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**Bovine Effector Cell Recognition of IBR Virus:
Evaluation by Monoclonal Antibodies,
Cellular Cloning and Viral Mutants**

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BARD FINAL REPORT

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ABSTRACT

The results of this work have characterized lymphocyte populations that participate in the response to bovine herpesvirus-1 (BHV-1). All BHV-1 strains including attenuated live vaccines can establish latency. Inactivated or live vaccines presently available can not prevent latency by a superinfecting virus. Many pharmaceutical companies have stopped manufacturing modified live virus vaccine. These serious statements support the need for improved understanding of host-viral interaction. Identification of the various kinds of immune cells that respond to the virus as well as the importance of particular viral proteins to these immune cells is a first key step in addressing the relevant components of the host and virus in this disease. The following four areas were studied:

First, four monoclonal antibodies (MAb) to bovine lymphocyte differentiation antigens (MAb 8-1E3 and 16-1E10 to CD2, 8-3F4 to CD5 and 8-10C5 to a novel leukocyte [non-lineage] antigen) were characterized phenotypically, biochemically and functionally. These antibodies were then used to enrich and characterize a population of peripheral blood lymphocytes expressing low levels of CD5 that exhibited non-MHC restricted cytotoxicity against BHV-1 infected cells following culture.

Second, natural killer-like cells were shown to lyse BHV-1 infected allogeneic and xenogeneic cells but not non-infected cells. The contribution of individual viral proteins were evaluated using inhibitor drugs and cells transfected with individual viral genes. The data indicate that the gI and gIV proteins are important recognition units for NK-like cell lysis while the gIII protein does not participate in NK-like cell recognition and lysis.

Third, cytotoxic T lymphocytes specific for BHV-1 infected cells were confirmed with an antigen specific role in the immune response of cattle against this virus. These T cell clones were specific for BHV-1 but not related BHV-2 and pseudorabies virus.

Fourth, with BHV-1 genes gI, gIII and gIV and the parallel development of an efficient cell-free translation system capable of producing viral proteins in vitro we have identified important T cell antigens encoded within the viral genome. Proteins from the gI, gIII, and gIV genes specifically stimulated T lymphocytes previously primed to BHV-1. A cell line developed to the gIV protein was CD2 and CD4 positive. In addition, a panel of precisely truncated peptides have allowed the identification of at least two T lymphocyte epitopes encoded within gIII.

The objectives of this study were to:

1. Define lymphocyte populations by monoclonal antibodies.
2. Using monoclonal antibodies, characterize BHV-1 responsive cells.
3. Characterize the specificities of cloned bovine lymphocytes for selected glycoproteins.

INTRODUCTION

Bovine herpesvirus-1 belongs to the alpha subgroup of herpes viruses associated with diseases of the respiratory or genital tracts. An important characteristic of this virus is its ability to produce latent infections of the nervous system (1, 2). Since the virus can become latent, it can persist in closed herds and be introduced to uninfected herds through a latently infected animal. The distinction between field and modified live vaccine strains has been difficult (3). The mechanism for protection of cattle by vaccination has been uncertain. Vaccine immunization can result in antibodies and such animals are protected from experimental challenge (4). However, other investigators have argued that antibody titers are not a reliable indicator of protection and correlate poorly with response to experimental challenge (5, 6).

The role of cell mediated immune responses in this disease have not been evaluated in light of known cytotoxic-and helper-T lymphocyte and natural killer cell populations. Identification of lymphocyte populations involved in these responses has been hampered by the lack of adequate reagents that identify bovine lymphocyte populations. Monoclonal antibodies specific for molecules present on the cell surface of different lymphocyte populations would provide the necessary reagents for identification and isolation of cell populations that may specifically recognize the virus.

Defining the cellular immune response to individual BHV-1 proteins may determine how or if immune cells contribute to protection against BHV-1 infection or disease. The isolation of BHV-1 genes that code for envelope proteins allow the characterization of specific host cell responses. Viral genes gI, which contributes to viral binding to the cell surface, gIII whose function is not clearly defined, and gIV, which allows penetration of the virus into the cell have been cloned and serve as useful genes to study the contribution of their respective proteins to cell mediated immunity in this disease.

In this study we have: 1. defined lymphocyte populations by monoclonal antibodies, 2. using these defined monoclonal antibodies, characterized BHV-1 responsive lymphocyte populations, and 3. characterized the specificities of bovine lymphocyte populations for selected BHV-1 glycoproteins using cells transfected with individual BHV-1 genes or genetically engineered BHV-1 proteins. Further, we have demonstrated the relevance of BHV-1 gI and gIV to the function of natural killer cells, and have mapped T cell epitopes of BHV-1 gIII recognized by bovine CD4 lymphocytes. Our results identify the contributions of cytotoxic- and helper- T lymphocytes as well as natural killer cells in the response to BHV-1 proteins.

MATERIALS AND METHODS

Animals. Mice were obtained from a BALB/c colony maintained at the Department. Cattle were Holstein-Friesian, Guernsey, Ayershire, Jersey and Brown Swiss cows and steers between 2 and 8 years of age from the Department of Dairy Science. Animals were immunized annually with killed BHV-1 vaccine (Fort Dodge, Fort Dodge, IA). Also, peripheral blood was obtained from Holstein calves and cows that were serologically negative to BHV-1 or Holstein steers vaccinated with BHV-1. Bone marrow, lymph nodes, thymus and spleen were obtained from healthy Holstein fetuses (third trimester of gestation) and cows at slaughter.

Cells. Peripheral blood mononuclear cells (PBMC) were obtained from fresh heparinized blood using Lymphoprep (Nycomed AS Diagnostics, Oslo, Norway) as described by the manufacturer. T cell enriched PBMC were isolated as previously described (7). Thymocytes were isolated from fetal thymi between 5 mo and term obtained from a slaughterhouse. Thymus was minced, passed through cheesecloth and separated using Lymphoprep. Fibroblasts were isolated from skin explants of various cattle. The BL-3 cell line was a gift from H. Lewin. Cells were resuspended in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml gentamicin, 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol and 10% heat inactivated fetal bovine serum (Sigma; medium).

Monoclonal antibodies. 8-IE3, 8-3F4 and 8-10C5 were generated from a fusion of splenocytes from BALB/c mice immunized with bovine T-enriched PBMC. on day -38 from fusion mice received 5×10^6 cells I.P., day -3 from fusion mice received 3×10^6 cells I.V. On day 0 cells were fused with P3X63Ag8.653 and maintained as described by Letchworth and Appleton (8). 16-IE10 was generated in a fusion of splenocytes from mice immunized with Concanavalin A (conA; Sigma Chemical Co., St. Louis, MO) lymphoblasts. On day 29 mice received 1.3×10^6 cells I.P., day -3 mice received 2.7×10^6 cells I.V. On day 0 cells were fused with P3X63Ag8.653 as described by Oi and Herzenberg (9). Supernatants were screened by ELISA using bovine PBMC, thymocytes, fibroblasts and BL3 cells. The isotype of each MAb was determined by gel diffusion using anti-isotype sera (Litton Bionetics, Inc., Kensington, MD). MAb 38-65 to SBU-T8 (10) was purchased from the University of Melbourne (Parkville, Victoria, Australia 3052). MAb M11 (11) to BoCD4 was a gift from W.Davis. MAb 33 to bovine IgM was a gift from K. Nielsen (12). Pl.17 and P3X63Ag8 myelomas were purchased from the American Type Tissue Collection (ATCC; Rockville, MD) and were used as isotype controls. MAb was purified by affinity chromatography using a recombinant protein A column (Beckman Instruments, Inc., Fullerton, CA) as described by the manufacturer. Monoclonal antibodies were biotinylated with Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, IL) as described by the manufacturer.

IL-A12 ascites (13) was a gift from C. Baldwin. Ascites containing MAb 19-19 specific for WC-1 (14) and 38-65 specific for BoCD8 (15) were purchased from the University of Melbourne (Parkville, Victoria, Australia). The MAb H4 to human HLA-DR is cross-reactive with bovine MHC class II (16) and was purchased from One Lambda (Los

Angeles, CA). The anti-bovine IgM secreting cell line 33 was supplied by K. Nielsen (12). The myeloma cell lines P1.17 and P3X63Ag8 were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and were used for isotype controls. Ascites containing these antibodies were prepared in our laboratory.

Additionally, MAbs to CD4 (CC30) and CD8 (CC58) have been previously reported (17). The cluster of differentiation for each antibody was as determined at the 1st International Workshop on Leukocyte Differentiation Antigens in Ruminants (July 23, 1989, Hannover, GR).

Flow microfluorimetry. Cells were suspended in PBS + 1% BSA + 0.02% NaN₃ at 10⁷ cells/ml and 100 µl of cell suspension and 100 µl MAb containing supernatant combined and incubated 30 min on ice then washed with PBS. The cells were resuspended in 50 µl of a 1:100 dilution of fluoresceinated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA) in PBS + 1% BSA + 0.02% NaN₃, incubated 30 min on ice and washed with PBS. For two colour analysis, stained cells were resuspended in the appropriate dilution of biotinylated MAb, incubated 30 min on ice and washed with PBS. Cells were then resuspended in 20 µl streptavidin phycoerythrin (Beckton Dickinson, Sunnyvale, CA) incubated 30 min on ice and washed with PBS. Cells were resuspended in 200 µl 1% paraformaldehyde and stored in the dark at 40C until analysed. All analysis were performed with the EPICS-C flow cytometer (Coulter Corp., Hialeah, FL). One colour histograms represent 10,000 signals, two-colour histograms represent 25,000 signals.

Transfected cells, grown to confluence in the presence of the G418, were released from the flask with 1X Trypsin-EDTA solution (Sigma). Cells, (1X10⁵) were pipetted into Lab-Tek chambered microscope slides (Nunc, Inc. Naperville, IL) 24 h. prior to examination. The cells were reacted with glycoprotein-specific murine monoclonal antibodies listed in Table 1, on ice, for 30 min. After 3 washes with PBS containing 1% BSA and 0.2% NaN₃, the cells were counterstained with a fluorescein-labelled goat anti-mouse secondary antibody, on ice for 30 min. The cells were examined by fluorescent microscopy and compared with BHV-1-infected cells.

Cell Sorting. The cell lines were labelled using the aforementioned primary and secondary antibodies as previously described. Following antibody binding, the transfectants were removed from their flasks by treatment with 0.35% EDTA and 0.5% glucose in PBS, for 5 minutes. Glycoprotein-expressing cells were separated from non-expressing cells by flow microfluorimetry using an EPICS (Coulter Co., Hialeah, FL). Cells expressing glycoprotein were cultured for later use as targets in cytotoxicity assays. At various time points after sorting (4-10 weeks), the recombinant cell lines were reexamined by flow microfluorimetry to verify maintenance of glycoprotein expression.

Radioimmunoprecipitation. Seventy-two hour ConA lymphoblasts were surface labeled with ¹²⁵I and immunoprecipitated as described by Knowles (18) except Sepharose 6MB-Protein A beads (Sigma) were used in place on S. aureus. Immunoprecipitates were separated by one dimensional SDS slab gel electrophoresis on 8, 10 or 12% acrylamide gels using the buffer system of Laemmli (19). Radio-labeled proteins were detected by exposure of dried gels to AR5 X-ray film (Kodak, Rochester, NY). Relative MW was determined by comparison

to proteins of known MW (Bio-Rad, Richmond, CA). Uninfected and infected Madin-Darby Bovine Kidney (ATCC# CCL22), uninfected and infected D17 cells and transfected cell lines were labelled with [35 S]-methionine (Dupont de Nemours & Co., Wilmington, DE) using a modification of the method of Fraker and Speck (20). Infected cells were cultured with virus at an MOI of 10 for 12 h. The cells were washed three times with NET buffer (150 mM NaCl, 5mM EDTA, 50mM Tris) containing 0.2mM phenylmethylsulfonyl fluoride (PMSF). The cells were subsequently lysed with NET containing 0.5% Triton X-100 on ice for 15 min. Cellular debris was pelleted by centrifugation and the lysates were immunoprecipitated using the glycoprotein-specific MAb listed in Table 1, adsorbed onto Protein-A sepharose 6MB beads (Sigma) as per manufacturer's instructions. The immunoprecipitates were released from the beads by boiling for 5 min. in 100 μ l of Sample Buffer (6.25mM Tris, 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and 1% Bromophenol Blue). A representative sample (1-5 μ l) of the immunoprecipitate was counted by scintillation spectrophotometry, total counts per sample calculated and equal counts loaded per well into an 8% polyacrylamide gel. The immunoprecipitates were resolved by SDS-PAGE using the procedure of Laemmli (19). The gels were dried and exposed on Kodak XAR-5 diagnostic film.

Determination of percent glycosylation. 125 I immunoprecipitates were treated with N-glycanase (Genzyme Corp., Boston, MA) or Endoglycosidase H (Boehringer-Mannheim, Indianapolis, IN) as described by the manufacturer. Proteins were separated on one dimensional SDS slab gel electrophoresis in gradient gels of 8-15% acrylamide, dried and MW determined as described above.

Determination of isoelectric point. 125 I labeled immunoprecipitates were separated in a tube gel apparatus under reducing conditions as described (21). Gels were cut into 5mm fragments and the location of the radiolabeled proteins determined in a gamma counter. Each piece of gel was allowed to stand in 0.5 ml H₂O overnight and the pH of each fraction determined using pH paper. For conformation, duplicate tube gels were separated in SDS slab gels and exposed to film as before.

Rosette formation inhibition assay. Rosette formation using AET treated SRBC was performed exactly as described by Belden and Strelkauskas (22). PBMC were incubated with a 1:100 dilution of ascites in medium for 30 min prior to the addition of treated SRBC. Individuals, blind to the antibody used, were asked to count 200 lymphocytes and determine percent rosette formation.

Proliferative assays. Response to mitogens, virus and alloantigens were conducted in 96 well, round bottom microtiter plates. Cells were cultured at 10^5 cells/well in RPMI 1640 (Grand Island Biological Company, Grand Island, NY) supplemented with penicillin (50 IU/ml), streptomycin (50 μ g/ml), L-glutamine (2 mM), HEPES (25 mM), 2 mercaptoethanol (0.1 mM) and 10% FCS (medium) in all assays. Final dilutions of mitogens were 5 μ g/ml phytohemagglutinin (PHA;Sigma) and 2.5 μ g/ml ConA. The final volume was 200 μ l/well. Mitogen assays were cultured 3 days at 37C, 5% CO₂. Mixed lymphocyte reaction (MLR) was performed by combining 10^5 /well PBMC with 10^5 /well gamma irradiated (2000 rad) autologous or allogeneic PBMC. Cells were incubated 5 days at 37C, 5% CO₂. Proliferation to virus was determined by mixing 10^5 PBMC with 10^5 UV inactivated PFU of bovine

herpesvirus-1, Cooper-1, (Colorado-1) (BHV-1; ATCC). Cells were incubated 5 days at 37C, 5% CO₂.

For blocking assays, 1:400, 1:2000 and 1:10000 dilutions of ascites containing approximately 10 mg/ml MAb were incubated with PBMC for 30 min at 37C prior to stimulation. Proliferation was determined by (³H) thymidine (1 µCi/well) uptake. All assays were performed in at least triplicate. Proliferation of cells in the absence of MAb was defined as 100%; proliferation in medium without stimulus was defined as 0% proliferation.

Cell lines responsive to cell-free translated lysate were maintained with UV-inactivated BHV-1 was included in cultures at a multiplicity of infection of five. Restimulation every four weeks was provided by ¹³⁷Cs irradiated PBM cells (2000 rads) and UV-inactivated virus. Before restimulation, dead cell debris was removed with Lymphoprep as above.

Alternatively for response to cell-free translated protein, one day prior to assay, antigens to be tested were added to round bottom 96-well plates at the concentrations noted in the results. Each well contained 1x10⁴ ³⁷Cs irradiated (2000 rads) PBM cells serving as feeder cells. Protein concentrations were tested in six replicate samples, in a final well volume of 200 µl and were incubated overnight at 37C. To initiate the assays, 2.5x10⁴ primed lymphocytes were added to each well and incubation continued for an additional 72 h. One µl of ³H-thymidine was included for the final six h, 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.

Isolation of the CD5^{dim} subpopulation. PBMC at 2 x 10⁷ cells/ml in medium were incubated overnight at 37°C in 5% CO₂. Non-adherent (NA) cells were removed from culture and resuspended in 10 ml of medium. NA cells were passed through a Sephadex G-10 (Sigma) column as previously described (23). The resulting lymphocyte population was incubated with 25 ml of medium containing MAb 16-1E10 (anti-CD2) and H4 (anti-MHC class II) at a final dilution 1:1000, for 30 min on ice. Cells were pelleted, resuspended in rabbit complement (Cedarlane Laboratories, LTD., Hornby, Ontario, Canada) diluted 1:10 in medium and incubated 30 min at 37°C. This complement treatment was repeated twice. The CD5^{dim}-enriched lymphocytes (CD2 and MHC class II depleted) were separated from dead cells by density gradient centrifugation as described above and resuspended medium.

IL-2. Recombinant bovine IL-2 (bov rIL-2), was supplied by P. Baker (Immunex Corp., Seattle, WA). The IL-2 (3000 units/µg) did not contain endotoxin as shown by the limulus lysate assay (P. Baker, personal communication). Other sources of IL-2 tested included human recombinant IL-2 (hu rIL-2; Biogen, Inc., Geneva, Switzerland), supplied by A. Abruzzini, and supernatant from the gibbon IL-2 producing cell line MLA-144 (24).

