Immunology 1992 **76** 367–372

Constitutively expressing cell lines that secrete a truncated bovine herpes virus-1 glycoprotein (gpI) stimulate T-lymphocyte responsiveness

T. P. LEARY, Y. GAO & G. A. SPLITTER *Department of Veterinary Science, University of Wisconsin-Madison, Madison, Wisconsin, U.S.A.*

Accepted for publication 27 February 1992

SUMMARY

The desire to obtain authentically glycosylated viral protein products in sufficient quantity for immunological study has led to the use of eucaryotic expression vectors for protein production. An additional advantage is that these protein products can be studied individually in the absence of their native viral environment. We have cloned a complementary DNA (cDNA) encoding bovine herpes virus-1 (BHV-1) glycoprotein 1 (gpI) into the eucaryotic expression vector, pZipNeo SVX1. Since this protein is normally embedded within the membrane of BHV-1 infected cells, we removed sequences encoding the transmembrane domain of the native protein. After transfection of the plasmid construct into the canine osteosarcoma cell line, D17, or Madin–Darby bovine kidney (MDBK) cells, a truncated BHV-1 (gpI) was secreted into the culture medium as demonstrated by radioimmunoprecipitation and SDS–PAGE. Both a $CD4^+$ T-lymphocyte line specific for BHV-1 and freshly isolated T lymphocytes could recognize and respond to the secreted recombinant gpI. Further, recombinant gpI could elicit both antibody and cellular responses in cattle when used as an immunogen. Having established constitutively glycoprotein producing cell lines, future studies in vaccine evaluation of gpI will be facilitated.

INTRODUCTION

Bovine herpes virus type 1 (BHV-1) is an alpha-herpes virus.[1] Other members of this subfamily include the herpes simplex virus (HSV), pseudorabies virus and equine herpes virus. BHV-1, capable of infecting both the respiratory and genital tracts of cattle, is the primary viral pathogen involved in bovine respiratory disease complex, an economically important disease syndrome.[2] Live BHV-1 vaccine strains establish neurological latency in immunized animals and may cause abortion if administered to pregnant females.[3] Therefore, identification of viral subunits that stimulate a protective immune response could be useful in understanding herpes viral immunity and immunopathology, in addition to identifying potential candidates for synthetic vaccines.

Both cellular and humoral responses against BHV-1 have been previously demonstrated.[4,5] Monoclonal antibodies, as well as antibody from naturally infected animals, identify at least three major glycoproteins (gp) that reside on the viral envelope.[6-9] Each of the three, gpI, gpIII and gpIV have been molecularly cloned and sequenced, identifying them as analogues of HSV envelope glycoproteins gpB, gpC and gpD respectively.[10-13] HSV gpB, gpC and gpD have been shown to elicit T-lymphocyte responsiveness in both human and murine models,[14-18] and immunization with recombinant gpB or gpD can protect mice from lethal HSV challenge.[19,20] Previous research has established a role for the individual BHV-1 glycoproteins in the protection of animals from disease following immunization,[21-23] in addition to a role for T lymphocytes in the immune response to these proteins.[24] Based on the lack of information regarding the role of gpI in bovine T-lymphocyte-mediated immunity, our desire was the development of a readily available BHV-1 gpI to assess its role as a viable vaccine candidate.

We have characterized the cellular and humoral immune response to a truncated BHV-1 gpI produced from a viral gene cloned into the eucaryotic expression vector, pZipNeo SVX1. In the process, we have removed sequences encoding the transmembrane anchor. Bovine and canine cell lines transfected with this plasmid secreted a truncated gpI identical to the native viral proteins in antibody recognition and glycosylation. Both a bovine $CD4^+$ T-lymphocyte line specific for BHV-1 and freshly isolated bovine T lymphocytes from animals immunized with killed virus responded to this protein. Furthermore, supernatants from these cell lines engendered antibody and T-lymphocyte responses in cattle immunized with the recombinant glycoprotein product.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli strain DH5α was used for plasmid propagation (BRL, Gaithersburg, MD). Plasmid pZipNeo SVX1 (pZN), a

Correspondence: Dr G. A. Splitter, Dept. of Veterinary Science, University of Wisconsin-Madison, Madison, WI 53706, U.S.A.

