutralization function of an scFv by multimerization to increase avidity, a recombinant scFv with a 2-amino-acid linker (scFv5-2L) was constructed and expressed in *P. pastoris* under the influence of the AOX1 promoter. The scFv was expressed as a secreted fusion protein with c-myc and His tags for subsequent purification and immunodetection. The presence of additional residues of a vector origin or the 2-amino-acid linker did not affect Ag recognition or hinder the multimerization required to form an Ag-binding site (6, 13). The functionality of scFv5-2L was confirmed by its BoHV-1 neutralization ability in *vivo* and antigen recognition in a Western blot. The scFv with a linker shorter than 12 amino acids cannot fold to form a functional domain but needs to pair with another scFv in order to form a functional Ag-binding site (2, 13). In addition to the linker size, scFv multimerization is influenced by factors such as the V~H~J~L~-V~H~P~M~H~ interface stability, concentration, and ionic strength (1, 5). The noncovalent formation of multivalent multimers clearly increased the *in vitro* BoHV-1 neutralization potency, as it was achieved at a lower molar concentration (1 μM) by scFv5-2L, similarly to scFv1-7L (14) but in contrast to monovalent scFv3-18 (2 μM). Whether the formation of trimers, tetramers, or higher multimers generates only functional Ag-binding sites is not known, but the increased avidity of such multimers likely enhances the virus neutralization potency. Evidence for scFv5-2L multimerization leading to increased avidity, however, is largely circumstantial, relying on mass spectrometry analyses and scFv functionality *per se*. Direct evidence for scFv5-2L multimerization using structural approaches, e.g., electron microscopy or crystallography, is needed to confirm the degree of multimerization in the context of valency. The *in vivo* protective ability of the scFvs is being experimentally tested in rabbit animal models and bovine calves.

The recombinant protein scFv5-2L, including monomeric scFv3-18L (data not shown), recognized the 32-kDa protein present in the envelope fraction of BoHV-1. The neutralizing epitope on BoHV-1 recognized by monoclonal IgG (HB9907) or the derived scFvs has not yet been identified. Based on theoretical molecular modeling (14), a probable carbohydrate moiety in the BoHV-1 epitope is predicted to be recognized by scFv5-2L or scFv3-18L present on the envelope glycoprotein. However, both scFv5-2L and scFv3-18L did not recognize immunodominant...
epitopes on essential gB and nonessential gE envelope proteins (20). Experiments aimed at an analysis of surface-accessible neutralizing epitopes on the envelope protein, essential for viral replication, are in progress.

Mass spectrum analysis revealed multiple scFv5-2L species at around 27 and 30.3 kDa but none within a 5-Da range of the expected mass of 30.261 kDa. Several peaks close to the expected molecular mass can be explained by the differential cleavage of the signal sequence and variable glycosylation. Some of the peaks representing molecular masses of around 27 kDa were found to be compatible with cleavage at position 259 of scFv5-2L, which would result in the loss of the c-myc and His tags, consistent with Coomassie blue-stained SDS-PAGE and Western blot observations. Purified His-tag-cleaved recombinant scFv5-2L, revealed during the mass spectrum analysis, could exist only because of noncovalent multimerization permitting copurification on Ni-chelating resin with properly His-tagged scFv5-2L. As the built-in expression vector His tag site resides outside the variable region of the heavy and light chains of the parental Ab, it does not interfere with the formation of the functional Ag-binding site. Such a cleavage of the His tag in some scFvs is consistent with the presence of a single but the absence of a separate protein band in the Western

FIG 5 Mass spectra of recombinant scFv5-2L (a) and scFv3-18L (b) expressed in P. pastoris. The expected molecular mass for properly processed and unglycosylated scFv5-2L is 30,261 Da, and that for scFv3-18L is 31,555 Da. A detailed description of separate peaks is provided in Table S1 in the supplemental material.

FIG 6 Schematic representation of possible cleavage and posttranslational modification sites of recombinant scFv5-2L expressed in P. pastoris. The signal sequence necessary for the secreted protein is cleaved by the Kex2 and Ste13 endoproteases; scFv5-2L amino acid positions 2 and 259 are likely sites of cleavage by an unidentified protease. Yellow boxes represent the sequence originating from the pPICZα vector. GS, glycine-serine (2-amino-acid linker).
blot. Mass spectrum analyses of scFv3-18L also showed protein heterogeneity with molecular masses of around 31.7 kDa containing a peak of 31.552 kDa, which seems to represent the expected theoretical molecular mass of 31.555 kDa. The observed scFv3-18L heterogeneity is likely because of differential cleavage, glycosylation, and the addition of minor adducts. The second cluster of peaks is absent as His-tag-cleaved recombinant scFv3-18L, as it will not be purified on nickel-charged affinity resin, confirming its monomeric form per se. Another research group (21) previously showed a similar cleavage of an scFv at the C terminus, resulting in the loss of the c-myc and His tags. Such a cleavage at the C terminus is probably caused by an unidentified protease that is not affected by the presence of the proteinase inhibitor Casamino Acids.