Analysis of proliferative function. PBMC and CD5^{dim}-enriched lymphocytes were added to round bottom microtiter plates (Costar, Cambridge, MA) at 5 x 10⁴ to 2 x 10⁵ cells/well. Cells were stimulated with bov rIL-2 (30 U/ml), UV-inactivated BHV-1 (2 x 10⁵ PFU/well), equal numbers of gamma irradiated PBMC (2000 rads, autologous or allogeneic) for 5 days or with 2.5 µg/ml Concanavalin A (ConA; Sigma) for 3 days at 37°C in 5% CO₂. During the last 4 hr cells were pulsed with 1 µCi [³H]thymidine deoxyribose. Cells were harvested and the radioisotope incorporation determined. The effect of accessory cells was determined

by adding gamma-irradiated (2000 rad) autologous adherent cells isolated from 1×10^6 PBMC/well cultured overnight. Following removal of the non-adherent cells, it was estimated 1×10^5 adherent cells/well remained.

Limiting dilution analysis. Doubling dilutions of lymphocytes were made in medium containing 1×10^6 /ml irradiated (2000 rads) autologous PBMC and added to round bottom plates (Costar, Cambridge, MA). A Poisson distribution was assumed, $n = 96$ for unstimulated control and $n = 24$ for each of the test points. A well was considered positive if proliferation was greater than two standard deviations above the mean of the unstimulated control. Limiting dilution analysis was performed using the Poisson single hit model as described by Taswell (25).

Target cells and NK-like cytotoxicity assay. The canine osteogenic sarcoma cell line D17 (ATCC) was maintained in monolayers in Eagles minimum essential medium (Sigma) supplemented with 2mM L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 25 mM HEPES and 10% FBS (Hyclone Laboratories, Inc., Ogden, UT). Cytotoxicity assays using D17 and BHV-1 infected D17 (Cooper strain ATCC VR 864) were conducted as previously described (26).

Subpopulation isolation. PBM cells were depleted of adherent cells by one hr incubation on plastic petri dishes. All incubations were at 37° C with 5% CO₂ in a humidified atmosphere. After gentle swirling, nonadherent (NA) cells were transferred to a second petri dish for an additional one hr incubation. NA cells were removed by gentle swirling, and the number of viable NA cells determined. From the first petri dish, adherent cells were removed with a cell scraper (American Scientific Products, McGaw Park, IL), and the number of viable cells determined. Adherent cells were greater than 80% esterase positive with alpha-naphthyl acetate esterase (27) and 95% viable. Alternatively, PBM cells were depleted of adherent cells by overnight incubation. NA cells were recovered and treated with MAbs H4 (anti-DR, 29), 3F4 (anti-CD5, 13) or both H4 and 3F4 (Table 2) followed by rabbit complement (C'). NA cells were less than 0.1% esterase positive and 90-95% viable. Percoll density gradient subpopulations were isolated from MAb H4 and C' treated cells. Cells were separated on a stepwise percoll density gradient of 30%, 35% and 40% percoll (Sigma). Cells were layered on top of the gradient and centrifuged for 30 min at 750 x g. Cells were removed from each interface and washed twice prior to use.

Target cells. The human lung carcinoma cell line A549 (ATCC CCL 185), human erythroleukemia cell line K563 (obtained from Dr. Ernest Borden), murine YAC-1 cell line (ATCC TIB 160), canine osteogenic sarcoma cell line D-17 (ATCC CCL 183), and normal bovine fibroblasts obtained from skin biopsies were used as target cells. Cells were maintained as monolayers in Eagle's minimum essential medium supplemented as described for RPMI 1640, except without 2-mercaptoethanol.

Transfected cells were cultured under identical conditions except with 1.0 mg/ml of the neomycin analog Geneticin-(G418) (GIBCO, Grand Island, NY) added to select for transformed cells. This level of selection represents 4-LD₁₀₀ G418 for D17 cells (data not shown).

Cytotoxicity assay. Target cells at 1×10^4 cells per well in 96 well round bottom microtiter plates (Costar, Cambridge, MA) were labeled overnight with 3 uCi/well of 51 sodium chromate (New England Nuclear, Boston, MA). Cells were infected with Cooper strain of BHV-1 at an MOI of 10 for two hr and then washed three times with phosphate buffered saline (PBS). Effector cells were added in quadruplicate to target cells at selected ratios. The cells were incubated for 20 hr at 37°C in 5% CO₂ because viral antigen expression was maximal at this time on target cells. After plates were centrifuged at 300 x g for 5 min, 100 µl of supernatant was collected from each well and counted in a gamma counter. Spontaneous release was determined by incubating target cells with medium alone and maximum release was obtained by adding 10% sodium dodecyl sulfate detergent solution (Sigma). Percent lysis was determined with the following formula:

$$\% \text{ lysis} = \frac{\text{mean sample cpm} - \text{mean spontaneous cpm}}{\text{mean maximum cpm} - \text{mean spontaneous cpm}} \times 100,$$

where cpm represents counts per minute.

BHV-1 glycoprotein expression as determined in an ELISA assay. Expression of BHV-1 major glycoproteins gI (55, 74, 130 kilodaltons), gIII (97 and 180 kilodaltons) and gIV (77 and 150 kilodaltons) were identified by the following IgG_{2a} mAbs: 510604 for gI, 290504 for gIII, and 110604 for gIV (29). The MABs at 1 mg/ml were diluted 1:500 in PBS with 1% bovine serum albumin (BSA). Five x 10⁴ A549 cells per well were incubated overnight in a round bottom microtiter plate. Cells were infected with Cooper strain of BHV-1 at an MOI of 10 for two hr, washed two times and incubated for 5, 10, or 20 hr. Infections were staggered so one assay included all time points. After incubation with virus, cells were washed twice with PBS. Monoclonal antibodies (100 µl) were added in triplicate to wells and incubated for one hr at 4° C. After centrifuging, the cells were washed three times with cold PBS, 50 µl of diluted horseradish peroxidase conjugated goat anti-mouse Ig (polyvalent) (Hyclone Laboratories, Inc., Logan, UT) was added to each well and incubated for one hr at 4° C. Cells were washed, followed by addition of the enzyme substrate. After 20 min, 150 µl of supernatant from each well was transferred to Immulon 1 MICROELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) and read on an ELISA reader (BIO-TEK EL-310, BIO-TEK Instruments Inc., Burlington, VT).

Drug treatment. Target cells alone were treated with phosphonoacetic acid (PAA) (Sigma). Expression of late BHV-1 glycoproteins were inhibited by 300 µg/ml of PAA in BHV-1-infected Madin-Darby bovine kidney cells (30). Therefore PAA, at 300 µg/ml was added to target cells at the same time as the viral infection or two hr post infection as stated in the results.

BoLA typing. BoLA typing was performed as described (31) using a panel of alloantisera produced in our laboratory and specificity substantiated using a panel of alloantisera provided by Domenico Bernoco, University of California-Davis. All antisera identifying BoLA A locus-encoded phenotypes have been confirmed by international BoLA typing workshops (32).

Viruses. BHV-1 (Cooper strain) and BHV-2 (New York strain) were obtained from ATCC #VR-864 (33) and ATCC #VR-845 (34), respectively. The pseudorabies virus, Sullivan/p3/4, was provided by Sandy McGregor (35). TCID₅₀-titered BHV-1 virus was diluted to 1×10^7 infectious units/ml in order to achieve a multiplicity of infection (MOI) of 10 for the target cells. Ultraviolet-inactivated virus was prepared using 5 ml aliquots of diluted stock exposed to germicidal ultraviolet light, at a distance of 15 cm, for 30 min. Inactivated virus was always prepared from the same batch of stock virus as that used for live virus infections.

Cloning peripheral blood lymphocytes with specificity for BHV-1. Peripheral blood mononuclear cells from immunized animals were cultured with UV-irradiated bovine herpesvirus 1, and clones were established from one of the animals by methods reported for human CTL (36). Briefly, peripheral blood mononuclear cells were isolated from Ficoll-hypaque density gradients centrifuged at $1000 \times G$ for 45 min and washed three times in PBS. The cells at 20×10^6 /ml were cultured for 10 days in the presence of UV-irradiated BHV-1 (Cooper strain) at 1×10^6 pfu/ml. At day 10 lymphocytes were restimulated with UV-irradiated BHV-1 and cloned on day 14 at 1 cell per well in the presence of 1×10^5 irradiated feeders (2000 rads)/well, 1×10^5 pfu/well of BHV-1 and a cocktail of 10% MLA 144 supernatant and 10% T cell growth factor (TCGF) containing supernatant. Evidence of clonality was provided by Possion distribution (25) where 76% of the wells were negative for cell growth. Clones were selected 3 weeks later, split into 2 wells and fed at 7-10 d intervals with 5% MLA 144, 5% TCGF containing supernatant, 1×10^5 irradiated feeders cells and 1×10^5 pfu UV-irradiated BHV-1.

The culture medium used throughout was RPMI 1640 with 25mM HEPES (GIBCO) containing 10% fetal bovine serum, 2mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin. The gibbon cell line MLA 144 constitutively produces interleukin 2 that can maintain bovine IL 2 dependent cell lines (37). TCGF containing supernatant was produced from bovine peripheral blood mononuclear cells cultured at a concentration of $2-5 \times 10^6$ /ml for 18 hr in the presence of 2.5 µg/ml concanavalin A (Con A) (Sigma). The supernatant was treated with α-methyl-D-mannoside (Sigma) at a concentration of 0.1M and filtered through a 0.22-µm filter.

Cytotoxic T lymphocyte mediated lysis. Day 3 Con A-stimulated lymphoblast targets were virally-infected for 2 hr at a multiplicity of infection (MOI) of 3 for BHV-1 and PRV and an MOI of 1 for BHV-2. Target cells were washed once and 250 µCi ⁵¹Cr added followed by a 1 1/2 hr incubation at 37°C and two washes. Infected and labeled target cells were plated in triplicate at 2×10^4 cells/100 µl. Plates were incubated with effector cells for 6 hr and supernatant was collected using a Skatron harvester (Skatron, Inc. Sterling, VA) and counted in a gamma scintillation spectrometer. Controls were included in assays to determine total and spontaneous release of isotope. Results were expressed as the percentage of specific lysis calculated from the formula:

% specific lysis =

c.p.m. experimental - c.p.m. spontaneous release

c.p.m. total release - c.p.m. spontaneous release

x 100.

Viral glycoprotein expression on the target cells was determined by a kinetics study. Target cells were incubated with virus for 2 hr, washed, then incubated for selected times. Following incubation, the cells were treated with a 1/10 dilution of a cocktail of 28 monoclonal antibodies, at 1 mg/ml, specific for BHV-1 glycoproteins (29). Fluoresceinated rabbit anti-mouse (Jackson Immuno Res. Lab., Avondale, PA.) or rabbit anti-swine antibody (provided by Sandy McGregor) was added for 30 min at 37°C, washed, and the percent fluorescence determined by flow cytometry.

Plasmids. The plasmid selected for these experiments was the murine retrovirus shuttle vector, pZipNeo SV(X)1. This plasmid was created when the long terminal repeats (LTR) of Maloney Murine Leukemia Virus and the neomycin phosphotransferase gene (neo) from Tn5 transposon were cloned into a pBR 322 plasmid. Between the 5' LTR and the neo gene resides a unique, BamHI cloning site (38- 40). Initially, pZipNeo was transfected into a number of cell lines, to assess its suitability as a transducing element in potential target cells prior to cDNA insertion. pZipNeo was prepared for glycoprotein gene insertion by opening the BamHI cloning site and dephosphorylating the sticky ends with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN). The glycoprotein-encoding genes were ligated into the BamHI site of pZipNeo following linearization and addition of synthetic BamHI linkers (Bethesda Research Laboratories, Gaithersburg, MD) to the ends of the inserts. The constructs were evaluated for the presence and orientation of the inserts by restriction endonuclease mapping with BamHI and SmaI. Plasmids containing the appropriate inserts were propagated in TB-1 strain

E. coli for large scale plasmid preparations (41); the respective DNA collected and purified twice with CsCl gradients. The purified DNA was rechecked by restriction endonuclease mapping prior to transfection into D17 cells.

Glycoprotein Genes. The BHV-1 genes encoding gI (also known as gB), gIII (gC) and gIV (gD) were obtained as sequenced cDNA's cloned into the Polink carrier plasmid (Molecular Genetics Inc., Minnetonka, MN). The 3.61 kbp fragment containing the gI gene, begins at a SalI site located 500 bp upstream (5') from the transcriptional start codon and terminates 275 bp downstream (3') of the transcriptional stop codon (42- 44). The 2.45 kbp fragment containing the gIII gene, begins at the BamHI site located 90 bp upstream of the start codon and terminates at the EcoRI site located 870 bp downstream from the stop codon (44). The 1.35 kbp fragment, containing the gIV gene, begins at the MaeI site located 46 bp upstream from the start codon and extends to the other MaeI site located 5 bp downstream from the stop codon (44).

Transfection. Recombinant plasmids containing inserts verified to be inserted correctly by restriction enzyme mapping were transfected into D17 cells using the calcium phosphate method of others (45). Briefly, 1-5 µg of supercoiled plasmid was mixed with 2X HBS (0.28M NaCl, 45.7mM HEPES, 0.7mM Na₂HPO₄) and 2M CaCl₂, added to a nearly confluent monolayer of cells and incubated four h. During this incubation period the DNA-calcium complexes were visualized by phase-contrast microscopy as a "dusty" overlay on the cells. After incubation the cells were washed with serum-free medium and osmotically shocked

with HBS-15% glycerol for two min. The cells were washed with serum-free medium and complete medium was replaced. After 48 h. the cells were split and G418 added to the cultures.

Strains and Plasmids. *Escherichia coli* strain DH5 α was used for plasmid propagation (BRL, Gaithersburg, MD). Plasmid pE5LVPO, a gift from A.C. Palmenberg, has been described in detail elsewhere (46). Briefly, pE5LVPO contains the 5' NCR, leader and VPO sequences of EMCV, and transcription of DNA sequences inserted downstream from the 5' NCR result in a chimeric RNA with the inserted gene. The EMCV 5' NCR conveys increased translational efficiency in rabbit reticulocyte lysates during cell-free reactions (47). Cloned and sequenced BHV-L cDNAs gl, gIII and gIV were provided in a carrier plasmid by Molecular Genetics, Inc. (Minnetonka, MN).

Plasmid Constructions. Standard cloning procedures were used for plasmid constructions (41); the noteworthy aspects of our constructions are illustrated by plasmid pE5GPIII in Figure 1. This plasmid contained a 2.6-kilobase-pair (kbp) NcoI to XbaI fragment spanning the entire BHV-L gIII coding region, cloned into the NcoI and XbaI sites of pE5LVPO. Following translation of the resulting T7-RNA polymerase directed mRNA, the synthesized protein contained the first 4 amino acids of EMCV leader protein, fused to the complete BHV-1 gIII protein beginning at the initiating methionine residue.

To-construct pE5GPIV, a 1.3 kbp MaeIII restriction fragment containing the gIV gene of BHV-1 was prepared, and the 5' overhanging sequences filled with Klenow polymerase. This fragment was then ligated into pE5LVPO that was Klenow polymerase blunted after NcoI digestion. The chimera that resulted from this construction encoded a protein containing the first 5 amino acids of EMCV leader protein, and a gIV protein which lacked BHV-1 gIV amino acids 1 thru 25.

pE5GPI was assembled in a two step process. First, a 2.2 kbp StuI fragment of BHV-1 gl was subcloned into the SmaI site of pUC 19. Then, the BamHI to EcoRI insert from this newly created plasmid was isolated, treated with Klenow, then ligated into the blunt-ended NcoI site of pE5LVPO. The resultant construction encoded a protein containing the first 5 amino acids of the EMCV leader protein, followed by 2 amino acids from the pUC 19 multiple cloning site, then a BHV-1 gl gene which lacked the first 133 amino acids. All plasmid constructions were sequenced through relevant regions using Sequenase (United States Biochemical Corp., Cleveland, OH) to verify that proper ligation had occurred.

In vitro RNA Synthesis. CsCl purified plasmid template was linearized with an appropriate restriction enzyme, extracted with phenol/chloroform, then precipitated with ethanol. After resuspension of the linearized plasmid in water, transcription with T7 RNA polymerase was in accordance with the manufacturers' instructions (BRL, Gaithersburg, MD). RNAsin (40 U; Promega, Madison, WI) was added to protect transcriptional products. After RNA synthesis was completed, the reaction mixtures were extracted with phenol/chloroform, then precipitated with ethanol. The RNA, resuspended in water, was added directly to rabbit reticulocyte lysates.

In vitro Translations. Rabbit reticulocyte lysate was prepared and used as described by Shih et al. (48). Reaction mixtures (30 μ l), containing 1 μ g of specific RNA, were incubated at 30C for 90 min. Translation efficiency was evaluated by incorporation of L-[35S]-methionine

into trichloroacetic acid (TCA) precipitable radioactivity. Typically, 5 μ l of reaction mixture was precipitated with acetone, resuspended in 100 μ l electrophoresis sample buffer, then boiled for 3 min prior to analysis by one-dimensional SDS-PAGE and autoradiography (19). Lysates for lymphocyte proliferation assays were treated in parallel reactions, and contained 100 μ M L-methionine instead of the radiolabeled substrate. Quantitation of unlabeled protein was based on the known methionine composition of the protein, and 35 S-methionine incorporation in parallel labeled reaction samples. Protein concentrations were standardized to one another on a molar basis using mock translated lysate as a diluent.