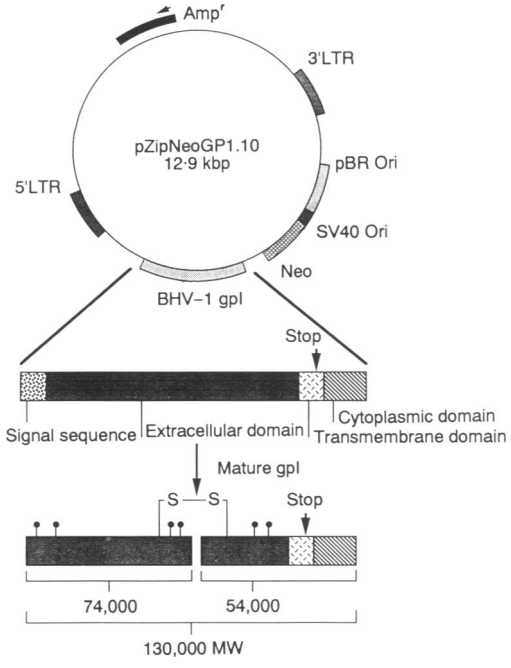

Figure 1. Plasmid map of the expression vector pZNGPI.10 and the BHV-1 gpI structural domains demonstrate noteworthy aspects of the construction. The Moloney murine leukaemia virus long terminal repeats (LTR), BHV-1 gene insertion site, neomycin gene and origins of plasmid replication are indicated. Additionally, the protein product encoded by the gpI gene demonstrates fragment sizes, disulphide bond positions, potential *N*-glycosylation positions and the recombinant translational stop codon.

gift from H. M. Temin (University of Wisconsin, Madison, WI) has been described in detail elsewhere.[25-27] Briefly, pZN contains the Moloney murine leukaemia virus long terminal repeats (LTR), and sequences derived from transposon *Tn5* which encode G418 resistance in eucaryotic cells, cloned into pBR322. The retroviral LTR contain sufficient information for transcriptional initiation and polyadenylation. A unique *Bam*HI cloning site resides between the 5′ LTR and the *neo* gene. In addition, pZN includes an SV40 origin of replication to enable plasmid propagation in mammalian cells. Cloned and sequenced BHV-1 viral cDNA gpI was generously provided in carrier plasmids by Tim Zamb,[10,12,28] formerly of Molecular Genetics, Inc. (Minnetonka, MN).

Plasmid constructions

Standard cloning procedures were used for plasmid construction,[29] the noteworthy aspects of our construction are illustrated by plasmid pZNgpI.10 in Fig. 1. This plasmid contained a 2·7 kilobase pair (kbp) fragment of BHV-1 gpI capable of encoding the first 742 amino acids (nucleotides 181–2787 of the published gene sequence)[11] of the 932 amino acid native gene product, cloned into the *Bam*HI restriction site of pZN. BHV-1 gpI is a 130,000 MW glycoprotein that is post-translationally cleaved to yield 74,000 and 54,000 MW products joined by a disulphide bond.[9] Translational termination within the transmembrane sequence was accomplished by inserting a nonsense stop codon contained within a *Spe*I linker (New England Biolabs, Beverly, MA) at the *Xmn*I restriction enzyme site of gpI. This prevented translation of the complete transmembrane anchor following plasmid transfection into mammalian cells. The transmembrane region of gpI was identified using the Hopp–Woods hydrophilicity plot.

Plasmid transfections

Recombinant plasmids were introduced into mammalian cells using calcium phosphate and glycerol shock.[30,31] Madin–Darby bovine kidney (MDBK) cells, and the canine osteosarcoma cell line D17, were used for transfection based on their previously established suitabilty for this purpose.[31] Transfected MDBK and D17 cell lines were cultured with 2 and 1 mg/ml, respectively, of the neomycin analogue Geneticin® (Gibco, Grand Island, NY) while under selection for drug resistance. After this time, surviving cells were cloned and maintained at 0·5 mg/ml Geneticin®. All cell lines were cultured (37° at 5% CO_2) in Minimal Essential Medium (Sigma, St Louis, MO) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol (2-ME) and 10% foetal bovine serum (FBS) (Hyclone, Logan, UT). Cloned lines were screened for the presence of specific BHV-1 viral RNA using Quick-Blot® (Schleicher and Schuell, Keene, NH) to ensure proper transformation had occurred in Geneticin®-resistant cells.

Monoclonal antibodies and virus

Anti-BHV-1 specific monoclonal antibody (5106) was kindly provided by G. J. Letchworth (University of Wisconsin, Madison, WI) as ammonium sulphate precipitated ascites.[7] Plaque-purified bovine herpes virus-1, Cooper strain (ATCC no. VR-864) was used for all viral experiments. Virus was titred by limiting dilution and diluted to achieve a multiplicity of infection (MOI) of 10 for all assays.