The scFv5-2L and scFv3-18L glycosylations related to a mass of 1 to 9 mannoses may reflect O-linked glycosylation, since a significantly higher mass would be expected if it were due to N-linked glycosylation. Since O-linked glycosylation has been described for P. pastoris on threonine and serine, these residues could also compete for the glycosylation of potential N-glycosylation sites (Asn-X-Ser/Thr). O-glycosylation is seen in both mammals and yeast, but glycosylation in P. pastoris can occur at sites not necessarily glycosylated in the native protein (4). Furthermore, the phosphorylation of mannose residues is common and could account for some of the unknown minor adducts (3). The relatively lower level of glycosylation of proteins expressed in P. pastoris can occur at sites not necessarily glycosylated in the native protein (4). Furthermore, the phosphor-ylation of mannose residues is common and could account for some of the unknown minor adducts (3). The relatively lower level of glycosylation of proteins expressed in P. pastoris can occur at sites not necessarily glycosylated in the native protein (4).

**FIG 7** Virus neutralization potency of recombinant scFv5-2L and scFv3-18L. BoHV-1 neutralization is indicated by the >50% plaque reduction in 3 to 8 individual experiments. Note that multimerized scFv5-2L significantly inhibits plaque formation by BoHV-1 compared to monomeric scFv3-18L at concentrations of 5 nM (P < 0.05) and 10 nM (P < 0.001). Various controls included 100 PFU BoHV-1 with media including 2% FBS, BSA (negative), and 1% or 2% anti-BoHV-1 serum (positive). Bars indicate means ± standard errors (*, P < 0.05 [significant]; ***, P < 0.001 [extremely significant] [determined by an unpaired t test, with a two-tailed P value]).

**FIG 8** Western immunoblot of BoHV-1 envelope and capsid protein fractions immunodetected by recombinant scFv3-18L. Lane 1, molecular mass marker; lane 2, BoHV-1 envelope fraction; lane 3, BoHV-1 capsid fraction.

**FIG 9** Blocking by recombinant scFv5-2L and scFv3-18L of the BoHV-1 envelope immunodominant epitope on gB using anti-gB-HRP monoclonal Ab (a) and on gE using anti-gE-HRP Ab (b) in an ELISA. Negative controls included bovine serum without gB or gE antibodies against BoHV-1 or phosphate-buffered saline (PBS), while bovine serum with a positive antibody titer for gB or gE provided the positive control.
not shown). These observations together with mass spectrometry data suggest an O-glycosylation of recombinant scFv that is not inhibited by tunicamycin treatment. This is consistent with the absence of an N-glycosylation site on recombinant scFv. Nevertheless, scFv heterogeneity due to posttranslational modifications deserves important consideration in the development of standardized therapeutics and immunodiagnostics. Future experiments should aim at the development of growth conditions to prevent the heterogeneous glycosylation or enzymatic deglycosylation of recombinant proteins. The effect of the differential glycosylation of Ag-binding sites on scFv function needs to be examined.

The recombinant scFvs against BoHV-1 potentially provide an adjunct immunotherapeutic, together with the available vaccines against BoHV-1, especially during transportation, when latent BoHV-1 becomes activated as a result of stress, leading to “shipping fever” and bovine respiratory disease complex. Recombinant scFv may be used as an intranasal spray in vaccinated cattle during transportation to prevent BoHV-1 infection by blocking viral transmission. These immunotherapeutics may also be used to prevent the spread of BoHV-1 infection by artificial insemination by treating high-quality semen to inhibit BoHV-1 replication. Furthermore, these immunotherapeutics may be used for local application to treat infectious pustular vulvovaginitis caused by BoHV-1. While antibody-based therapy currently may be costly, technological advances, e.g., new expression systems, optimized antibody fragments, or combination therapies, are likely to make these therapies cost-effective in the future. Apart from their therapeutic significance, recombinant scFvs may be used for the rapid, specific, and sensitive immunodetection of BoHV-1 infection, for example, by immunofluorescence (14, 15). Overall, multimerized scFv5-2L has a high virus neutralization efficacy due to increased valency and is potentially suitable for passive immunization and topical application.

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