RESULTS

Monoclonal Antibodies

Determination of tissue distribution. We have examined the distribution of BoCD2, BoCD5 and 8-10C5 using our MAb. Representative one-colour profiles are shown in Fig. 2. BoCD2 was present on $43 \pm 11\%$ of periperal blood lymphocytes (PBL; Fig. 2A, panel 1a), $79 \pm 9\%$ of T lymphocyte enriched periperal blood lymphocytes (Fig. 2A, panel 1b), $73 \pm 9\%$ of thymocytes (Fig. 2A, panel 1c) and $50 \pm 2\%$ of lymph node lymphocytes (data not shown) using MAb 8-1E3. The reactivities of both our MAb were nearly identical; 16-1E10 bound $46 \pm 11\%$ of PBL and $78 \pm 3\%$ thymocytes. The MAb 8-3F4 to BoCD5 identified three populations of PBL (Fig. 2A, panel 2a). We have termed these populations "bright", "dim" and "negative". While the total number of BoCD5 cells was constant, $67 \pm 10\%$ ($n=38$), the number of bright and dim cells varied from animal to animal (Fig. 2B). Animals from 6 mo to 8 yrs were used in these studies, and the ratio of bright to dim cells could not be correlated to age. As shown in Fig. 2, BoCD5 was found on $87 \pm 1\%$ thymocytes (Fig. 2A, panel 2c). The MAb 8-10C5 bound T lymphocytes as well as a variety of other cells and was present on $84 \pm 15\%$ PBL (Fig. 2A, panel 3a); however, only $23 \pm 10\%$ of thymocytes expressed this molecule (Fig. 2A, panel 3c).

The phenotype of the cells was confirmed using two-colour flow microfluorimetry and supported the observation that these molecules are not present on all cells. BoCD2 population of PBL contained all the BoCD4⁺ cells and most of the BoCD8⁺ (Fig. 3A, panels 1a to 3a). Most BoCD2 tytmocytes were dual positive for BoCD4 and BoCD8 (Fig. 3B, panel 1a to 1c). 8-1E3 and 16-1E10 were each capable of blocking the binding of the other (data not shown) and thus bound the same functional determinant of BoCD2. BoCD5 was of particular interest because of the observed differences in surface concentration. The brightest population contained T lymphocytes, BoCD4⁺ and BoCD8⁺, but not B lymphocytes (Fig. 3A, panels 1b to 4b). The dim and negative populations contained both IgM-bearing cells and non-T/non-B lymphocytes based on the expression of CD4, CD8 and IgM. The cell populations bound by 8-10C5 included the BoCD4⁺ and BoCD8⁺ lymphocytes as well as IgM-bearing lymphocytes (Fig. 3A, panel 1c to 4c). Most thymocytes that were stained by 8-10C5 coexpressed either BoCD4 or BoCD8 (Fig. 3B, panel 1a to 3c). The thymocytes expressing the immature T cell marker CD1 did not overlap with those expressing the 8-10C5 molecule (Fig. 3B, panel 5c). This MAb also bound granluocytes, the B-lymphoblastoid cell line BL-3 and plastic adherent PBMC, but was not present on fibroblasts (data not shown). Further, the expression of 8-10C5 molecule increased following activation with lectin or antigen (data not shown).

The ability of the antibodies to bind human, ovine, porcine or caprine PBMC was examined using flow microfluorimetry. MAb 8-3F4 and 16-1E10 bound only bovine PBMC, 8-1E3 bound bovine and caprine PBMC and 8-10C5 bound PBMC from all species tested except human.

Biochemical characteristics of the molecules. To confirm that the molecules identified by these antibodies were the equivalents of human CD2 and CD5, we examined their biochemical characteristics. Immunoprecipitations of the molecules from ¹²⁵I surface labeled ConA

lymphoblasts are shown in Fig. 4. Both antibodies to BOCD2 precipitated a broad band with MW Of 60 kd. The antibody to BoCD5 immunoprecipitated a molecule with a MW of 65 kd. These antibodies precipitated molecules of similar MW from thymocytes and resting PBMC under reducing and non-reducing conditions (data not shown). The antibody 8-10C5 precipitated a heterodimer with MW Of 120 kd (α chain) and 89 kd (β chain) from ConA blasts (Fig. 4) and PBMC (data not shown) under reducing and non-reducing conditions. We were unable to precipitate this molecule from thymocytes. In addition, a molecule of identical size was precipitated from ^{35}S -methionine labeled BL-3, a lymphoid cell line. The molecule precipitated by 8-10C5 was compared with molecules precipitated by CD18 (LFA-1 family β chain) and was found to be distinct (data not shown).

Immunoprecipitates of ^{125}I labeled ConA lymphoblasts were used to examine the glycosylation and isoelectric points of the three molecules. The results are summarized in Table 3. All three molecules were found to be highly glycosylated (10-20%). All molecules had neutral isoelectric points with the exception of the beta chain of the 8-10C5 which was acidic (pI=4.0- 4.6).

We also examined the cell surface concentration of the molecules using equilibrium binding as described by Trucco and de Petris (49). It was of interest that in both attempts the antibodies 8-3F4 (BoCD5) and 8-10C5 lost all binding activity following ^{125}I labeling, suggesting that a tyrosine residue(s) is present in the binding sites of these antibodies. Surface concentrations for BoCD2 were determined using 16-IE10 at 100,000 molecules/cell for ConA lymphoblasts, 30,000 molecules/cell for PBTL and 12,000 molecules/cell for thymocytes.

Functional characteristics of the molecules. Many surface molecules function in cell-cell interactions; therefore, we examined the ability of our antibodies to inhibit these known functions. Antibodies to the human CD2 TII₁ epitope have the ability to block E rosette formation. Bovine T lymphocytes do not form stable rosettes with untreated SRBC (22); therefore, we examined the ability of the antibodies to block AET-treated SRBC rosette formation which has been reported to be T cell associated (22). Table 4 shows that only the antibodies to CD2 are able to block E^{AET} T rosette formation.

We also examined the ability of these MAb to block proliferative and cytotoxic function. A representative experiment of 7 experiments is shown in Fig. 5. At dilutions of 1:400 (approximately 25 ug/ml) to 1:10000 (approximately 1ug/ml) of ascites, the MAb 16-IE10 to BoCD2 was able to block proliferation to alloantigen in all experiments ($83 \pm 19\%$ inhibition at 1:400 n=7) and virus in 6 out of 7 experiments ($86.6 \pm 17\%$ inhibition at 1:400, n=6) when compared to isotype controls. The MAb 8-IE3 did not block as efficiently, blocking proliferation to alloantigen and virus greater than 50% only at high dilutions. The antibody to BoCD5 was variable in its ability to block function, inhibiting 50% of the proliferation to alloantigen in 3 out of 7 experiments and proliferation to virus in none. Unlike the other antibodies, 8-3F4 slightly enhanced proliferation when included at high concentrations with ConA (Fig. 5). This was also observed with MAb OX-19 to the rat CD5 equivalent (50). The MAb 8-10C5 was able to block proliferation to alloantigen ($95 \pm 7\%$ inhibition at 1:400, n=6) and virus ($89 \pm 11\%$ at 1:400, n=6). These MAb were not able to block proliferation to lectin (ConA or PHA), nor were they mitogenic alone or in the presence of

PMA or anti-murine IgG as a cross-linking reagent (data not shown). Further, these MAb did not block alloantigen-generated CTL or NK-like activity (data not shown).

Characterization of a CD5^{dim} subpopulation and functional activity.

Effectiveness and efficiency of CD5^{dim} subpopulation enrichment. T lymphocytes vary in the levels of CD5 expressed on their surface. The bovine CD5^{dim} population composed 10 to 30% of the PBMC population. This population is BoCD2⁻ and BoCD4⁻ and contains some BoCD8⁺ and T19⁺ lymphocytes as determined by two colour flow microfluorimetry (Fig. 6). CD5^{dim}-enriched lymphocytes contained a small population of IgM⁺ lymphocytes. Our interest was in examining the non-CD2/non-B lymphocytes, therefore, we used anti-BoCD2 and anti-MHC class II and complement to deplete BoCD2⁺ (CD5^{bright}) and B lymphocytes (mature and immature). Using the enrichment method described above, $1-3 \times 10^6$ PBMC yielded $1-8 \times 10^7$ CD5^{dim}-enriched lymphocytes. These cells had lymphocyte morphology as determined by Giemsa staining and contained no observable esterase positive cells.

To determine the effectiveness of the CD5^{dim} enrichment, the phenotype of the PBL and CD5^{dim} lymphocytes was determined. The CD5^{dim} population expressed lower surface levels of BoCD5 (Fig. 7a) and was heterogeneous for T19 and CD8 ranging from 30 to 80% and 5 to 35%, respectively. A portion of this population (7 to 20%) expressed low levels of CD2; these cells were not removed by the complement treatment (Fig. 7b). CD5^{dim} cells obtained using this procedure were IgM⁻ ($\leq 3\%$) but contained small numbers of contaminating CD4⁺ lymphocytes ($\leq 13\%$). Mackay *et al.* (51) reported a portion of ovine T19⁺ lymphocytes coexpressed CD8. In a preliminary experiment with cow 759S, we found 3% of the T19+ CD5^{dim}-enriched lymphocytes also expressed CD8 (data not shown). The results of four animals are shown in Table 5.

Proliferative ability of CD5^{dim} lymphocytes. The function of CD5^{dim} or T19⁺ lymphocytes has not been determined. We examined the ability of the CD5^{dim}-enriched lymphocytes to proliferate to lectin (ConA) and T cell antigens including alloantigen, viral antigen (BHV-1, UV-inactivated) and the cytokine IL-2. The results of two representative experiments are shown in Table 6. In eight experiments, CD5^{dim}-enriched lymphocytes did not proliferate to alloantigen (Table 6) or BHV-1 (data not shown). However, in a single experiment, CD5^{dim}-enriched lymphocytes responded at a high level to autologous PBMC (data not shown), the reason for this proliferation is not known. Both PBMC and CD5^{dim}-enriched lymphocytes proliferated at a high level to ConA and IL-2 in the absence of exogenous antigen (Table 6). Recombinant bovine IL-2 was used in these experiments; however, in other experiments these cells were able to proliferate at equivalent levels to hu rIL-2 and MLA 144 supernatant. The proliferation in the presence of bov rIL-2 was dose dependent (Fig. 8).

The ability of PBMC to proliferate to IL-2 in the absence of exogenous antigen has been reported previously in human (52), mouse (53) and bovine (26). Limiting dilution analysis showed that the population of cells responsible for this proliferation was enriched in the CD5^{dim}-enriched lymphocytes population from 1/2668 cells (PBMC) to 1/632.

Requirement for accessory cells. While CD5^{dim}-enriched lymphocytes proliferated at high levels to rIL-2 and ConA, they proliferated poorly to alloantigen or virus in any of the assays.

Antigen presenting cells were removed by depleting macrophages (esterase⁻, MHC class II⁻) and B cells (Ig⁻, MHC class II⁻) from the CD5^{dim}-enriched lymphocytes population during the enrichment procedure; therefore, we investigated the effect of accessory cells on proliferation. The addition of antigen presenting cells had no effect on CD5^{dim}-enriched lymphocytes proliferation to alloantigen or virus, but interestingly enhanced the proliferation to ConA and bov rIL-2. Representative results are shown in Table 6. In two experiments, the proliferation of CD5^{dim} lymphocytes to ConA was enhanced significantly by the addition of adherent cells ($p < 0.05$ by Student's t test). In both experiments, the proliferation in the presence of IL-2 was increased although the difference was not statistically significant. Additional antigen presenting cells had a slight suppressive effect on PBMC.

The phenotype of the proliferating cells. PBMC and CD5^{dim}-enriched lymphocytes stimulated with ConA or rIL-2 were examined with a panel of mAb to determine the phenotype of the proliferating cells. Fig. 9 indicates the change in size (y-axis) and granularity (x-axis) using forward angle light scatter and 90° light scatter, respectively. Lymphoblasts occurred in response to both ConA (Fig. 9b) and IL-2 (Fig. 9d) as compared to the unstimulated cells (Fig. 9a and 9c). The phenotype of the resting and proliferating cells is shown in Table 7. CD5^{dim}-enriched lymphocytes that proliferated to bov rIL-2 expressed a CD11c⁺, T19⁺ phenotype while the cells proliferating in response to ConA were more numerous and heterogeneous.

Examination of the lytic capability of IL-2 stimulated CD5^{dim} lymphocytes. T19 lymphocytes have been shown to be $\gamma\delta$ TCR bearing in sheep (14) and this antibody binds an analogous molecule in cattle (54). In humans, $\gamma\delta$ TCR⁺ T cells isolated from peripheral blood are non-cytotoxic but can lyse NK sensitive targets following activation (55, 568). Because the CD5^{dim} lymphocytes that proliferated in response to IL-2 possessed a T19⁺ phenotype, we examined the ability of these cells to function in an NK-like manner. The results are shown in Fig. 10 and Table 8. The low recovery of CD5^{dim} lymphocytes made large studies on many animals difficult and was the reason for the low effector to target ratios in Table 8. However, even at low effector to target ratios ($\leq 1:2$) killing of virus infected D17 cells at levels greater than 20% was seen with CD5^{dim} lymphocytes cultured in the presence of IL-2. This killing was not due to an inherent weakness in the virus infected D17 as freshly isolated, Sephadex G-10 non-adherent cells were unable to kill (Fig. 10, panel 2a; Table 8). Further, no lysis was observed following addition of undiluted culture supernatants from these cells to D17 or virally-infected D17 (data not shown).

Natural killer-like cells mediate lysis of BHV-1 infected cells.

Freshly isolated bovine PBM cells from BHV-1 immune or nonimmune animals lysed BHV-1-infected A549 xenogeneic cells (Fig. 11). Therefore, non-MHC restricted, naturally cytolytic effector cells were capable of mediating lysis regardless of the immune status of the animal. Bovine cells failed to lyse A549 tumor target cells that were not virally infected (Fig. 11) or human K563 or murine YAC-1 tumor cells (data not shown). This observation confirms that bovine natural cytolytic cells require a virally infected target cell for lysis to occur. To optimize effector cell recognition of BHV-1-infected cells the kinetic pattern of

BHV-1 major cell surface glycoprotein expression on target cells was determined (Fig. 12). Importantly, all three glycoproteins were expressed at 20 hr. Although nonadherent cells could lyse target cells as early as 4 hr, maximal lysis was at 20 hr (data not shown).

To determine the organ distribution of this natural cytolytic activity against BHV-1-infected cells, mononuclear cells from bone marrow, lymph node, spleen and thymus from a fetus in the third trimester of gestation were analyzed. Cells from lymph node and spleen had high lytic activity, while cells from bone marrow and thymus had low lytic activity (Table 9). Similarly, cells from lymph node, spleen and peripheral blood of an adult animal had high lytic activity, while cells from bone marrow and thymus had low lytic activity (Table 9).

PBM cells were separated to determine if the effector cell population(s) were present in the monocyte enriched adherent population or the NA population. Both adherent and NA populations from freshly isolated cells mediated lytic activity, with increased lytic activity in the adherent population and decreased lytic activity in the NA population when compared to the whole population (Fig. 13, Panel A). In contrast, both NA and adherent cells from overnight cultures maintained levels of killing comparable to the freshly isolated whole population (Fig. 13, Panel B). From these results it was clear both adherent and nonadherent populations mediated lytic activity. However, overnight culture enhanced the lytic capabilities of the NA population. Culturing may have removed a suppressive population or may have activated the NA cells, adherent cells or both populations.

Having determined lytic activity was present in both the adherent and NA populations, the NA cells were further divided and characterized by two procedures. First, NA cells were treated with MAb and C'. Monoclonal antibody H4 (anti-DR,) and C' treatment depleted cells expressing MHC class II molecules, including activated monocytes and B cells. In experiment #1 of group I, no C5B6⁺ cells (anti-CD11c on bovine monocytes) were detected after MAb H4 and C' treatment (data not tabulated). Therefore, when B cells and macrophages were removed from the NA population, lytic activity similar to controls was maintained (Table 10, group I). Lytic activity was still strongly expressed after removal of only T cells and small null cells in group II using MAb 3F4 (anti-CD5) and C' or T cells, small null cells and MHC class II positive cells in group III (Table 10).

Second, NA cells depleted of MHC class II positive cells, were further separated on a stepwise percoll density gradient. High density cells obtained from the 40% percoll interface had no lytic activity, while lower density cells obtained from the 30 and 35% percoll interfaces had enhanced lytic activity over the control population (Table 11). Fluorescent antibody staining with the pan T cell MAb 1E3 (anti-CD2) indicated again that T cells were not mediating this lytic activity since the T cell enriched fraction (P40) had no lytic activity. The percoll density gradient findings together with the MAb and C' depletion studies, indicate the lytic activity was not mediated by T cells, B cells or activated monocytes, but by a large, low density NA cell population.

Natural killer-like cells do not require late viral glycoproteins.

PBM cells lysed BHV-1-infected A549 and D-17 xenogeneic target cells, and allogeneic fibroblasts (Table 12). PBM cells also lysed PI-3-infected A549 cells (Table 12). Lysis of

BHV-1-infected D-17 cells was consistently the highest, followed by BHV-1-infected A549 cells, with lysis of BHV-1-infected fibroblasts and PI-3-infected A549 cells the lowest. This study concentrated on the effector and target cell requirements for lysis of BHV-1-infected A549 cells for two reasons. Firstly, lysis of BHV-1-infected fibroblasts was inconsistent from assay to assay, and secondly, D-17 cells were not evaluated as a target cell until late in the study. Lysis of a number of cell types was examined to determine if killing was limited to BHV-1-infected A549 cells or if a number of different virally-infected cells could be lysed. Lysis of PI-3-infected fibroblasts was evaluated to determine if the effector cells would lyse target cells infected with another virus.

The kinetics of BHV-1 cell surface viral glycoproteins I, III, and IV expression was evaluated previously, and importantly, all three glycoproteins were expressed at 20 hr. Having observed an increase in overall glycoprotein expression with time, the kinetic pattern of BHV-1-infected A549 cell lysis was determined. Freshly isolated PBM cells and overnight cultured NA cells had similar levels of lytic activity (Fig. 14, Panels A and B). When target cells were infected for 22 hr, effector cells present for the last 10 hr of culture showed low levels of lytic activity (Fig. 14). Effector cells present for the last 15 hr of culture had high lytic activity and maximal lytic activity was observed at 20 hr of culture. This indicated that even with the same level of glycoprotein expression for the last 10 hr of culture, the longer the effector cells were with the target cell the greater the lytic activity.