Radioimmunoprecipitation

Transfected, infected and uninfected D17 or MDBK cell lines were labelled with [^{35}S]methionine (Dupont de Nemours and Co., Wilmington, DE) for 6 hr as previously described.[6] Cells were infected by culturing with BHV-1 at an MOI of 10 for 12 hr prior to radiolabelling. Labelling was terminated by washing three times with NET buffer (10 mM Tris base, 1 mM EDTA and 0·5 mM NaCl), pH 8·0, containing 0·2 mM phenylmethyl sulfonyl fluoride (PMSF) and subsequently the cells were lysed with NET-0·5% Triton X-100 for 15 min on ice. Cellular debris was removed by centrifugation, and the desired proteins immunoprecipitated using specific mAb adsorbed onto protein-A Sepharose 6MB beads (Sigma) as per manufacturer's instructions. Immunoprecipitates were released by boiling for 5 min in 100 μl of sample buffer (6·25 mM Tris, 10% glycerol, 5% 2-ME, 2·3% SDS, and 1% bromophenol blue), resolved by 10% SDS–PAGE using the procedure of Laemmli,[32] then exposed on Kodak XAR-5 film.

Animals and cells

Donors of peripheral blood mononuclear cells (PMBC) were cows from the university dairy herd. All animals were clinically normal and immunized annually against BHV-1 with a formalin-inactivated vaccine preparation (Triangle-3, Fort Dodge Laboratories Inc., Fort Dodge, IA). Several months following vaccination, PBMC were obtained by layering heparinized venous blood over Lymphoprep® (Nycomed, Oslo, Norway), followed by interface collection after centrifugation (22°, 40

min, 1700 *g*). Recovered cells were resuspended in RPMI-1640 (Sigma) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 5×10^{-5} M 2-ME and 10% FBS (Sigma). The CD4+ T-lymphocyte line has been previously reported.[24] Animals immunized with recombinant gpI were approximately 7 months of age and fed BHV-1 negative colostrum at birth to maintain their BHV-1 seronegative status. Calves were immunized by intramuscular injection of 17 μg of concentrated recombinant gpI produced in D17 cells emulsified in an equal volume of Freund's incomplete adjuvant (Sigma). Glycoprotein I from D17 cell culture supernatants was dialysed against water to remove medium salts, concentrated by lyophilization, then resuspended in a minimal volume of water. Following dialysis against phosphate-buffered saline (PBS), recovered protein was quantitated by ELISA[22] and used as an immunogen. Two weeks following the initial protocol, animals were reimmunized intranasally with 100 μg of recombinant gpI with 20 μg choleratoxin B subunit (Sigma) as an adjuvant.[5,33,34] Both neutralization and cellular assays were performed at least 2 weeks following the second immunization. Mock immunized animals were given adjuvant with D17 cell supernatant concentrated identical to gpI containing cell supernatant.

Proliferative assay

Freshly isolated PBMC diluted in RPMI-1640 were added to round bottom 96-well plates (2×10^5 cells/well) with the appropriate antigens. When conducting viral assays, UV-inactivated BHV-1 (15 cm, 10 min) was added at an MOI of 10, while recombinant supernatants were supplemented to a concentration of 25% (v/v). Antigens assayed in six replicate samples at a final well volume of 200 μl, were incubated for 6 days at 37°. One microCurie of [^{3}H]thymidine was included for the final 6 hr at which time the cultures were harvested onto glass filters, then counted by scintillation spectrophotometry. Data were expressed as the mean counts per minute ± standard deviation and differences between samples were assessed using the independent Student's *t*-test.

Virus neutralization

Assays were performed in duplicate using 96-well flat-bottom microtitre plates. Twofold serial dilutions of heat-inactivated bovine serum (56°, 30 min) were incubated at 37° with 100 $TCID_{50}$ of BHV-1 in the presence of guinea-pig complement. After 1 hr, 2×10^4 MDBK cells were added per well and incubated an additional 4 days at 37°. Serum titres were determined as the highest dilution at which 50% microscopic cytopathic effect (CPE) was observed.