Knowing that PBM cells had high lytic activity in a 20 hr assay and BHV-1-infected A549 cells had high levels of viral glycoprotein expression at 20 hr, adherent and NA effector cells were evaluated for lytic activity against target cells. Adherent and NA cells of freshly isolated and overnight cultured cells were placed in a 4 hr assay for lytic activity against target cells infected with virus for 22 hr. Freshly isolated PBM cells did not lyse the target cells. However, overnight cultured adherent and NA cells had low but consistent lysis of virally-infected A549 cells in a 4 hr assay (Fig. 15). These results suggested that the freshly isolated cells required activation to mediate lysis or that by overnight culturing a regulatory population was inactivated or depleted.

Requirement of viral glycoproteins for lysis of BHV-1-infected target cells was evaluated using PAA to inhibit DNA polymerase activity in the target cells. An ELISA determined that PAA treatment abrogated expression of the late protein gIII, while early glycoproteins gI and gIV were present but at reduced levels (Fig. 16, panel A).

Lysis of drug treated target cells by freshly isolated PBM cells (Fig. 16, panel B) was determined in a 20 hr assay. PAA treated target cell lysis was only slightly lower than lysis of nondrug treated but infected target cells. Similar results were obtained when freshly isolated cells were separated into adherent and NA cell populations (Data not shown).

To insure these results were not due to the presence of the drug in the culture and to evaluate recognition requirements of the overnight cultured effector cells, lysis of 22 hr virally-infected target cells was determined in a 4 hr assay. Similar to the 20 hr assay with freshly isolated effector cells, effectors cultured overnight lysed target cells expressing viral antigens. PAA treated target cells that lacked expression of the late glycoprotein gIII had less than a

two-fold reduction in lysis compared to untreated virally-infected target cells (Fig. 17) confirming that gIII was not essential for target cell lysis.

Natural killer-like cells recognize individual BHV-1 glycoproteins expressed by transfected cells.

Immunofluorescence. Transfected cells grown under G418 selective pressure appeared as plaques of cells after 10-14 days in culture. It was anticipated that all of the cells would express the transfected glycoprotein gene. Examination of the cells by fluorescent microscopy and comparison with BHV-1-infected cells, indicated that not every transfected cell expressed detectable level of glycoprotein. Also, infected cells were not uniformly positive, but the transfected cells were obviously more heterogeneous with regard to glycoprotein expression. Therefore it was necessary to further purify the transfected cells.

Cell Sorting. The transfected cells were sorted on the basis of fluorescence intensity. As seen in Fig. 18, prior to cell sorting, 18.7% of the pZipNeo-gB transfected cells (Panel A), 28.9% of the pZipNeo-gC transfected cells (Panel B), and 10.8% of the pZipNeo-gD transfected cells (Panel C) exhibited brighter fluorescence than the gated value. The gate value was determined based on the fluorescence observed from noninfected, nontransfected D17 cells that were reacted with the fluorescein-labelled secondary antibody. The percentages of glycoprotein expressing cells above the gated values was much lower than expected and lower than suggested by direct immunofluorescent microscopy.

Following EPICS sorting, the cells were again cultured in the presense of G418. After 30-60 days, depending on the cell line, the transfected cells were re-analysed by flow microfluorimetry for maintenance of expression level. Figure 19 shows the comparison of histograms representative of the control and transfected cells that were maintained in culture after sorting. Glycoprotein expression was indicated by the shift to the right of the peak fluorescence for the transfected cells when compared with the peak fluorescence of the control cells.

Furthermore, the shoulder in the histogram representative of transfected cells extends farther to the right than does the control cells. The shift of the peaks and shoulders is more apparent in panel B which is an overlay of the two histograms. It is interpreted that the transfected cells were nearly 100% positive since the peak and shoulder representing the transfected cells lies to the right of the control cell peak.

Radioimmunoprecipitation. To determine that the glycoproteins expressed on transfected cell lines were similar to native viral proteins, immunoprecipitations were performed. The autoradiograph in Fig. 20 demonstrates the electrophoretic mobilities of the immunoprecipitates. Lanes 1-6 were loaded with equal counts of the indicated lysates, immunoprecipitated with monoclonal antibody (MAb) 110504, specific for gIV of BHV-1. In Lanes 1,3 and 5, a very faint band, representative of nonspecific binding, can be visualized. Lane 2 represents gIV from infected MDBK cells. The intense bands in this lane correlate with the 77kDa protein which make up the gIV homodimer, the lower band represents a partially glycosylated precursor to the 77 kDa protein.

Lane 4 represents gIV on infected D17 cells. The bands indicating the 77 kDa protein and its precursor are both present but not as well resolved. Comparing lanes 1 & 2 to lanes 3 & 4 indicates that D17 cells and MDBK cells express similar molecular weight gIV. Similar molecular weight suggests similar glycosylation of the protein cores. This methodology does not indicate that the same residues are glycosylated, but that approximately the same number of sugar moieties are added to the glycoproteins.

Lane 6 shows the gIV molecule expressed on cells transfected with pZipNeo-gD. This lane contains one intense band at 60 kDa relative molecular weight. This molecular weight represents low level glycosylation of gIV, completely nonglycosylated gIV protein has a molecular weight of 38 kDa (44).

Lanes 7-12 were loaded with equal counts of lysate from the indicated cell lines, immunoprecipitated with MAb 510604, specific for gI of BHV-1. Lane 8 represents gI immunoprecipitated from infected MDBK cells. Three characteristic bands of the gI heterodimer of 133 kDa, 74 kDa and 55 kDa Mr were visualized. A precursor molecule, m=not fully glycosylated, was observed immediately below the 133 kDa band. Lane 10, representing infected D17 cells, contained the characteristic bands of gI, though the relative intensity of the bands were obviously different from lane 8. This difference probably results from the quantity of glycoprotein available for chemical reduction, by 2-ME, of the glycoproteins into subunits, since the lanes were loaded based on radioactivity rather than protein amounts.

Lane 11 represents cells transfected with pZipNeo-gI. The three bands characteristic of gI were present but the 133 and 74 kDa bands were faint. Presence of the 74 and 55 kDa bands are more meaningful than the relative intensity of each because the lower bands are reduction products of the 133 kDa dimer. Comparison of lanes 8, 10 and 11 demonstrates that gI is expressed in a very similar fashion on infected MDBK, infected D17 and the transfected cells. Lanes 7, 9 and 12 contained a rather intense band at approximately 110 kDa Mr and a faint band at 50 kDa, these bands represent nonspecific binding to host proteins. This observation is similar to the reports using this MAb (57).

Cytotoxicity Studies. When glycoprotein expression on the surface of the transfectants was established, transfected and nontransfected D17 cells were used as targets for bovine NK cells in cytotoxicity assays to examine responses directed against individual glycoproteins. The data in Table 13 presents representative experiments demonstrating cytotoxicity associated with the transfected cell lines.

The cell line D17-pZipNeo-gB was used to demonstrate cytotoxicity directed at gI. The experiment shown in Table 13 is representative of cytotoxicity for 11 of 17 experiments using effector cells from 6 different animals. The the observed responses in all experiments examining responses against gI, when the E:T ratio was 100:1, ranged from 0.7 times background (Control) cytotoxicity to 5.75 times background level. In the same experiments, the responses against infected D17 cells ranged from 8.9 to 12.7 times background cytotoxicity.

The cell line D17-pZipNeo-gC was used to demonstrate cytotoxic responses directed at gIII. No significant response against gIII was detected in any of the four experiments, using effector cells from three different cows, in which it was examined.

The cell line D17-pZipNeo-gD was used to demonstrate cytotoxicity directed against gIV. Table 13 presents data representative of cytotoxicity observed in 8 of 15 experiments using effector cells from five different cows. The observed responses in all experiments that examined cytotoxicity directed against gIV ranged from 0.5 times background (Control) cytotoxicity to 8.8 times background level. Concurrent responses against BHV-1-infected D17 cells ranged from 9.5 to 14.2 times background cytotoxicity.

Although cytotoxicity directed against the transfected cell lines could not be demonstrated in every experiment, responses were observed in a majority of the experiments. Other than in the case of responses directed against gIII, representative experiments were presented in order to avoid the confusion encountered when averaging data from many experiments using cells from several animals with large variations in the responses against both control and transfected cells.

Cytolytic T lymphocytes recognize BHV-1 in a genetically restricted, antigen-specific manner.
Genetic restricted killing of BHV-1-infected cells. In mice (58) and humans (59), CTL activity against herpes simplex virus (HSV) has been concluded to be not only antigen specific but also genetically restricted. In contrast, early experiments in cattle examining CTL activity against bovine herpesvirus 1 concluded that CTL activity functions in a genetically nonrestricted manner (60). Using cloning methodology now currently available, it was important to discern whether cattle possessed MHC restricted killing of BHV-1 or whether MHC nonrestricted cytolytic killing occurred as reported in other species with herpesviruses (61). To determine whether BHV-1 immunized cattle have CTL directed against the virus (Cooper strain), T cell clones were assessed for reactivity to virally-infected autologous target cells as shown in Table 14. Nine of 12 clones possessed cytolytic activity against BHV-1-infected cells at an effector to target ratio of 1:1. Clones were cytolytic for virally-infected cells but not noninfected cells with lysis ranging from 20 - 58%. Three clones had either low or no cytolytic activity. These results are consistent with findings from bulk culture maintained bovine cells that CTL can kill virally-infected cells, data not presented. However, the findings in Table 14 do not demonstrate whether the cytolytic cells are genetically restricted or function as lymphokine activated killer cells capable of killing cells infected with other viruses. To address this question, 6 clones were assayed on autologous as well as allogeneic BHV-1-infected Con A-stimulated blast cells. Results in Table 15 indicate a dose dependent cytolysis of BHV-1-infected autologous target cells (BoLA MHC class I w6/11) but not BHV-1-infected allogeneic target cells (BoLA MHC class I w20/-). MHC restricted killing was demonstrated with all clones tested. These findings support the concept that bovine CTL function in a genetically restricted manner in killing herpesvirus-infected cells.

Specificity of CTL clones for BHV-1. Kinetics of viral glycoprotein expression on infected target cells was determined by flow cytometry using a cocktail of 28 monoclonal antibodies with specificity for three glycoproteins of BHV-1 (180K/97K, 150K/77K, and 130K/74K/55K),

Table 16. Con A-stimulated blast cells from 2 different animals infected with virus produced maximal glycoprotein expression between 8 and 10 hr after infection. These data indicate the optimal period for BHV-1 glycoprotein expression as detected by monoclonal antibodies and the most likely time for assessing CTL activity against expressed viral glycoproteins. Separate cultures infected with pseudorabies virus expressed pseudorabies virus glycoproteins in a similar time period as indicated in Table 16.

Specificity of the T cell clones for viral determinants of BHV-1 but not BHV-2 is shown in Table 17. Ten of the 11 clones killed only BHV-1 infected autologous target cells. Cytolysis ranged from 10 to 35% at an effector-to-target ratio varying from 1:1 to 4:1. Clone E7 which had previous cytolytic activity against BHV-1-infected autologous cells was unable to kill. BHV-1-specific T cell clones were unable to recognize determinants present on an additional herpesvirus, pseudorabies virus, Table 18. Lysis of BHV-1-infected autologous target cells ranged from 4 to 20% at effector-to-target ratios of 1.5:1 to 3:1. Low to no lysis by the CTL clones was observed on BHV-1-infected allogeneic target cells, reconfirming the genetic restriction of the clones. These data support the idea that bovine CTL clones are antigen specific. Further, these results suggest that BHV-1 does not contain epitopes recognized in common with other bovine herpesviruses.

Helper T lymphocytes recognize BHV-1 proteins and peptides produced by cell-free translation.

Cell-free translation of RNAs using rabbit reticulocyte lysate. After establishing by Northern blot and sequencing analysis that our constructed RNAs were in fact chimeric sequences between the EMCV 5' NCR and each of the BHV-1 genes, we next addressed whether the transcribed RNAs could produce appropriate protein products. Each recombinant viral RNA was translated using a cell-free RRL, then analyzed by SDS-PAGE and autoradiography. As shown in Fig. 21, all three RNAs directed synthesis of the expected proteins as predicted by their nucleotide sequences. Minor protein bands appeared in the translation samples in addition to the anticipated products. This is a phenomenon not uncommon to in vitro protein synthesis and is believed to be the result of premature translational termination. Actin, a cell protein common to RRL, appeared in many of the translated samples. Immune precipitation was used to demonstrate that each of the engineered RNAs had indeed translated properly, and that the synthesized proteins were actually analogous to those authentically produced by BHV-1 (data not shown).

Lymphocyte responses to translated proteins. To determine whether bovine lymphocytes specific for native viral antigens could recognize and respond to the RRL translated proteins, two-fold serial dilutions of each protein were incubated with lymphocytes primed against UV-inactivated BHV-1. As demonstrated in Fig. 22, each of the cloned BHV-1 proteins specifically stimulated primed lymphocytes to varying degrees of responsiveness as assessed by ³H-thymidine uptake. For gl, gIII and gIV translated samples, maximum thymidine uptake was 5.3, 18.8 and 15.2-fold higher, respectively, than control samples containing mock translated RRL alone. In addition, the lack of a response in a sample which had translated the EMCV-derived LVPO protein demonstrated the antigen specificity of these cells. The less than maximal response observed when adding 3 ul of RRL was a result of proliferation

inhibition by the reticulocyte lysate. This could be demonstrated by adding mock translated lysate at 3 μ l in both viral and test samples. The proliferative response observed was dependent upon the quantity of translated lysate added to the assay sample. Similar responses have been observed by others using affinity-column purified gI, gIII and gIV, and freshly isolated PBM. (K. O'Reilly, unpublished observations). Therefore, we believe the responses by our cultured lymphocytes to recombinant proteins correlate well with those observed using fresh PBMs and native viral proteins.

Lymphocyte specificity to translated protein. To further explore specific T lymphocyte responses induced by the individual engineered proteins, cell specificity to pE5GPIV synthesized protein was evaluated. This analysis would also dismiss the possibility that the responses observed in Fig. 22 were due simply to the engineered proteins acting as mitogens. Our interest in further examination of this protein was based on its previously established immunological prominence in viral infections with the HSV analog of this protein (gD) in both human and murine models (62, 63). Lymphocytes previously primed to UV-inactivated BHV-1 were stimulated with the protein product translated from pE5GPIV mRNA, then allowed to achieve the resting stage after proliferation. These cells were then evaluated for their ability to respond to each of the three RRL translated BHV-1 proteins (Fig. 23). pE5GPIV protein was the only one of the three engineered antigens that significantly stimulated 3 H-thymidine uptake by these cells. Similar results were obtained with pE5GPI and pE5GPIII in analogous experiments. In addition, none of these RRL translated proteins stimulated a T lymphocyte cell line previously primed to, and specific for *Brucella abortus* antigens (data not shown). These experiments demonstrated that lymphocytes responded specifically to proteins generated in the RRL, and that the recombinant viral protein products did not function as mitogens.

Selective truncation of gIII allows the identification of T lymphocyte epitopes. Having established that each of the three engineered BHV-1 proteins could be specifically recognized by a previously primed T lymphocyte line, we next wanted to determine which regions of the proteins were involved in stimulation. Since each DNA sequence has a unique pattern of restriction enzyme digestion sites, a panel of similar RNAs that differ in length can be generated by restriction enzyme digestion of the plasmid DNA prior to transcription with T7 RNA polymerase. Using this strategy, eight different RNAs of BHV-L gIII were produced (Fig. 24). As before, in addition to the specific gIII sequences, each RNA contained the 5' NCR of EMCV and thus allowed translation in our cell-free RRL. Therefore, a panel of eight peptides were produced, the length of each being determined by the encoding RNA. All peptides were related in that each contained an identical N-terminus, only differing from the preceding one at the carboxyl-terminal end. As demonstrated in Fig. 25, two epitopes on gIII can be detected by culturing each of the peptides with the T lymphocyte line. The first epitope, detected by peptide six is contained within an 88 amino acid region; while the second is within an 84 amino acid region of peptide eight. Differences in proliferation between the peptides was assessed using the independent student test ($p < .001$). Extensive analysis of these regions in an identical manner using different restriction enzymes will further define these epitopes. In fact, we have used this method to identify a single 20 amino acid epitope within

BHV-1 gIV (data not shown). Further analysis of gIII may disclose more than one epitope within each of the responding regions.

Flow cytometric analysis of cultured lymphocytes. Finally, to examine the phenotype of our BHV-1 specific lymphocyte cell line, flow cytometric analysis was performed. Three days following stimulation with UV-inactivated virus, cells were stained for their expression of CD2, CD4, or CD8 molecules. As shown in Table 18, 99.5% of the cultured cells were CD2 positive, while 91.9% were CD4 positive. The portion of cells expressing the CD8 phenotype was not significant when compared to the control antibody, P3X63Ag8. Therefore, the responding lymphocytes in our cell line that could recognize both BHV-1 and genetically engineered proteins from this virus were of the T helper phenotype.

DISCUSSION

These studies have investigated cattle immune cells capable of responding to the proteins of bovine herpesvirus-1. To identify the immune cells monoclonal antibodies were produced and characterized. Assays were developed to assess immune cell proliferation and lytic activity following the recognition of the virus. Lastly, the contribution of individual viral proteins was studied by transfecting cells with individual viral genes or producing proteins or peptides from the genes.

Monoclonal antibody production and characterization.

Three molecules on the surface of bovine T lymphocytes, CD2, CD5 and an unclustered molecule identified by MAb 8-10C5 were characterized using monoclonal antibodies prepared in our laboratory. The bovine CD2 and CD5 have biochemical characteristics comparable to those of their human counterparts based on size, glycosylation and charge. These molecules are also important in T cell interactions as they block proliferation to alloantigen and virus.

These results indicate bovine CD2 has tissue distribution nearly identical to that of the human CD2 (64), found on all T4+ cells and most T8+ lymphocytes but absent from B lymphocytes. Surface concentrations of the molecule were comparable to those on the human CD2+ cells, where 20,000 molecules/cell on resting T lymphocytes increases to 200,000 molecules/cell following activation with mitogens or antigens (65) compared with 30,000 and 100,000 for cattle, respectively. These differences can be accounted for by the purity of lymphocyte populations.

The biochemical characteristics of CD2 are also similar to those reported for the human molecule. The native 60 kd molecule is slightly larger than the human (50 kd), but has near identical glycosylation (20%) (65), making the peptide backbone larger as well. The murine molecule is a more heavily glycosylated, smaller peptide (55 - 66 kd and decreases to 35 kd following treatment with endo-B-acetyl-glucosaminidase F) than the human. These results suggest that the bovine molecule may be more closely related to the human molecule. This will be confirmed when the sequence of the molecule is known.

Functionally, the bovine CD2 behaves similarly to the human CD2, having a role in proliferation to alloantigen and virus. Interestingly, both of our antibodies appeared to be T11_i equivalents. Both blocked E^{EAT} rosette formation and the binding of each other in fluorescence studies. However, these antibodies do not bind identical sequences. MAb 8-1E3 bound caprine PBMC and 16-1E10 was far superior in its ability to block proliferation. These antibodies probably bind different epitopes in the T11_i functional determinant. The inability of these antibodies to block in a cytotoxicity assay is also of interest. The T11_i determinant of the CD2 molecule is known to function as an adherence epitope in these assays through its ligand LFA-3 on the target cell; however, our antibodies were unable to block CTL activity. Other adhesion molecules are known to increase following activation (e.g. LFA-1, MHC class I and MHC class II), these adhesions mechanisms may be able to overcome the MAb blocking of CD2 in these assays if the CTL cells are highly activated. However, the possibility that the bovine CD2 is functionally different cannot be eliminated.

The tissue distribution of bovine CD5 is similar to its human equivalent (64). In this study, we found the CD5 molecule was expressed at different levels on CD4 and CD8 cells but also at much lower levels on peripheral blood CD2⁺ IgM⁺ lymphocytes when compared to CD4 and CD8 T lymphocytes. While the absolute number of these of these nonT/nonB lymphocytes varies from individual to individual, all cattle tested possess this "dim" population. Howard et al., (66) reported that the numbers of "dim" cells dropped with age relative to the "bright" population, but we found wide variation between animals regardless of age, although we did not test animals under 6 months of age. CD5^{dim} appears to be a good marker for bovine "null" cells and characterization of this population is under investigation.

Biochemically BoCD5 (65 kd) is also very similar to the human (67 kd; 67), rat (69 kd; 50) and murine (67 kd; 68) analogues. The BoCD5 is glycosylated at levels near that of the human molecule (16%; 67). The human and murine both contain 491 amino acids and the molecules have 63% identity, however the identity of BoCD5 to either molecule has not yet been determined.

The CD5 molecule is known to be polymorphic (69) and involved in sustaining T lymphocyte activation (70, 71) this has not yet been determined for BoCD5. However, while our antibody bound the same percentage of PBMC in all animals, it was variable in blocking proliferative function. Charmot *et al.*, al. (72) reported discordant blocking with three of the 5 MAb he tested in MLR. This functional variability may be due to polymorphism in the BoCD5 molecule; the MoAb 8-3F4 may be binding its respective epitope more strongly in some animals than others. Also, 8-3F4 enhanced proliferation to lectin when added at high concentration to mitogen stimulated cultures, confirming that BoCD5 has a role in activation. Increased proliferation induced by MoAb to CD5 in the rat (50) and human (71, 73) has been correlated to increased levels of IL-2. This has not been examined in the bovine system.

The molecule identified by the antibody 8-10C5 is highly conserved between species and is present on peripheral T and B lymphocytes, monocytes and granulocytes, but absent from immature thymocytes. It binds a heterodimer under reducing and non-reducing conditions, the α chain having a MW of 120 kd and the β chain having a MW of 90 kd.

MoAb 8-10C5 was unable to induce proliferation alone or in the presence of PMA or rabbit anti-murine IgG but inhibited proliferation to alloantigen and virus. Taken together, these results suggest that this molecule is involved in T lymphocyte activation, but the epitope bound by this antibody is not involved in signal transduction; this molecule may function in some adhesion event. This antibody was submitted to the First International Workshop on Lymphocyte Differentiation Antigens of Cattle and Sheep and was unclustered with any of the other antibodies submitted. There were two molecules reported in the Third International Workshop on Human Leucocyte Differentiation Antigens (1986) that were found on T cells, B cells and granulocytes but were not found on immature thymocytes: CD44 (Pgp-1) and the unclustered molecule 2H1 (74).

The 2H1 molecule, unclustered in the Third or Fourth International Workshops is found on human T lymphocytes and less than 10% of thymocytes (74). This molecule is involved in T cell activation, stimulating cells in the presence of PMA (74, 75). Biochemically this molecule is a heterodimer with a MW of 140 kd and 105 kd under

reducing and 120 kd, 90 kd under non-reducing conditions (74). It is not known if 2H1 binds the α or the β chain. Unlike 8-10C5, both chains of this molecule have acidic pI of near 4.5 (74). Based on the pI of the β chain and the inability of 8-10C5 to stimulate cells in the presence of PMA, it is unlikely that 8-10C5 is the bovine equivalent of 2H1.

The murine Ly24 (Pgp-1; human CD44) is expressed on most leukocytes, but is present on only 5-10% of adult thymocytes (76, 77). The surface concentration of this molecule increases following activation (78). CD44/Ly24 is highly conserved; MoAb cross-reacting between unrelated species (79). These results are consistent with the distribution of 8-10C5, but 8-10C5 did not cluster with MoAb 25-32 to the cross-reactive ovine Pgp-1 equivalent produced by Mackay *et al.* (79) in the International Workshop. Also, 8-10C5 precipitated a heterodimer compared with the single 95 kd peptide precipitated by MoAb to Pgp-1 in the human (77) and sheep (79).

In summary, the BoCD2 and BoCD5 are similar to the equivalent human and murine molecules based on the tissue distribution, function and biochemical characteristics of the molecules. Although a human or murine equivalent of 8-10C5 has not been defined, this analogy may be true of the 8-10C5 molecule as well. DNA or protein sequence information will elucidate the relationship of the bovine T cell molecules to those of other species as well as suggest information regarding their function. The biochemical and functional information provided in this study will aid in this characterization.

CD5^{dim} peripheral blood lymphocytes proliferate to exogenous IL-2 in the absence of antigen and kill in an NK-like manner.

Others have reported high percentages of CD5^{dim} lymphocytes in the peripheral blood of cattle (80). In addition, Mackay *et al.* (14) reported that T19 lymphocytes coexpress CD5 at low levels. However, no information is available regarding the ability of these cells to respond to antigens, cytokines or exhibit cytotoxicity. Therefore, we examined the functional capabilities of these cells.

To determine the function of small populations of lymphocytes expressing a particular phenotype most studies have used cloned cells. However, cloning of lymphocytes from cattle has been difficult. Problems have included loss of specificity (81) and difficulty in freezing and long term maintenance (82). Also, it is uncertain whether CD5^{dim} T19⁺ cells constitute a heterogeneous group of cells with varied functions or a particular cell lineage. Cloning may bias the interpretations regarding these populations. For these reasons, we examined a population CD5^{dim}-enriched lymphocytes from PBMC.

The large numbers of cells required for functional analysis and phenotyping of non-cloned lymphocytes made fluorescence activated cell sorting and panning techniques for positive selection impractical. We had observed that BoCD2, BoCD4 and most BoCD8 cells coexpressed CD5^{bright}. Therefore, to isolate CD5^{dim} lymphocytes we used a negative selection method comprised of anti-BoCD2 and anti-MHC class II antibodies and complement to enrich for CD5^{dim}/non-B lymphocytes.

As in the whole PBL population, there was wide variation between animals in the phenotype of the CD5^{dim}-enriched lymphocytes. CD5^{dim} lymphocytes increased from 10-

30% to 65-85% following enrichment. Unexpectedly, we observed a low level (<20%) of the cells in this population that expressed lower levels of CD2. The low level of CD2 on the surface of these cells probably accounts for their resistance to lysis, but they were CD5^{dim} and did not coexpress BoCD4, BoCD8, BoCD11c or T19 (data not shown). In most isolations, 50% of the CD5^{dim} lymphocytes were T19⁺, however a large population of CD8⁺ (16-36%) were present as well. A study with a single animal (759S) showed a small population of CD8⁺ T19⁺ (3%) was present in this enriched population (data not shown). Mackay *et al.* (51) also reported that a subpopulation CD8⁺ T19⁺ lymphocytes could be isolated from afferent and efferent lymph of sheep. The enrichment procedure also yielded a CD5⁻ IgM⁻ population (15-36%) in all animals. Together, this data shows that CD5^{dim} -enriched lymphocytes are a heterogeneous population.

Functionally, we found the CD5^{dim} population proliferated in response to ConA and bov rIL-2 but was unresponsive to alloantigen or UV-inactivated BHV-1, and addition of antigen presenting cells did not restore the response. This is in contrast to reports that some human γ/δ TCR bearing T cell blasts are generated in an allogeneic MLR (83). However, those studies were conducted using undepleted murine lymph node lymphocytes, and the interaction of lymphocytes is a complex balance of cells and lymphokines. We examined a fraction of the whole population, and presently, we can not eliminate the possibility that an additional accessory cell population or vital cytokine was missing in the CD5^{dim} -enriched lymphocytes.

The proliferation of the CD5^{dim} -enriched lymphocytes to ConA and bov rIL-2 was enhanced by adherent cells. A cytokine, such as IL-1, produced by the adherent cells could augment the response of these cells to ConA or IL-2. A second possibility, based on the heterogeneity of the CD5^{dim} -enriched lymphocytes, is that another previously unstimulated population was activated. CD5^{dim} -enriched lymphocytes activated with rIL-2, and to a lesser extent, the smaller, non-lymphoblast cells, had a CD5^{dim}, T19⁺, CD11c⁺ phenotype. These cells were not induced to express CD2, suggesting they were not immature T lymphocytes. Further, CD11c expression was induced on these cells. CD11c, a member of the LFA family of molecules, is present on fresh monocytes and a population of CD2⁻, IgM⁻ lymphocytes in all species examined including cattle (Eskra, O'Reilly and Splitter, submitted). This molecule is present on freshly isolated NK (17, 84) and T cell clones (85, 86). Analysis with T cell clones suggests CD11c is involved in conjugate formation with the target cells (86); however, the role of CD11c in NK lysis is controversial (84). The absence of BoCD11c prior to stimulation, suggests that the precursors are not fully differentiated NK cells and that this molecule maybe involved in the function of these CD5^{dim} -enriched lymphocytes. Gray and Horwitz (87) reported that only lymphocytes bearing the related molecule CD11b (Mac-1, OKM1) proliferated to IL-2 in the absence of antigenic stimulation and developed lymphokine activated killer (LAK) activity and that these cells could be either CD5⁺ or CD5⁻. Additionally, Grant, Merchant & Hall (88) reported that CD11c appears on the surface of LAK cells following stimulation with IL-2.

Human γ/δ TCR bearing lymphocytes activated with IL-2 have been reported to kill in an MHC non-restricted, NK-like manner (55). Additionally, LAK cells can kill a broader

range of targets in an MHC non-restricted manner. Since the phenotype of the proliferating lymphocytes was $T19^+$ ($\gamma\delta$ TCR $^+$), CD11c $^+$ we examined the ability CD5 dim -enriched lymphocytes to kill in an assay measuring NK-like and LAK activity. During the isolation of CD5 dim -enriched lymphocytes, the ability of PBL to lyse in an NK-like assay was removed by passage over Sephadex G-10. Following culture in the presence or absence of exogenous IL-2, the CD5 dim -enriched lymphocytes from one animal were able to kill virally-infected D17, and uninfected D17 at a much lower level (Fig. 10). Since CD5 dim -enriched lymphocytes from this animal had higher levels of CD2 $^+$ and CD4 $^+$ cells, it is possible that IL-2 was endogenously produced by a subpopulation of cells. In all three animals tested, cultured CD5 dim -enriched lymphocytes were very active, exhibiting >20% cytotoxicity even at effector to target ratios of 1:2 or less (Table 8). Culture supernatants from these cells had no effect on either target. Together, these results suggest that a population(s) present in the CD5 dim -enriched lymphocytes is capable of MHC non-restricted killing. Also, while exogenous IL-2 was not required for cytotoxicity, culture supernatant failed to lyse virally-infected target cells suggesting cells, not cytokines, are responsible for the killing observed.

In conclusion, we feel that in the absence of lymphocyte clones, enrichment is a useful means of examining subsets of lymphocytes from peripheral blood. Although the CD5 dim -enriched population was heterogeneous, we demonstrated that the CD5 dim T19 $^+$ population proliferated to IL-2 in the absence of antigen and was driven to express the BoCD11c molecule on its surface. Further, CD5 dim -enriched lymphocytes were able to kill in an MHC-nonrestricted manner; however, this activity could not be assigned to a single phenotype. Additional studies are required to identify the population(s) responsible for cytotoxicity. Starting with this CD5 dim -enriched lymphocyte population, fluorescence activated cell sorting may be possible, yielding information on the phenotype of the cytotoxic effector.

Characterization of Natural Killer-like activity against BHV-1.

Freshly isolated or overnight cultured adherent and nonadherent PBM cells from immune or nonimmune animals lysed BHV-1-infected xenogeneic target cells and not uninfected target cells. BHV-1 major cell surface glycoproteins, gI, gIII and gIV were expressed during the 20 hr assay. Organ distribution of cattle natural cytolytic activity was similar to NK activity in human (89), mouse (90), rat (91), and dog (92). All have high natural killing activity in cells obtained from peripheral blood and spleen and low activity in bone marrow cells. Also similar to cattle, no NK cell activity was found in mouse (90) or rat (91) thymus cells. However cattle lymph node cells had high lytic activity while little or no NK activity was detected in mouse (90) or rat (91) peripheral lymph nodes.

In contrast to other species, bovine naturally cytolytic cells failed to lyse tumor target cells alone. In the present study bovine effector cells failed to lyse A549, K563, YAC-1 or allogeneic fibroblasts that were nonvirally infected. Others have failed to observe the lysis of YAC-1 target cells by bovine effector cells (93); however, bovine macrophages can kill YAC-1 cells (94). This would support our observation that the nonadherent effector cell in the present study is not a macrophage.

Lysis of herpes simplex virus 1 (HSV-1)-infected target cells by an adherent, monocyte/macrophage enriched population has been documented in human (95) and mouse (96) systems. Bovine peripheral blood monocytes lysed BHV-1-infected fibroblasts (94). Consistent with the results of this study, lytic activity was not dependent on age or immune status of the animal (94). However, others (97) detected lysis of BHV-1-infected bovine testicular cells only with adherent effector cells from immune or hyperimmune animals. With effector cells from nonimmune animals, there was no lysis of BHV-1-infected target cells (97). The same workers found adherent cells from PI-3 nonimmune animals lysed PI-3-infected bovine embryo (BE) cells, Georgia bovine kidney (GBK) cells and bovine testicular (BT) cells (98). These conflicting results are difficult to resolve, but may be due to differences in isolation of effector cell populations, cell surface determinants on target cells or in vitro culture conditions.

Bovine natural cytolytic activity by NA cells against virally-infected cells has been detected in other systems. Nonadherent, nonphagocytic PBM cells from PI-3 nonimmune cattle lysed PI-3-infected BE cells, GBK cells and BT cells (98). Also, nonadherent, nonphagocytic PBM cells from nonimmune cattle lysed fetal lamb kidney cells persistently infected with bovine leukosis virus and expressing bovine leukosis viral antigens on the cell surface (93). However, nonadherent, nonphagocytic PBM cells from BHV-1 nonimmune cattle did not lyse BHV-1-infected GBK cells or BT cells (98). These results differ from the findings obtained in the present study where NA cells from BHV-1 nonimmune animals lysed BHV-1-infected cells.

Treatment of the nonadherent population with mAb and C' allowed the characterization of the effector cell population. When B cells, T cells, small null lymphocytes and activated esterase positive monocytes were removed, lytic activity was maintained. This indicated the NA effector cells mediating lysis of BHV-1-infected A549 target cells were large null cells. Additional evidence for this interpretation was provided by the results from percoll density gradients results. The effector cells were large, low density mononuclear cells, with no lytic activity in the high density (P40) fraction.

Differentiating between a large null lymphocyte and an immature monocyte is difficult. There is evidence for both cell types mediating natural cytolytic activity in various species. Macrophages, from the spleens of cyclophosphamide treated mice, mediated natural cytolytic activity and were characterized as low density, nonadherent, nonphagocytic and in an early stage of differentiation (97). Similarly, NK cells in human (98), mouse (99), rat (91), and dog (92) are described as nonadherent and nonphagocytic but determined to be low density lymphocytes.

NK cells are phenotypically a very heterogeneous population (98). Although some human NK cells express CD2 (sheep erythrocyte receptor), the majority of NK effector cells are non T, non B, CD3⁻ CD16⁺, and Leu 19⁺ lymphocytes (100). Monoclonal antibody 1E3 identifies the CD2 equivalent molecule on bovine T cells (101). Bovine PBM cells identified by mAb 1E3 are a subset of the CD5 population (Lyt 1⁺) recognized by mAb 3F4 (K. O'Reilly, personal communication). Although some mouse NK cells reacted with a MAb to Lyt 1 (102) the major mouse effector cell with NK activity is an Lyt 1⁻ Lyt 2⁻ asialo G_{m1}⁺ cell (100).