RESULTS

Radioimmunoprecipitation

Transfected D17 and MDBK cells appeared as distinct plaques after 10–20 days under G418 selective pressure, at which time the cells were cloned.[28] Clones were analysed by slot-blot and ELISA[22] to establish that both specific mRNA and the recombinant glycoprotein product were present. To analyse the fidelity of recombinant gene products as compared to the native viral glycoprotein, radioimmunoprecipitation, SDS-PAGE and autoradiography were performed. As shown in Fig. 2, recombinant gpI precipitated with specific monoclonal antibody from

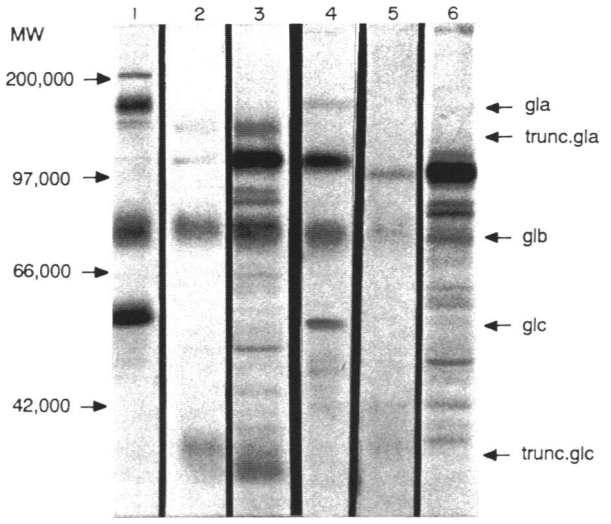

Figure 2. SDS-PAGE analysis of viral and recombinant BHV-1 gpI from D17 (1–3) and MDBK (4–6) cells. Radiolabelled lysates from cells or tissue culture supernatants were precipated with a monoclonal antibody specific for BHV-1 gpI, followed by separation under reducing conditions on a 10% gel. The migration positions of protein molecular weight markers are indicated. The nomenclature gIa, gIb and gIc represent the 130,000 MW native and 74,000 and 54,000 MW subunits of gI as previously reported.[8,9] Truncated (trunc.) proteins are also identified.

D17 or MDBK cell lines was similar in migration to gpI precipated from virally infected cells. BHV-1 gpI is a 130,000 MW glycoprotein gIa that is post-translationally cleaved to yield 74,000 gIb and 54,000 gIc MW products joined by a disulphide bond.[9] Bands representing partially glycosylated precursors of each migrate slightly lower than the mature proteins.[9] With recombinant gpI harvested from tissue culture supernatants, peptides of 110,000, 74,000 and 33,000 MW were visualized. The 110,000 MW band represented a truncated, disulphide-linked protein, while the 74,000 and 33,000 MW bands are reduced versions of the larger product. The 74,000 MW peptide, not being affected by the truncation event, migrates identically to the native gene product, while the 33,000 MW peptide, normally containing the transmembrane and cytoplasmic domains of the native protein, migrates 21,000 MW lower than the 54,000 MW native gene product. This is exactly as would be predicted by the removal of 190 carboxy-terminal amino acids without affecting native glycosylation. Recombinant gpI precipitated from both transfected cell lines had identical migration patterns to the secreted gene product, in addition to partially glycosylated precursors being evident. The conclusion that glycosylation was not affected by the truncation event is further substantiated using the deglycosylating enzymes endoglylcosidase F and *N*-glycosidase F. Observed migration patterns when using this procedure are as predicted by the amino acid sequences. The band slightly greater than 97,000 MW is a cellular gene product that is non-specifically precipitated by this procedure.

Lymphocyte responses to recombinant proteins

Having established that herpes viral glycoprotein was secreted by both D17 and MDBK cell lines, we next wanted to determine whether bovine lymphocytes specific for native viral antigen

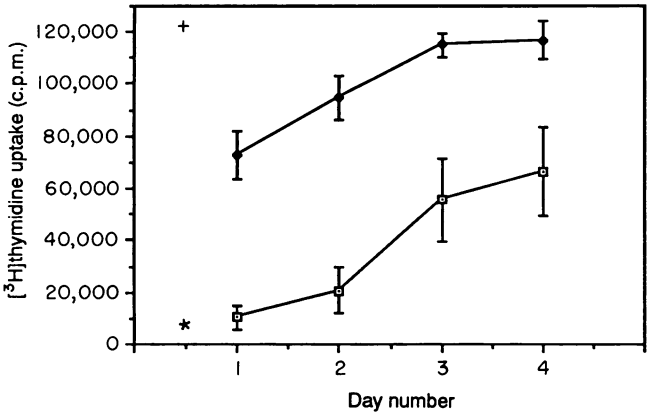

Figure 3. T-lymphocyte proliferation to proteins produced by plasmid transfected cells. BHV-1 specific bovine lymphocytes were cultured at $2{\cdot}5 \times 10^4$ cells/well with 25% (v/v) culture supernatants harvested on each of 4 successive days from recombinant MDBK (□) or D17 (◆) cell lines. Proliferation was measured on Day 3 by [^{3}H]thymidine incorporation, and the data expressed as mean c.p.m. ± SD. Specific antigen concentration is indicated in μg. Medium (*); BHV-1 (+).