Results in the present study indicated natural cytolytic activity was present in populations depleted of CD5⁺ cells (with MAb 3F4 and C'). The natural cytolytic population(s) detected in these studies have many characteristics in common with NK cells. Until MAbs to bovine immature monocytes and NK cell surface determinants become available, it will be difficult to classify this natural cytolytic activity.

NK-like activation does not require late viral glycoproteins.

The requirement for cell surface viral glycoprotein expression is important for NK cell and monocyte mediated lysis of virally-infected cells in a variety of systems. Findings of the present study demonstrate that late viral proteins of BHV-1 including one of the major envelope glycoproteins, are not required for NK cell lysis of target cells. In contrast, human NK cell mediated lysis of HSV-infected cells required late viral glycoprotein (gC) expression as well as early HSV-1 glycoproteins (gB and gD) (103). Evidence for this conclusion was elucidated using viral glycoprotein mutants to infect target cells or drug treated target cells (103), and using mAbs to HSV-1 gB or gC to block lysis of HSC-1-infected target cells (104). These findings which indicate the importance of the late HSV-1 gC differ from our findings regarding the late BHV-1 gIII (gC).

Similar to results in the present study, human NK cell lysis of human cytomegalovirus-infected cells required expression of early proteins but not late proteins (105). PAA treated target cells expressed BHV-1 early glycoproteins I and IV but not late gIII confirming earlier work by Ludwig & Letchworth, (31). These PAA treated target cells in the present study were lysed at only slightly lower levels than untreated controls, indicating target cells were recognized and lysed in the absence of gIII. Although PAA treated cells expressed glycoproteins I and IV below control levels (Fig. 16, panel A), the treated target cells were lysed at levels near controls (Fig. 16, panel B). These findings suggest a late glycoprotein expression is not required for target killing to occur.

Target cells lysed in the present study were BHV-1-infected xenogeneic and allogeneic cells and PI-3-infected xenogeneic cells. The target cell A549 is resistant to human NK cell lysis (106) and bovine NK cell lysis (Fig 11), but as shown in the present study, when these cells are virally infected with BHV-1, cytolysis can occur by bovine NK cells. Therefore, lysis is dependent on viral proteins or viral alteration of the host to permit cytolysis. Our data support the participation of early viral proteins in this process.

BHV-1-infected target cells expressed very low levels of early and late viral cell surface glycoproteins I, III and IV and little or no lysis was observed when treated with tunicamycin (data not presented). Results of others indicate tunicamycin blocks the transport of all viral glycoproteins to the cell surface of BHV-1-infected Georgia bovine kidney cells (107). However, it is not entirely clear that tunicamycin affected only viral protein expression in the target cells. When K562 cells (the human NK cell target cell) were treated with tunicamycin, lysis by large granular lymphocytes or natural killer cytotoxic factor was significantly reduced (108).

Both NK cells (109) and activated monocytes (110) mediate lysis of virally-infected cells. However, the contribution of target cell lysis by monocytes and adherent NK cells requires

further distinction. Human NK cells lysed HSV-infected human embryonic lung fibroblasts in a 4 hr assay (103). If the assay time was increased to 18 hr, the level of lysis increased three-fold. Human monocytes lysed HSV-infected fibroblasts, with lysis increasing as assay time was increased from 4 to 24 hr (111). Similar results were attained in the present study, target cells infected with virus for 2, 7, or 12 hr were cultured with effector cells in a 20, 15 or 10 hr assay, respectively. As with HSV-infected target cells, the longer the assay time the greater the killing. These same results were obtained with freshly isolated PBM cells and with overnight cultured NA cell population. Since the effector cells were exposed to the same level of viral glycoprotein expression on the target cells, these results suggest effector cells were activated, had differentiated or matured during culture, or a regulatory population was depleted.

To further analyze activation requirements, lysis by freshly isolated effector cells and overnight cultured NK and monocyte populations against 22 hr infected target cells was determined in a 4 hr assay. There was no lysis by freshly isolated effector cells, but cultured effector cells lysed virally-infected target cells indicating that culturing overnight could induce activation of both NA and adherent cell populations. Similar to results in the present study, in both human (112) and mouse (113), NK cells were activated during the assay to kill virally-infected cells.

In summary, patterns of lysis were consistent whether NA or adherent cell populations of freshly isolated cells or overnight cultured cells acted as effector cells. Target cells were lysed when early gI and gIV were expressed and the late gIII was not present. To further address the importance of individual glycoproteins on target cells required for effector cell recognition, studies using BHV-1 glycoprotein genes transfected into target cells will be required.

NK-like cell responses against cell lines expressing recombinant BHV-1 glycoproteins.

The results presented indicate that two of the three major surface glycoproteins of BHV-1, gI and gIV, induce cytotoxic responses by bovine NK cells. This study is in agreement with earlier work indicating that bovine NK cells respond against virally-infected cells (114), and the presence of late glycoproteins, e.g., gIII, are not required for NK cell lysis (26). Further, the present study, using genetically engineered cells expressing single BHV-1 glycoproteins establishes the importance of these molecules to NK cell cytolysis in the absence of viral infection and its concurrent effects on the target cell.

The mechanisms of target molecule recognition by NK cells is not well understood. Studies using target cells expressing recombinant viral proteins and cytotoxic T lymphocytes have demonstrated the importance of antigen participation (115). Considering the role of each glycoprotein in the interaction between virus and target cell, it seems likely that the immune response against BHV-1 would focus on gI and gIV rather than gIII.

gI of BHV-1, like gB of HSV, is the molecule used by the virus for attachment to target cell membranes (42). gIV, like gD, facilitates penetration of the virion into cells (116, 117). gIII, just as gC, has not been shown to play a role in target cell infection (114). It is

easy to speculate that the gI or gIV molecules could play a further role in the target-effector cell interaction by stabilizing conjugation between the two cells. Further studies using cloned effector cells or isolation of the viral attachment molecule(s) on host cells would contribute to understanding how viral glycoproteins function in this process.

Previous studies have concluded that participation of the target cell is of primary importance to NK cell lysis of a variety of unrelated viruses, tumor cells and spontaneously transformed cell lines without prior exposure (118, 119). This implies that host cell proteins or interactions between host and viral proteins is central to NK cell recognition and lysis of target cells. Relying on target cell infection, previous experiments have used mutant viruses that lack one or more genes (120), antibody blocking of viral determinants (121) or drug treatments to block protein expression or processing (114, 122). These approaches have examined NK cell recognition and killing when a particular protein is absent, and such methods are hampered by the confounding effects of virus-host cell interactions. The present study also suggests that host cell proteins normally induced by stress, e.g., induced during selection for G418 resistance, contribute minimally to the observed cytotoxicity. As supported in Table 13, the effector cells do not respond against target cells transfected with the vector, pZipNeo, alone. In addition to the viral protein, neomycin phosphotransferase is the only other protein encoded by the plasmids that is expressed in eucaryotic cells. The presence of the transducing elements (LTR) in the plasmids apparently does not induce abnormal synthesis of host proteins that promote NK cell lytic activity as supported by the lack of response against D17-pZipNeo. Therefore, NK cell activity is strongly directed by viral glycoprotein expression on the transfected cells.

The recombinant glycoprotein molecules were evaluated for abundance and fidelity of expression as compared to virally-infected cells. Initially, direct examination of the transfectants by fluorescent microscopy revealed the presence of glycoprotein, but not on every cell. Further scrutiny by flow microfluorimetry indicated that a minority of cells expressed detectable glycoprotein. However, the gate value established to analyse and sort transfected cells lead to an underestimation of the population expressing glycoproteins. This underestimate was due, primarily, to two factors: 1) a broad range of cell sizes and complexities, and 2) nonspecific binding of fluorescein-conjugated secondary antibody to control D17 cells. Heterogeneity in D17 cell size was indicated by forward light scatter (data not shown). Large or more complex cells produce a higher level of autofluorescence than small or less complex cells. Therefore, small glycoprotein-expressing cells may not have exhibited fluorescence greater than the gate value set using uninfected D17 (Control) cells. The influence of these two factors suggests that transfectants considered negative or marginally positive for surface glycoprotein were expressing higher levels than was indicated by the histograms in Fig. 18 and 19.

The immunoprecipitation studies revealed that BHV-1 glycoproteins were expressed on infected D17 cells very similarly to those on infected MDBK cells, of bovine origin. Additionally, gI expression on the transfected cell line was comparable to gI expression on infected MDBK and D17 cells. The gIV molecule expressed on the transfected cell line had a lower M_r , indicating partial glycosylation relative to gIV on infected cells. Glycosylation

may not be an important attribute for recognition of target molecules by bovine NK cells. This is suggested by cytotoxic responses against the fully glycosylated gI molecule that were no greater than those directed against the partially glycosylated gIV molecule on the transfected cells. Expression of gIII transcribed from the same cDNA insert has been previously characterized as similar to native gIII expression (57).

Relative importance of a specific glycoprotein to NK lysis when compared to lysis directed at virally-infected targets is difficult to assess using the present data from 8 different animals over a 13 month period. Variation in lysis levels was less for D17 cells treated with UV inactivated virus or D17 cells infected with live virus when compared with transfected cells, Table 13. At least four factors contributed to the variations observed for responses against the transfected cell lines. First, trypsin treatment may have stripped varying amounts of glycoprotein off the surface of the transfectants. Each of the glycoproteins contain trypsin cleavage sites that would lead to truncation of the surface proteins (43, 44). Cells expressing greater glycoprotein amounts would repopulate their surface glycoprotein more quickly than weak glycoprotein expressing cells. Therefore, manipulation of the transfected target cells with trypsin would increase heterogeneity of glycoprotein levels within the transfected cell line. Second, clinically normal cows exhibit variability in lymphocyte, and perhaps NK cell, populations over time (123, 124, 125). Third, individuals may vary in their response against an agent on different days. Considering the current knowledge regarding the network of lymphocyte and cytokine interactions (126, 127), it is likely that population dynamics could influence NK cell responses on a given day. Individuals functioning at peak physical levels often display marked variation in NK response levels (128). Considering that milking animals were used in these experiments, it is possible that NK cell levels fluctuated during this physiologically demanding period.

Fourth, NK cell lysis of transfected cells may be less than virally-infected cells because these genetically engineered cells present a limited repertoire of antigens for NK cell recognition when compared with virally-infected cells. This limited repertoire theory is supported by the observations that transfected target cells expressing gI or gIV, subsequently infected with live virus were lysed to a greater level than virally-infected D17 cells alone. gIII-expressing transfectants were not lysed at a higher level than control cells and gIII-expressing cells were infected with BHV-1 were not lysed to a greater extent than were infected D17 cells.

Although these studies demonstrate that BHV-1 glycoproteins can stimulate NK cell cytotoxic responses, further experiments are needed to establish the relative importance of BHV-1 proteins, including the contribution of other early (β) proteins (129). Cloning of bovine NK cells and identification of phenotypic markers on bovine effector cells would be instrumental in more clearly addressing effector cell interactions with their targets. The present study provides direct examination of the response against individual proteins using recombinant DNA technology. This approach may prove more pertinent to understanding how cells of the immune system react with viral determinants. This understanding is central to formulation of strategies for protecting animals from pathogenic agents.

Cytolytic T lymphocytes recognize BHV-1 in a genetically restricted, antigen-specific manner.

These results indicate two important findings. First, antigen specific CTL can be derived from peripheral blood of BHV-1 immunized cattle. This is important because a commonly held conclusion, without documented evidence, is that T cell mediated defense mechanisms are more important than those mediated by antibody in controlling BHV-1 infections. Second, the CTL clones that can be produced function in a genetically restricted manner. This evidence supports the role of CTL as a cellular effector mechanism, although other cellular mechanisms, e.g., NK cells, may also participate. By identifying effector mechanisms that participate in killing BHV-1-infected cells, construction of alternative vaccines that stimulate these effector mechanisms is possible.

Antigen specific CTL induction against herpesviruses in domestic animals has received insufficient attention. Previously, cytotoxicity by effector cells was reported against BHV-1-infected cells; however, the same effector cells were cytotoxic against vaccinia-infected target cells (60) suggesting an effector cell other than a cytotoxic lymphocyte. More recently, natural killer cells have been proposed as the effector cell in these previous studies (130). No other studies have adequately addressed bovine cytotoxic T cell effector mechanisms specific for BHV-1-infected cells. Recent attempts by others to detect cytotoxic T cells against BHV-1 have not been successful (131). Instead, these workers identified natural cytotoxic cells that were not genetically restricted and preferentially lysed BHV-1 but not PRV-infected cells. The present study documents the existence of BHV-1 specific cytotoxic T cell clones that can readily destroy virally-infected cells in a genetically restricted manner. The role of these effector cells in terminating established primary and recrudescant infection in cattle has yet to be determined.

HSV-specific CTL play a role in recovery from HSV infections in mice, based on studies in which mouse HSV-specific CTL populations and a CTL clone caused rapid clearance of HSV after adoptive transfer to infected mice (132- 135). Human CTL have been shown to recognize antigens in common between HSV-1 and -2. Potentially, immunization against one virus would provide an effector mechanism against both herpes simplex strains (136). It is, therefore, important to determine if CTL generated to BHV-1 can recognize determinants in common with other herpesviruses that might infect cattle. Data from the work presented here indicates that bovine CTL clones do not recognize determinants shared by other herpesviruses examined. This suggests that CTL clones primarily recognize determinants unique to BHV-1 or that BHV-1 does not contain a large number of determinants in common with other herpesviruses. Antibodies recognize shared determinants among HSV-1, BHV-2 and pseudorabies virus by agar gel immunodiffusion (137) and HSV-1 glycoproteins B and D are antigenically similar to BHV-2 (138). Therefore, considerable conservation of peptides exist between human and certain bovine herpesviruses. However, antisera from BHV-1 seropositive animals fails to identify determinants on BHV-2 and vice versa (139).

T cell recognition of common viral determinants between BHV-1 and HSV has been demonstrated using human peripheral blood mononuclear cells (140). Cells from HSV seropositive but BHV-1 seronegative individuals could proliferate to BHV-1. Therefore, antibody and T cell recognition of determinants in common among different herpesviruses

suggests that bovine T cell clones might be found that recognize several herpesviruses. Such was not the case in the present study. Bovine T cell clones recognize determinants specific for BHV-1 even when assayed against other herpesviruses that naturally infect cattle. The particular viral determinants recognized by the T cell clones have not yet been characterized, but efforts to clone the genes that code for specific viral glycoproteins should address this question. It has been concluded previously that HSV glycoprotein(s) serve as recognition structures for mouse (141, 142) and human (36) CTL, based on studies performed with HSV-infected or glycoprotein specific target cells. Demonstrating antigen specificity and genetic restriction of bovine cytolytic T cell clones are important steps in defining the antigenic requirements of a BHV-1 subunit vaccine that can stimulate cellular effector mechanisms.

Findings in the present study illustrate that genetic restriction by bovine CTL occurs in cattle as has been demonstrated in humans for HSV-1 (136). Recognition of BHV-1 glycoproteins in association with MHC class I or II molecules is not conclusive in the present studies. MHC class II disparity was evident between the animal from which the clones were derived and the animal from which the allogeneic targets were produced because of bidirectional mixed leukocyte reactivity, data not presented. In humans, MHC class II molecule recognition of HSV-1 and -2 has been reported (36, 136). However, a preference for MHC class I molecule recognition for genetic restriction between effector and target cells for killing HSV-infected cells has been reported for mice (58). Demonstration of MHC class I or II restriction by CTL may depend on the method of *in vitro* antigen priming or cloning to select for effector cells. More interestingly, it has been suggested that HSV replicates only in human lymphocytes that express class II antigens (143) and that the predominance of class II restricted CTL may serve as an important defense against infection (36). In cattle, BHV-1 has a preference for epithelial cells and fibroblasts, and *in vitro* BHV-1 will replicate in cells from a wide spectrum of organs (144). The virus binds in high amounts to lectin stimulated lymphocytes suggesting a possible *in vivo* role of proliferating lymphocytes in viral pathogenesis (145). However, we have been unsuccessful in routinely obtaining specific CTL lysis of virally-infected fibroblasts. Therefore, if bovine CTL have an important protective role *in vivo* it is presently not clear if MHC restriction would be class I, class II or both.

The methods used here should provide the basis for identification of viral glycoprotein antigens important as major foreign determinants present on infected cells. Vaccines developed as alternatives to the currently used modified live virus must consider the importance of cell mediated immunity. Production of cytolytic T cell clones specific for selected viral determinants on infected cells should facilitate further identification and characterization of BHV-1 antigens recognized by T cells. Once these viral antigens are expressed in bovine cells either by transfection with cloned viral genes or by using purified viral glycoproteins, it should be possible to evaluate the relative importance of particular glycoproteins to the T-cell repertoire of BHV-1 immune cattle and to examine the role of each glycoprotein in protection against infection.

Helper T lymphocytes recognize recombinant BHV-1 proteins produced by cell-free translation.

In this study, we have described the production of chimeric plasmid constructions which produced RNAs that were capable of being translated in rabbit reticulocyte extracts. Data also indicate that each of the three engineered peptides specifically stimulated T lymphocytes of the CD4 phenotype previously primed by native viral antigens. Manipulation of the synthesized peptide allows dissection of the relevant regions recognized by the responding lymphocytes. To our knowledge, this is the first report of T cell responsiveness to antigens synthesized in cell-free extracts.

We chose to study viral-surface proteins gI, gIII and gIV based on comparative data using HSV-1. With this virus, the analogous proteins, gB, gC and gD were shown to elicit T lymphocyte responsiveness in both human and murine models (62, 63, 146- 148). Additional immunological relevance was demonstrated by showing that immunization with purified gD, (149) or adoptive transfer of HSV-1 specific murine T lymphocytes provided protection from lethal HSV-1 infection (150). The responses we observed in this study with individually cloned BHV-1 proteins produced by cell-free translation, parallel the responses obtained with affinity-column purified BHV-1 glycoproteins (K. O'Reilly, unpublished observations) and with cloned cytotoxic T lymphocytes to BHV-1 (82). Further, our results suggest that T lymphocytes of the CD4 phenotype maybe an important subclass of cells in eliciting immune responsiveness to this herpesvirus. It would be of interest to determine if other T cell subpopulations, expressing different phenotypic molecules, could also respond to proteins produced in this system. Alternatively, it is possible that these particular proteins may contain predominately helper-cell epitopes, or that the manner of antigen presentation may preferentially stimulate cells of the CD4 phenotype. The possibility that our cell population may contain CD4⁺, MHC class II restricted cytotoxic lymphocytes as previously reported in humans with HSV (151) has yet to be investigated.

Relevant to the development of a subunit vaccine effective in controlling primary herpesvirus infections is understanding the role of individual glycoproteins in the induction of various immune components. Efforts in vaccination against analogous HSV infection has concentrated on gD, including the cloning (152) and synthesis of peptides corresponding to neutralization (153) and T cell epitopes (154, 155). However, few studies have compared the cellular responses to isolated herpesvirus proteins, and little is known regarding the T cell epitopes of these proteins beyond gD. The present system offers a practical approach to produce viral proteins and subsequently identify epitopes within the protein. We have demonstrated that three herpesviral proteins can be recognized by the T cell repertoire from its natural host. Further, the methodology of the present study would allow the exploration of epitope recognition frequency among a large number of animals within a population. In the present study, the two epitopes of gIII recognized by T cells provides the basis to explore other proteins using a similar approach and to characterize the responding cell types.

For immune studies, RRL generated protein has a number of advantages over other synthesis techniques. First, the cloned gene product is free of LPS. This result is in direct contrast to bacterial protein synthesis systems such as λ gt11. When lymphocyte responsiveness to the protein product is desired, the absence of LPS is critical due to its known mitogenic properties. Second, superfluous viral antigens are not present as is the case

with vaccinia virus produced-protein. Finally, due to the in vitro nature of this technique, mRNAs can be selectively truncated to produce a spectrum of diverse, but related proteins upon translation. This type of flexibility can allow precise epitope mapping of antigens by testing lymphocyte responsiveness to a panel of specifically truncated proteins of decreasing length. Should this strategy ultimately prove successful, epitope mapping could in the future be performed more rapidly, with little or no subcloning of the gene of interest. In addition, previous studies on T lymphocyte epitope mapping using synthetic peptides or alternative expression vectors have proven costly and labor intensive. This is in direct contrast to the in vitro antigen synthesis described in the present study.

Future definition of T lymphocyte epitopes encoded within each of the BHV-1 proteins may provide important insights for understanding host immunity and disease development. It will be of interest to expand our studies to multiple cell lines derived from different animals to assess the degree of antigenic conservation within a population. Some variation would be expected, much like that observed between strains of mice. We think this novel engineering approach for the production of viral proteins will have broad application to future studies on T lymphocyte recognition and epitope mapping of a wide variety of antigens.

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TABLE I

Bovine herpesvirus-1-specific monoclonal antibodies^a

Designation	Isotype	Specificity	Molecules recognized (M _r)
510604	IgG _{2a}	gI (gB)	133, 74, 55 kDa
290504	IgG _{2a}	gIII (gC)	180, 97 kDa
110604	IgG _{2a}	gIV (gD)	150, 77 kDa
P1.17	IgG _{2a}	None	Isotype Control

^aAntibody suspensions were at 1mg/ml and were diluted 1:100 for cell labelling.

TABLE 2

Monoclonal Antibodies to Bovine Mononuclear Cell Subsets

mAb	MW (kDa)	Reactivity	Reference
H4	32, 28	Human MHC class II framework determinant cross-reactive with the bovine MHC class II DR-like molecule ^a	(12)
C5B6	150, 95	CD11c molecule, monocytes	— ^a
IE3	60	CD2 molecule, pan T lymphocytes	(13)
3F4	67	CD5 molecule (Lyt 1 ⁺), T lymphocytes	(13)

^a A. M. Janzer-Pheil and G. A. Splitter, Identification of two independent MHC class II antigens in a bovine lymphoblastoid cell line. Manuscript submitted.

^b L. Eskra and G. A. Splitter, A 150/95 kDa molecule present on bovine monocytes that participates in antigen recognition. Manuscript in preparation.

Table 3
Summary of the biochemical characteristics of
BoCD2, BoCD5 and 8-10C5^a

Characteristic	BoCD2	BoCD5	8-10C5
MW (glycosylated)	60 kd	65 kd	120 kd (α) 90 kd (β)
MW (deglycosylated)	48 kd	55 kd	117 kd (α) 75 kd (β)
% glycosylation	20%	15%	10% (α) 16% (β)
pI	6.2-6.5	6.4-6.7	6.1-7.0 (α) 4.0-4.6 (β)

a. ¹²⁵I labeled 72 hour ConA lymphoblasts used in all determinations.

Table 4

The effect of monoclonal antibody
E^{AET} rosette formation

MoAb	Specificity	%E ^{AET} Rosettes ^a	% Inhibition
Pl.17	none	56.2±9.1 ^b	0
8-1E3	BoCD2	0.3±0.4 ^b	99.5
16-1E10	BoCD2	1.8±1.8 ^b	96.9
8-3F4	BoCD5	51.5 ^c	8.4
8-10C5	novel	48.5 ^c	13.7

a. Percent of lymphocytes forming rosettes (>3 SRBC) ± standard deviation.

b. n=3

c. n=1

Table 5
Phenotype of CD5^{dim} enriched lymphocytes

antigen	% Fluorescent Cells ^a							
	549M ^b		420A ^c		755G ^d		759S ^e	
	PBL	CD5 ^{dim}	PBL	CD5 ^{dim}	PBL	CD5 ^{dim}	PBL	CD5 ^{dim}
CD5	80	64	66	80	47	85	54	85
CD2	69	9	67	20	47	7	52	6
CD4	33	3	42	13	32	10	30	4
CD8	30	28	23	16	19	36	15	5
CD11c	17	<1	ND ^f	ND	8	2	11	<1
classII	ND	ND	9	11	33	15	22	2
IgM	12	0	10	0	11	3	8	1
T19	9	54	9	34	8	51	18	77

- a. Percent fluorescent cells as determined by flow microfluorimetry, corrected by the percent staining of second antibody (< 10% all cases).
- b. Jersey steer, age 5 yr. These results are representative of 5 experiments conducted with this animal.
- c. Ayershire cow, age 6 yr.
- d. Guernsey cow, age 5 yr.
- e. Brown Swiss cow, age 3 yr.
- f. Not determined.

Table 6
Proliferation to ConA and IL-2 CD5^{dim} Lymphocytes

Days in Culture	Stimulator	Experiment 1 ^a		Experiment 2 ^b			
		- adherent cells		- adherent cells		+ adherent cells	
		PBMC	CD5 ^{dim}	PBMC	CD5 ^{dim}	PBMC	CD5 ^{dim}
3 days	None	943±54	1232±111	3143±446	2698±630	740±156	2576±167
	ConA	550224±25899	422374±27272	160806±10263	243030±6993	161013±3732	282748±13237
5 days	None	2638±510	833±372	4805±837	2679±736	3005±260	4649±549
	rIL-2	123350±4710	296678±20576	12330±826	28065±21086	17429±3322	19994±1070
	allogeneic	12778±2570	3617±789	12795±900	1909±214	8924±2874	3987±1064
	BHV-1	ND ^c	ND	11945±634	4879±285	8939±1531	3499±622

- a. 5 yr old Jersey steer, 2 x 10⁵ cells/well
b. 5 yr old Guernsey cow, 4 x 10⁴ cells/well
c. Not determined.

Table 7

Phenotype of Activated CD5^{dim} Lymphocytes^a

Antigen	Experiment 1 ^b			Experiment 2 ^b			
	day 0		day 3	day 0		day 5	
	Fresh	Untreated	ConA	Fresh	Untreated	rIL-2	
						small ^c	large ^c
CD5	83	83	88	64	64	70	88
CD2	11	20	28	9	3	9	12
CD4	2	8	15	3	<1	1	<1
CD8	23	24	56	28	8	13	21
CD11c	8	15	25	<1	<1	42	89
MHC classII	12	31	64	ND ^d	<1	21	49
IgM	1	9	14	<1	<1	4	3
T19	59	72	83	54	33	53	90

- a. Percent fluorescence as determined by flow microfluorimetry, corrected for background staining of secondary antibody, less than 13% in all cases.
- b. Cells from Jersey steer 549M.
- c. Small and large cells as defined in Fig. 6, panel d.
- d. Not determined.

Table 8
NK-like Cytotoxicity of CD5^{dim} Lymphocytes

Population	Experiment 1 ^a			Experiment 2 ^b		
	E:T	% cytotoxicity		E:T	% cytotoxicity	
		D17	D17+BHV1		D17	D17+BHV1
fresh PBMC	3:1	3 ± 2	13 ± 2	10:1	0 ± 3	25 ± 4
G10 ⁺ PBL		ND ^c	ND	10:1	2 ± 3	-12 ± 3
PBMC (cultured)	1:5	1 ± 2	20 ± 4	8:1	3 ± 2	49 ± 5
PBMC + rIL-2	1:5	11 ± 7	34 ± 8	10:1	-3 ± 4	34 ± 0
CD5 ^{dim} (cultured)	1:10	4 ± 1	9 ± 5	1:2	-2 ± 7	15 ± 1
CD5 ^{dim} + rIL-2	1:5	21 ± 6	49 ± 7	1:2	-3 ± 3	21 ± 5

a. Cells from Jersey steer 549M, cells cultured 21 days.

b. Cells from Brown Swiss cow 759S, cells cultured 11 days.

c. Not done.

TABLE 9

Lysis of BHV-1-Infected A549 Cells by Mononuclear Cells from Fetal and Adult Bone Marrow, Lymph Node, Spleen, Thymus, or Peripheral Blood

Effector cells	Percentage of cells staining with mAb				% Cytotoxicity of BHV-1-infected A549 cells		
	H4 ^a Ia	C5 CD11c	3F4 CD5	IE3 CD2	100:1 ^b	50:1	25:1
From third trimester fetus							
Lymph node	15	3	57	62	25	12	7
Spleen	13	5	48	61	48	33	16
Bone marrow	7	5	3	0	9	1	1
Thymus	9	0	88	83	9	-1	0
From adult animal							
Lymph node	26	8	72	68	13	5	2
Spleen	37	11	35	35	19	5	3
Bone marrow	5	11	0	1	3	0	-1
PBM cells	33	11	67	46	28	17	11
Thymus ^c	ND ^d	ND	ND	ND	4	1	1

^a Monoclonal antibody and its specificity.

^b Effector to target cell ratio in a 20-hr assay.

^c Adult thymus was from a 1-year-old heifer ($n = 1$); the other tissues were from a separate animal ($n = 3$).

^d Not determined.

TABLE 10

Lysis of Virally Infected A549 Cells by mAb and C'-Depleted NA Cells

Group no.	NA effector cells treated with mAb and complement ^a	% Cytotoxicity of BHV-1-infected A549 cells					
		Control ^b			Depleted		
I.	Removal of Ia ⁺ (mAb H4) cells ^c	100:1 ^d	50:1	25:1	100:1	50:1	25:1
	Expt 1	25	17	12	22	14	13
	Expt 2	45	38	24	39	29	18
	Expt 3	50	40	29	40	22	9
II.	Removal of CD5 ⁺ (mAb 3F4) cells ^c						
	Expt 1	25	17	12	31	27	16
	Expt 2	44	36	29	65	52	45
III.	Removal of Ia ⁺ and CD5 ⁺ cells ^c						
	Expt 1	25	17	12	28	28	20

^a Effector cells were obtained from PBM cells incubated overnight on plastic petri dishes to select for the nonadherent (NA) cell population.

^b NA cell population.

^c Analysis of the depleted subpopulation by flow cytometry showed group I < 2% H4⁺ cells, group II < 1% 3F4⁺ cells, and group III < 6% H4⁺ cells and < 1% 3F4⁺ cells.

^d Effector to target cell ratio in a 20-hr assay.

TABLE 11

Lysis of Virally Infected A549 Cells by NA Cells Separated on a Density Gradient

Experiment	Cell population ^a	Percentage of cells staining with mAbs				% Cytotoxicity of BHV-1-infected A549 cells			
		H4 ^b Ia	C5 CD11c	3F4 CD5	1E3 CD2	100:1 ^c	50:1	25:1	12:1
No. 1	Control ^d	5	7	73	71	19	4	1	-4
	P30	9	10	40	36	ND ^e	12	5	4
	P35	10	13	51	40	25	15	6	2
	P40	2	3	78	74	-4	-6	-4	-6
No. 2	Control	3	6	63	53	21	10	4	5
	P30	ND	14	21	ND	ND	ND	ND	5
	P35	8	9	27	29	34	27	9	3
	P40	4	6	67	49	1	0	-1	-2

^a Nonadherent cells from PBM cells incubated overnight on plastic petri dishes were treated with mAb H4 and C' and separated on a stepwise density gradient, including 30% (P30), 35% (P35), and 40% (P40) Percoll.

^b Monoclonal antibody and its specificity.

^c Effector to target cell ratio in a 20-hr assay.

^d NA population treated with mAb H4 and C'.

^e Not determined.

Table 12 PBM cells lyse a variety of BHV-1- and PI-3-infected target cells

Cow no.*	Target cell	% cytotoxicity			
		100:1†	100:1	50:1	25:1
	A549	No virus		+ BHV-1	
755	Allogeneic fibroblasts	7	44	24	9
756		5	41	30	24
715		5	26	11	1
728		1	14	5	6
	D-17				
651		6	71	70	56
658		5	75	71	37
748		3	73	65	39
	A549	+ PI-3			
755		5	18	8	6
756		3	22	12	8

* Freshly isolated PBM cells were used as effector cells.

† Effector to target cell ratio.

TABLE 13
Cytotoxicity^a assays demonstrating responses directed against cell lines expressing recombinant
BHV-1 glycoproteins^b

Target cell	Effector : Target Cell Ratio		
	100:1	50:1	25:1
<u>EXPERIMENT 1</u>			
Control (D17 cells)	4	3	1
D17-pZipNeo-gI	23	16	13
Control + UV-BHV	16	8	3
Control + BHV-1	50	43	28
D17-pZipNeo-gI + BHV-1	94	81	57
<u>EXPERIMENT 2</u>			
Control (D17 cells)	5	ND ^c	3
D17-pZipNeo	8	ND	5
D17-pZipNeo-gIII	9	ND	7
Control + BHV-1	32	ND	26
D17-pZipNeo-gIII + BHV-1	37	ND	25
<u>EXPERIMENT 3</u>			
Control (D17 cells)	8	6	5
D17-pZipNeo-gIV	24	17	14
Control + UV-BHV	43	26	11
Control + BHV-1	53	49	27
D17-pZipNeo-gIV + BHV-1	95	78	48

^aCytotoxicity is expressed as % lysis.

^bTransfectants were cultured without G418 for 48 h. prior to assay. Target cells at 1×10^4 cells/well, were added to 96 well plates, cultured overnight in the presense of ^{51}Cr . Live or UV-inactivated BHV-1 was added 1 h. prior to effectors cells. Target and effector cells were cocultured for 19 h.

^cNot Done.

Table 14 Specificity of CTL clones for BHV-1-infected cells

Clone no.	% cytotoxicity of Con A-stimulated blast cells			
	Animal 686		Animal 686 + virus	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
C3	6.4 ± 0.3	0.6 ± 2.3	25.3 ± 0.6*	31.2 ± 7.4*
D5	7.6 ± 0.8	3.0 ± 1.0	40.0 ± 6.2	14.9 ± 4.1†
C10	3.7 ± 1.4	ND	57.7 ± 9.4	43.0 ± 4.1
F7	4.2 ± 3.3	-1.0 ± 0.5	32.8 ± 3.2	3.1 ± 0.3
G10	4.7 ± 0.2	-2.0 ± 1.2	25.1 ± 4.3	ND
D2	0.3 ± 2.6	ND	32.8 ± 0.6	21.2 ± 2.9
E2	2.4 ± 2.1	0.6 ± 4.1	25.9 ± 1.1	7.8 ± 0.8
E3	2.7 ± 2.5	-0.5 ± 2.2	26.2 ± 4.0	13.3 ± 4.0
E7	5.5 ± 1.6	-2.4 ± 1.1	19.8 ± 3.0	3.4 ± 0.3
E4	2.4 ± 2.9	ND	-2.5 ± 2.3	ND
D7	4.1 ± 1.8	0.0 ± 0.7	7.7 ± 2.4	10.7 ± 0.8
F2	0.8 ± 1.8	ND	3.0 ± 1.3	2.3 ± 1.6
				8.0 ± 3.0
				1.6 ± 0.5
				5.0 ± 1.4
				0.6 ± 1.8
				ND
				18.4 ± 1.0
				4.3 ± 1.9
				ND

* E:T ratio of 1:1, except for clone E2 in Exp. 2 which was at 0.5:1 and 0.25:1. Cultures of effector and target cells were incubated for 6 hr.

† E:T ratio of 0.5:1.

ND, not determined.