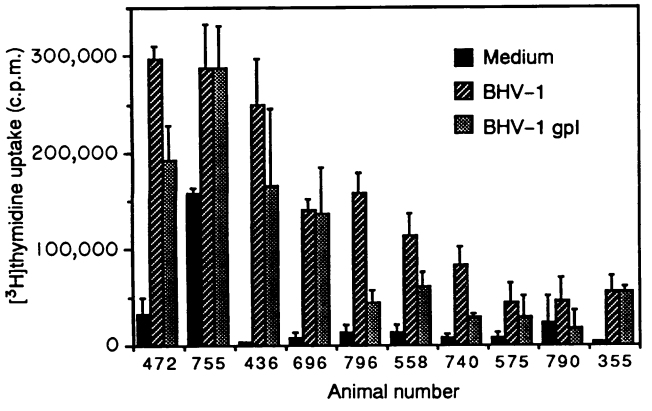

Figure 4. Peripheral blood mononuclear cell proliferation to recombinant cell supernatants or BHV-1. Freshly isolated PBMC were cultured at 2×10^5 cells/well with 25% (v/v) Day 4 culture supernatants from recombinant D17 cells or UV-inactivated BHV-1. Proliferation was measured on Day 6 as described in Fig. 3.

could recognize and respond to the recombinant products harvested from cell supernatants. This was determined by assaying the supernatant from (1×10^6) transfected cells for the presence of specific glycoprotein using a CD4$^+$ T-lymphocyte line specific for BHV-1.[24] As shown in Fig. 3, proliferation against the supernatants increased dramatically from Days 1–4 as anticipated due to the accumulation of secreted glycoprotein in the recombinant cell supernatants. Proliferation against supernatants from transfected D17 cells was always significantly higher than proliferation against transfected MDBK supernatants. This result could be reconciled using a gpI specific ELISA[22] to demonstrate that 10-fold more gpI (0·31 μg/ml versus 0·03 μg/ml in Day 4 supernatants) was contained within the recombinant D17 cell supernatants as compared to the recombinant MDBK cell supernatants. Control samples were either supernatants collected from the D17 cell line transfected with pZN lacking a cDNA insert or UV-inactivated BHV-1 as described in Materials and Methods.

To demonstrate that lymphocytes from animals immunized with a killed virus vaccine could respond *in vitro* to the recombinant protein product, we cultured freshly isolated PBMC with Day 4 culture supernatants from transfected D17 cells. Animals that proliferate to UV-inactivated BHV-1 also proliferate to the recombinant gpI (Fig. 4). Though the magnitude of the observed response in some animals was significantly lower to the recombinant gpI as compared to the virus response, this result would be expected due to the limited lymphocyte repertoire responding to a single protein as compared to multiple proteins in the later case.

Secreted BHV-1 gpI as an immunogen

To ascertain the immunogenicity of recombinant gpI *in vivo*, we immunized calves intramuscularly with 17 μg of recombinant gpI followed 14 days later by an additional 100 μl of recombinant protein administered intranasally. Control calves were mock immunized using D17 cell supernatant treated identically to gpI containing recombinant cell supernatant. At least 14 days after the second immunization, PBMC and blood serum were collected to evaluate antigen-specific lymphocyte proliferation and BHV-1 neutralization antibody from the immunized animals. As shown in Table 1, those animals immunized with recombinant protein (animals 1 and 2) had significantly greater lymphocyte proliferation ($P < 0{\cdot}001$) to gpI as compared to medium controls. Lymphocyte proliferation to gpI from mock immunized animals (3 and 4) was not significantly different from medium controls ($P > 0{\cdot}3$). Comparable proliferative responses were observed using BHV-1 as an antigen in these assays. Additionally, BHV-1 serum neutralization titres from gpI immunized animals were much greater than those obtained from mock immunized animals (Table 1), and comparable with the 1:8–1:256 titre observed following immunization of calves using a killed virus vaccine (data not shown). Therefore, it appears from these data that a truncated form of BHV-1 gpI can successfully serve as an immunogen when administered to cattle.

DISCUSSION

In this report, we describe the production of a recombinant, secreted version of BHV-1 gpI synthesized by cells from two different species transfected with the eucaryotic expression vector, pZipNeo SVX1. This protein could be recognized by both fresh and cultured T lymphocytes from animals previously immunized using a BHV-1 killed virus vaccine. Additionally, the recombinant protein engendered both antibody and T-lymphocyte responses in cattle. These results will be important in the future assessment of this protein as a genetically engineered vaccine candidate.

Given that cell membrane and secreted proteins must proceed through a specific intracellular pathway to reach their destination, it is clear from our data that recombinant gpI compartmentalization is not affected. This is supported by the fact that glycosylation of the secreted gpI is not altered. Others in the past have used similar strategies to construct recombinant protein that are secreted into the extracellular environment by removing predicted hydrophobic sequences thought to be involved in membrane anchoring.[20,35–37] Indeed, the only difference between secreted and membrane-anchored proteins appears to be the presence or absence of a hydrophobic

Table 1. PBM proliferation and virus neutralization capacity of animals immunized with recombinant BHV-1 gpI*

Animal	Treatment†	Proliferation		BHV-1 serum neutralization titre	
		Medium	Recombinant gpI	Pre-immunization	Post-immunization
1	Recombinant gpI	8210±1395	67,720±2198	<1:2	<1:8
2	Recombinant gpI	5474±1910	68,969±10,696	<1:2	1:32
3	Medium	6763±893	6388±3117	<1:2	<1:2
4	Medium	9087±3581	21,644±11,695	<1:2	<1:2

* PBM cell proliferation was performed and evaluated as described in Fig. 4. Virus neutralization titres were determined as described in Materials and Methods.

† Animals were immunized both intramuscularly and intranasally with recombinant gpI or D17 cell supernatants as described in Materials and Methods.

anchoring domain. Natural proteins in which both a secreted and membrane-anchored version exists exploit this concept. Immunoglobulin molecules that serve as antigen receptors in immature and memory B cells, are later secreted into the extracellular environment upon B-lymphocyte differentiation into plasma cells. This is accomplished by differential splicing of the same mRNA molecule to remove sequences necessary for encoding the hydrophobic transmembrane domain in the later case.[38,39]

We have undertaken the study of BHV-1 envelope glycoproteins in T-lymphocyte immunity based on previous studies using analogous proteins from HSV.[14-18] With this virus, glycoproteins gpB, gpC and gpD were shown to elicit T-lymphocyte recognition and activation in both human and murine models. Additionally, immunization with recombinant gpB or gpD,[20] or adoptive transfer or immune T lymphocytes[40] could protect mice from lethal challenge with live virus. In earler reports, our laboratory has established a potential role for BHV-1 envelope glycoproteins,[24] T lymphocytes[4] and natural killer (NK) cells[31] in the bovine immune response to this virus. Further, others have reported a role for these same glycoproteins in the protection of animals from disease following immunization.[9,21,22]

Prior to developing subunit vaccines effective at controlling primary herpes viral infections, understanding viral antigens that stimulate immune responsiveness is essential. Included is the development of antigen sources free of superfluous viral proteins in sufficient quantity for immunological study. Glycoproteins purified by affinity chromatography from virus-infected cell lysates, in addition to being labour intensive, have the potential of being contaminated by minute quantities of other viral proteins which have unknown consequences in immunization studies. This dilemma is resolved using recombinant cell lines that produce a single viral protein. Secreted gpI synthesized by our recombinant cell lines can provide milligrams of this viral antigen with relative ease. Therefore, we are now in the process of undertaking extensive studies on the use of this antigen as an immunogen. Insight into the success of these studies was recently provided by the demonstration that cattle immunized with native gpI were protected from primary viral infection upon BHV-1 challenge.[5]

Although these studies demonstrate that BHV-1 gpI can be readily obtained and has great potential as a subunit vaccine candidate, further studies are needed to clarify the immunogenicity of this and other BHV-1 proteins.[42] Though questions on the protective capacity of recombinant gpI are presently being addressed in our laboratory, these data are an important first effort to address the immunogenicity of recombinant gpI that is a prerequisite to the success of these trials. Future definition of how immune and viral components interact could prove invaluable in understanding disease progression and abrogating primary herpes viral infections.

ACKNOWLEDGMENTS

The authors wish to express gratitude to Michael J. van der Kooy, Jerome S. Harms, Karen M. Everlith and Linda Eskra for excellent technical assistance, thoughtful discussions and helpful advice. Additionally, Drs Kevin Schultz and Geoffrey Letchworth for careful review of the manuscript. This work was supported in part by the College of Agricultural and Life Sciences, the USDA grant 91-37204-6734 and BARD-US-1649.89C.

REFERENCES

1. Roizman B. (1990) Herpesviridae: a brief introduction. In: *Virology*, Vol. 2 (eds B. Field and D. Knipe), p. 1787. Raven Press, New York.
2. Ludwig H. (1983) Bovine herpesviruses. In: *The Herpesviruses*, Vol. 2 (ed. B. Roizman), p. 185. Plenum Press, New York.
3. Yates W.D.G. (1982) A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Can. J. Comparative Med.* **46,** 225.
4. Splitter G.A., Eskra L. & Abruzzini A.F. (1988) Cloned bovine cytotoxic T cells recognize bovine herpes virus -1 in a genetically restricted, antigen-specific manner. *Immunology*, **63,** 145.
5. Israel B.A., Herber R., Gao Y. & Letchworth G.J. (1992) Induction of a mucosal barrier to bovine herpesvirus 1 replication in cattle. *Virology*, **88,** 256.
6. Marshall R.L., Rodriguez L.L. & Letchworth G.J. (1986) Characterization of envelope proteins of infectious bovine rhinotracheitis virus (Bovine Herpesvirus 1) by biochemical and immunological methods. *J. Virol.* **57,** 745.
7. Marshall R.L., Israel B.A. & Letchworth G.J. (1988) Monoclonal antibody analysis of bovine herpesvirus-1 glycoprotein antigenic areas relevant to natural infection. *Virology*, **165** 338.
8. van Drunen Littel-van den Hurk S., van den Hurk J.V., Gilchrist J.E., Misra V & Babiuk L.A. (1984) Interactions of monoclonal antibodies and bovine herpesvirus type 1 (BHV-1)

glycoproteins: characterization of their biochemical and immunological properties. *Virology*, **135,** 466.

9. VAN DRUNEN LITTEL-VAN DEN HURK S., VAN DEN HURK J.V., & BABIUK L.A. (1985) Topographical analysis of bovine herpesvirus type 1 glycoproteins: use of monoclonal antibodies to identify and characterize functional epitopes. *Virology*, **144,** 216.
10. FITZPATRICK D.R., BABUIK L.A. & ZAMB T.J. (1989) Nucleotide sequence of bovine herpesvirus type 1 glycoprotein gIII, a structural model for gIII as a new member of the immunoglobulin superfamily, and implications for the homologous glycoproteins of other herpesviruses. *Virology*, **173** 47.
11. MISRA V., NELSON R. & SMITH M. (1988) Sequence of a bovine herpesvirus type-1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. *Virology*, **166,** 549.
12. TIKOO S.K., FITZPATRICK D.R., BABIUK L.A. & ZAMB T.J. (1990) Molecular cloning, sequencing and expression of functional bovine herpesvirus 1 glycoprotein gIV intransfected bovine cells. *J. Virol.* **64,** 5132.
13. WHITBECK J.C., BELLO L.J. & LAWRENCE W.C. (1988) Comparison of the bovine herpesvirus 1 gl gene and the herpes simplex virus type 1 gB gene. *J. Virol.* **62,** 3319.
14. CHAN W.L., LUKIG M.L. & LIEW F.Y. (1985) Helper T cells induced by an immunopurified herpes simplex virus type I (HSV-1) 115 kilodalton glycoprotein (gB) protect mice against HSV-1 infection. *J. exp. Med.* **162,** 1304.
15. MARTIN S., MOSS B., BERMAN P.W., LASKEY L.A. & ROUSE B.T. (1987) Mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: cytotoxic T cells. *J. Virol.* **61,** 726.
16. ROSENTHAL K.L., SMILEY J.R., SOUTH S. & JOHNSON D.C. (1987) Cells expressing herpes simplex virus glycoprotein gC but not gB, gD or gE are recognized by murine specific virus-specific cytotoxic T lymphocytes. *J. Virol.* **61,** 2438.
17. ZARLING J.M., MORAN P.A., BURKE R.L., PACHL C., BERMAN P.W. & LASKY L.A. (1986) Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. *J. Immunol.* **136,** 4669.
18. ZARLING J.M., MORAN P.A., LASKY L.A. & MOSS B. (1986) Herpes simplex virus (HSV)-specific human T-cell clones recognize HSV glycoprotein D expressed by a recombinant vaccinia virus. *J. Virol.* **59,** 506.
19. CANTIN E.M., EBERLE R., BALDICK J.L., MOSS B., WILLEY D.E., NOTKINS A.L. & OPENSHAW H. (1987) Expression of herpes simplex virus 1 glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal herpes simplex virus 1 infection. *Proc. natl. Acad. Sci. U.S.A.* **84,** 5908.
20. LASKY L.A., DOWBENKO D., SIMONSEN C.C. & BERMAN P.W. (1984) Protection of mice from lethal herpes virus infection by vaccination with a secreted form of cloned glycoprotein D. *Biotechnology*, **2,** 527.
21. BABIUK L.A., L'ITALIEN J., VAN DRUNEN LITTEL-VAN DEN HURK S., ZAMB T., LAWMAN M.J.P., HUGHES G. & GIFFORD G.A. (1984) Protection of cattle from bovine herpesvirus type 1 (BHV-1) infection by immunization with individual viral glycoproteins. *Virology*, **159,** 57.
22. ISRAEL B.A., MARSHALL R.L. & LETCHWORTH G.J. (1988) Epitope specificity and protective efficacy of the bovine immune response to bovine herpesvirus-1 glycoprotein vaccines. *Vaccine*, **6,** 349.
23. VAN DRUNEN LITTEL-VAN DEN HURK S., GIFFORD G.A. & BABIUK L.A. (1990) Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. *Vaccine*, **8,** 358.
24. LEARY T.P. & SPLITTER G.A. (1990) Recombinant herpesviral proteins produced by cell-free translation provide a novel approach for the mapping of T lymphocyte epitopes. *J. Immunol.* **145,** 718.
25. CEPKO C.L., ROBERTS B.E. & MULLIGAN R.C. (1984) Construction and applications of a highly transmissible murine retrovirus shuttle vector. *Cell*, **37,** 1053.
26. HOFFMAN J.W., STEFFEN D., GUSELLA J., TABIN C., BIRD S., COWING D. & WEINBERG R.A. (1982) DNA methylation affecting the expression of murine leukemia proviruses. *J. Virol.* **44,** 144.
27. MURPHY A.J.M. & EFSTRATIADIS A. (1987) Cloning vectors for the expression of cDNA libraries in mammalian cells. *Proc. natl. Acad. Sci. U.S.A.* **84,** 8277.
28. CHASE C.C.L., CARTER-ALLEN K. & LETCHWORTH G.J. (1989) The effect of bovine herpesvirus type 1 glycoproteins gI and gIII on herpesvirus infections. *J. Gen. Virol.* **70,** 1561.
29. MANIATIS T., FRITSCH E.F. & SAMBROOK J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
30. GRAHAM F.L. & VAN DER EB A.J. (1973) A new technique for the assay of human adenovirus 5 DNA. *Virology*, **52,** 456.
31. PALMER L.D., LEARY T.P., WILSON D.M. & SPLITTER G.A. (1990) Bovine natural killer-like cell responses against cell lines expressing recombinant bovine herpesvirus type 1 glycoproteins. *J. Immunol.* **145,** 1009.
32. LAEMMLI U. (1990) Cleavage of structural proteins during the assembly of the head of bacteriophage T4: *Nature*, **227,** 680.
33. LIANG X., LAMM M.E. & NEDRUD J.G. (1988) Oral administration of cholera toxin-sendai virus conjugate potentiates gut and respiratory immunity against sendai virus. *J. Immunol.* **141,** 1495.
34. NEDRUD J.G., LIANG X., HAGUE N. & LAMM M.E. (1987) Combined oral/nasal immunization protects mice form sendai virus infection. *J. Immunol.* **139,** 3484.
35. GETHING M.J. & SAMBROOK J. (1982) Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. *Nature*, **300,** 598.
36. PACHL C., BURKE R.L., STUVE L.L., SANCHEZ-PESCADOR L., VAN NEST G., MASIARZ F. & DINA D. (1987) Expression of cell-associated and secreted forms of herpes simplex virus type 1 glucoprotein gB in mammalian cells. *J. Virol.* **61,** 315.
37. ROSE J.K. & BERGMANN J.E. (1982) Expression from cloned cDNA of cell-surface secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. *Cell*, **30,** 753.
38. ALT F.W., BOTHWELL A.L.M., KNAPP M., SIDEN E., MATHER E., KOSHLAND M. & BALTIMORE D. (1980) Synthesis of secreted and membrane-bound immunoglobulin Mu heavy chains is directed by mRNAs that differ at their 3′ ends. *Cell*, **20,** 293.
39. EARLY P., ROGERS J., DAVIS M., CALAME K., BOND M., WALL R. & HOOD L. (1980) Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. *Cell*, **20,** 313.
40. SETHI K.K., OMATA Y. & SCHNEWEIS K.E. (1983) Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus-specific and H-2-restricted cytotoxic T lymphocytes. *J. Gen. Virol.* **64,** 443.
41. MISRA V., GILCHRIST J.E., WEINMASTER G., QUALTIERE L., VAN DEN HURK S. & BABIUK L.A. (1982) Herpesvirus-induced "early" glycoprotein: characterization and possible role in cytolysis. *J. Virol.* **43,** 1046.