Table 5 Genetic restriction of cytolytic T-cell clones

% cytotoxicity of Con A-stimulated blast cells				
Clone no.	686*	686 + virus	679†	679 + virus
D7	0 ± 0.7	18.4‡ ± 1.0 4.3§ ± 1.9	-0.6 ± 0.2	1.0 ± 4.3
D5	3.0 ± 1.0	42.9 ± 4.0 3.1 ± 0.3	0 ± 1.2	-0.8 ± 1.5
F7	-0.9 ± 0.5	21.2 ± 2.9 7.8 ± 0.8	NT	-1.5 ± 1.0
G10	-2.0 ± 1.2	13.3 ± 4.0 9.3 ± 7.4	0.5 ± 1.4	-0.3 ± 1.0
E2	0.6 ± 4.1	10.7 ± 0.8 2.3 ± 1.6	-0.3 ± 0.6	-0.4 ± 0.3
E3	-0.5 ± 2.2	9.6 ± 2.2 1.6 ± 0.5	-0.8 ± 0.4	-3.4 ± 0.6

* BoLA class I 6/11.

† BoLA class I 20/-.

‡ E:T ratio of 1:1.

§ E:T ratio of 0.5:1.

NT, not tested.

Table 16 Expression of BHV-1 and pseudorabies virus glycoproteins on Con A-stimulated blast cells*

Total hr infected	% fluorescence			
	Anti-BHV-1		Anti-PRV	
	Rabbit anti-mouse	+ rabbit anti-mouse	Rabbit anti-swine	+ rabbit anti-swine
12	0.9 ± 0.7	33.7 ± 16.0†	ND	ND
10	1.0 ± 0.0	44.0 ± 12.9	ND	ND
8	0.7 ± 0.8	40.8 ± 17.7	0	22.5
6	0.7 ± 1.0	35.6 ± 14.5	ND	ND

* Con A-stimulated blast cells were infected with BHV-1 or PRV at an MOI of 3 for 2 hr. Cells were washed once and cultured for the indicated times. Following incubation, the cells cultured with BHV-1 were treated with a 1/10 dilution of a cocktail of 28 monoclonal antibodies with specificity for three glycoproteins of BHV-1. Cells cultured with PRV were treated with polyclonal swine anti-PRV antibody. Following a 30-min incubation, fluorescein-labelled rabbit anti-mouse or rabbit anti-swine was added for 30 min at 37°, washed and the percentage fluorescence determined by flow cytometry.

† Results are from two animals.

Table 1. Specificity of CTL clones for BHV-1 but not BHV-2*

Clone	% cytotoxicity of target cells			E:T ratio
	Animal 686	Animal 686 + BHV-1	Animal 686 + BHV-2	
E2	0.7 ± 1.2	12.2 ± 3.9	0.9 ± 1.2	2:1
E3	0 ± 0.9	14.9 ± 5.0	-1.9 ± 1.6	2:1
E7	0.3 ± 0.5	1.8 ± 3.1	1.4 ± 3.1	2:1
D7	0 ± 0.5	15.2 ± 1.2	0 ± 1.5	2:1
D5	-1.1 ± 1.8	34.5 ± 6.6	-0.4 ± 2.3	2:1
E8	-1.9 ± 0.7	14.9 ± 2.1	-0.8 ± 0.4	4:1
D5	-0.3 ± 0.5	22.3 ± 1.4	3.7 ± 2.8	2.5:1
G10	-0.4 ± 0.4	23.0 ± 0.6	-1.0 ± 0.7	2:1
D7	0.7 ± 1.0	17.1 ± 2.2	-0.2 ± 1.6	2:1
D2	-1.9 ± 0.1	10.1 ± 1.8	-1.6 ± 1.2	2:1
E2	-1.4 ± 0.6	17.1 ± 3.8	-2.1 ± 1.7	1.4:1
E3	-1.5 ± 0.6	13.8 ± 3.1	-2.1 ± 0.9	1:1

* Con A-stimulated blast cells were cultured with BHV-1 (MOI of 3) or BHV-2 (MOI of 1) for 2 hr, washed once and labelled with ^{51}Cr . Cultures of effector and target cells were incubated for 6 hr.

Table 1 Absence of cytolytic activity of T-cell clones for pseudorabies virus*

Clones	% cytotoxicity of target cells					E:T ratio
	Animal 686	Animal 686 + BHV-1	Animal 686 + PRV	Animal 679	Animal 679 + BHV-1	
D2	-2.0 ± 1.2	6.4 ± 3.4	-11.3 ± 1.4	ND	ND	2:1
G10	-1.9 ± 1.7	9.1 ± 2.1	-12.2 ± 0.1	ND	ND	2:1
E9	-2.2 ± 0.7	3.6 ± 0.6	-12.6 ± 0.3	ND	ND	2:1
D5	-1.7 ± 0.5	10.3 ± 5.4	-12.9 ± 1.0	ND	ND	1.5:1
E2	1.3 ± 0.9	3.9 ± 1.4	11.3 ± 0.6	ND	ND	1.5:1
E3	-0.2 ± 1.6	11.2 ± 3.7	0.9 ± 5.2	ND	ND	2:1
E8	0.3 ± 1.1	9.1 ± 1.7	1.1 ± 8.2	6.7 ± 2.1	6.6 ± 1.9	2.5:1
C4	2.6 ± 0.3	20.3 ± 1.0	-4.6 ± 1.6	1.3 ± 1.5	0.4 ± 1.5	3:1
C3	1.6 ± 1.0	14.0 ± 1.0	0.9 ± 2.8	-1.5 ± 1.3	-2.5 ± 1.3	1.8:1

* Con A-stimulated blast cells were cultured with BHV-1 (MOI of 3) or PRV (MOI of 3) for 2 hr, washed once and labelled with ^{51}Cr . Cultures of effector and target cells were incubated for 6 hr.

TABLE 19

Expression of CD2, CD4 or CD8 on
cultured lymphocytes as analyzed by flow cytometry^a

mAb	Isotype	Ag	% Positive
8-1E3	IgG1	CD2	99.45
CC30	IgG1	CD4	91.93
CC58	IgG1	CD8	4.62
PJX63Aq8	IgG1	?	2.05

^a BHV-1 stimulated lymphocytes (2×10^6) were incubated with mAb (1/200 dilution) for 30 min on ice. Cells were washed and incubated with FITC-labeled goat anti-mouse IgG (1/1000) for 30 min on ice. Following wash, cells were resuspended in 200 μ l PBS and analyzed by flow cytometry.

Figure 1 The plasmid map of EMCV-derived transcription vector pE5GP111, demonstrates the noteworthy aspects of the construction. The T7 promoter, 5' noncoding region of encephalomyocarditis virus and the BHV-1 gene insertion site between NcoI and XbaI are indicated.

Figure 2 Representative flow microfluorimetry profiles of MoAb 8-1E3 to BoCD2 (1), 8-3F4 to BoCD5 (2) and 8-10C5 (3) on peripheral blood lymphocytes (a), T-enriched PBL (b) and thymocytes (c) are indicated in panel A. Panel B shows representative profiles of the binding of BoCD5 on PBL from three animals: a 3 yr old Jersey cow(a), a 6 yr old Holstein cow(b), a 4 yr old Brown Swiss cow (c).

Figure 3. Representative two-colour flow microfluorimetric scatter plots of MoAb on PBL (panel A) and thymocytes (panel B). MoAb 8-1E3 (a), 8-3F4 (b) and 8-10C5 (c) stained with phycoerythrin (red fluorescence) are shown on the Y axis. MoAb M11, anti-BoCD4 (1), 38-65, anti-BoCD8 (2), a combination of M11 and 38-65 (3), 33, anti-IgM (4) and 20-27 to BoCD1 (5) are stained with fluorescein (green fluorescence) and are shown on the X axis.

Figure 4 Immunoprecipitations of antigens from radiolabeled bovine lymphocytes. ^{125}I labeled ConA lymphoblasts were immunoprecipitated using MoAb and separated on 10% SDS-PAGE under reducing conditions. The MoAb used were P3x63AgS (lane a), 8-1E3 (lane b), P1.17 (lanes c, e, g), 16-1E10 (d), 8-3F4 (f) and 8-10C5 (h)

Figure 5 Representative experiments demonstrating the effect of MoAb to BoCD2, BoCD5 and 8-10C5 on proliferation to ConA, alloantigen or BHV-1.

Figure 6 The phenotype of $CD5^{dim}$ lymphocytes determined by two-colour flow microfluorimetry on PBL. BoCD5 stained red is shown on the Y-axis and BoCD2, T19 (WC-1), IgM, BoCD4 and BoCD8 stained green are shown on the X-axis. Cells were scored "dim" positive for red fluorescence if they were between the channels indicated by the horizontal lines in each panel.

Figure 7 A representative experiment showing the reactivity of 8-3F4 (BoCD5; a) or 8-1E3 (BoCD2; b) with PBMC and CD5^{dim}-enriched lymphocytes as measured by flow microfluorimetry. The second antibody control is indicated by the dotted line.

Figure 8 The ability of bovine rIL-2 to stimulate PBMC (closed) and CD5^{dim} lymphocytes (open) from steer 549M.

Figure 9 Size and granularity as determined by forward angle light scatter (y-axis) and 90° light scatter (x-axis) respectively of CD5^{dim} lymphocytes from steer 549M cultured in the presence of medium alone (a) or with ConA (b) for three days or medium alone (c) or with 30 U/ml bov rIL-2 (d) for 5 days. Frames indicate bit maps used to determine percent of cells staining in Table 3.

Figure 10 NK-like cytotoxicity of D17 (hatched) and BHV-1 infected D17 (solid) by PBMC (1), G10⁺ PBL (2) or lymphocytes (3) from cow 420A, freshly isolated (a) or cultured in medium alone (b) or with 30 U/ml bov rIL-2 (c).

FIG. 11 Cells from BHV-1 immune (No. 4) or nonimmune (Nos. 11, 63, 66) animals lysed A549 cells infected with BHV-1 in a 20-hr assay. Values are at effector to target (E:T) cell ratios of 100:1, 50:1, and 25:1. Error bars represent standard deviation from the mean of four samples.

FIG 12 Kinetic pattern of expression of BHV-1 glycoproteins (gI, gIII, and gIV). A549 cells were infected with BHV-1 for 2 hr, washed, and incubated for 10, 15, or 20 hr. Expression of viral glycoproteins was determined by ELISA using monoclonal antibodies (mAb) to BHV-1 gI ($\blacklozenge$), gIII ($\blacksquare$), and gIV ($\blacklozenge$). The IgG₂₄ isotype control value was negative. The pool ($\square$) was composed of the above mAbs to gI, gIII, and gIV. Values are expressed as the mean ($n = 3$) optical density $\pm$ SD.

FIG. 13 Lysis of BHV-1-infected A549 cells by freshly isolated or overnight cultured PBM cells. All cells were isolated from BHV-1 nonimmune animal No. 966. In A, freshly isolated cells were separated into NA and adherent (ADH) populations by culturing for 2 hr (representative experiment, $n = 8$). Lysis at E:T cell ratio of 100:1 for ADH population was not determined. In B, cells were cultured overnight, separated into NA and adherent populations, and compared with freshly isolated unseparated PBM cells (representative experiment, $n = 3$). Lysis was determined in a 20-hr assay at E:T cell ratios of 100:1, 50:1, and 25:1. Error bars represent SD from the mean of four samples. Lysis of noninfected target cells was $<2.5\%$ at the highest E:T ratio for each effector cell population.

Figure 14 Kinetic patterns of BHV-1-infected A549 cell lysis by PBM cells. All target cells were infected with BHV-1 for a total of 22 hr. The last 10, 15 or 20 hr freshly isolated (a) or overnight-cultured (b) NA effector PBM cells were present. All reported values are the mean ($n=4$) $\pm$ SD at E:T ratios of 100:1. Lysis of uninfected target cells at: 10 hr, no lysis; 15 hr, <5%; and 20 hr, <16%.

Figure 15 Lysis of 22 hr BHV-1-infected A549 cells by freshly isolated PBM cells (unseparated: W) or overnight-cultured NA cells (NA) and adherent cells (ADH) in a 4-hr assay. Target cells were infected with BHV-1 for 2 hr, washed, cultured 20 hr and then washed. Effector cells from two animals (nos 653 and 965) were added and cells were cultured for 4 hr. Values are at effector to target (E:T) cell ratios of 100:1 and 50:1. E:T cell ratio of 100:1 was not determined for adherent populations (representative example, $n = 3$).

Figure 16 (a) Pattern of BHV-1 glycoprotein expression on drug-treated A549 cells. A549 cells were infected with BHV-1 for 2 hr, washed and medium with or without 300 µg/ml of phosphonoacetic acid (PAA) was added to each well. After 20 hr, expression of viral glycoproteins was determined by ELISA using mAbs to BHV-1 gI, gIII and gIV. The pool (glypools) consisted of the above mAbs to gI, gIII and gIV. Values are expressed as the mean ($n=3$) optical density $\pm$ SD. (b) Lysis of BHV-1-infected A549 cells by PBM cells in the presence of phosphonoacetic acid. Fresh PBM cells were added to the drug-treated or untreated target cells for 20 hr. Shown are results from animal no. 755. Drug treatment was at 2 hr post-infection (representative example, $n=5$).

Figure 17 Lysis of phosphonoacetic acid-treated BHV-1-infected A549 cells by PBM cells in a 4-hr assay. PBM cells were separated into NA and adherent (ADH) cells after overnight culture. Shown are results from one of two animals. Target cells were treated for 22 hr with medium or 300 μ g/ml of phosphonoacetic acid (PAA). After target cells were washed, effector cells were added and cells were incubated for 4 hr. Values are expressed as the mean ($n=4$) $\pm$ SD at E:T cell ratios of 100:1 and 50:1. Values for monocytes at E:T cell ratio of 100:1 were not determined.

Figure 18 Flow microfluorimetric analysis of non-sorted cells expressing recombinant glycoproteins, gI (Panel A), gIII (Panel B) or gIV (Panel C). The vertical lines indicate the gate values used to sort glycoprotein-expressing cells from the population.

Figure 19 Flow microfluorimetric analysis of sorted glycoprotein-expressing transfectants. gI-expressing cells at 47 days following initial sorting are indicated by the shaded histogram and control D17 cells by the open histogram.

Figure 20 Autoradiograph from immunoprecipitates of radiolabelled cells. Lanes 1-6 were immunoprecipitated using mAb 110604 (anti-gIV). Lanes 7-12 were immunoprecipitated using mAb 510604 (anti-gI). Lane 1, uninfected MDBK cells; Lane 2, BHV-1-infected MDBK cells; Lane 3, uninfected D17 cells; Lane 4, BHV-1-infected D17 cells; Lane 5, D17-pZipNeo-gI cells; Lane 6, D17-pZipNeo-gIV cells; Lane 7, uninfected MDBK cells; Lane 8, BHV-1-infected MDBK cells; Lane 9, uninfected D17 cells; Lane 10, BHV-1-infected D17 cells; Lane 11, D17-pZipNeo-gI cells; Lane 12, D17-pZipNeo-gIV cells.

Figure 21 SDS-PAGE analysis of RRL translated proteins. RRL translated protein samples separated under reducing conditions on a 10% gel as described in Materials and Methods: lane 1, mock translated RRL; lane 2, BHV-1 gI; lane 3, BHV-1 gIII; lane 4, BHV-1 gIV. The migration positions of protein molecular weight markers are indicated.

Figure 22 T lymphocyte proliferation to proteins generated by cell-free translation. BHV-1 primed bovine lymphocytes were cultured at 2.5×10^4 cells/well, with two-fold serial dilutions of each protein sample as indicated. Proliferation was measured on day 3 by ^3H -thymidine incorporation, and the data expressed as mean counts per minute $\pm$ standard deviation. Numbers within parentheses () indicate in μl 's the concentration of RRL added to each well.

Figure 23 Antigen specificity of lymphocytes primed to BHV-1 gIV. Resting lymphocytes previously primed to the protein product of pE5GPIV mRNA were tested for responsiveness to the indicated antigens. Proliferation was measured as described in Figure .

Figure 24 Restriction enzyme map of transcription vector pE5GP111. Restriction enzyme sites within BHV-1 gp111 are noted, as is the RNA molecules generated following transcription with T7 RNA polymerase. The size of each RNA molecule in bases is denoted within parentheses.

Figure 25. Proliferation of T lymphocytes to truncated peptides of BHV-1 gIII. Truncated peptides (1.5 μ l each) were cultured with BHV-1 primed bovine lymphocytes as in Figure 3. The length of each peptide is denoted within parentheses, except peptide one which is 21 amino acids. Lymphocyte proliferation to each peptide is illustrated by a data point in the above line graph.

Description of cooperation.

Having produced a panel of monoclonal antibodies and developed the cellular methodology, we exchanged reagents and examined the reagents in our respective laboratories. Ruth Meirom from the Israel lab visited the U.S. lab to further characterize the monoclonal antibodies she produced. The cooperation conducted on the present grant has placed us in a strong collaborative position for a second BARD proposal which has been submitted.

Evaluation of the research achievements and the benefit to agriculture.

All BHV-1 strains including attenuated live vaccines can establish latency, even thermosensitive or thymidine kinase negative mutants. None of the inactivated or live vaccines presently available or undergoing clinical trials are able to completely prevent establishment of latency by a superinfecting challenge virus. Presently, many of the major pharmaceutical companies have stopped manufacturing modified live BHV-1 vaccine. These serious statements support the need for improved understanding of host-viral interaction. Proteins of the virus important for viral infection that are recognized by T cells may be useful immunogens. The production of monoclonal antibodies specific for immune cell populations (Objective 1) is a first step in understanding the participation of selected cell populations in for viral antigens (Objective 2). At the project's beginning well characterized antibodies were not available. Development of these mAb reagents has been essential for defining the roles of bovine immune cells against relevant pathogens. Characterizing the function of T lymphocyte populations and natural killer cell following recognition of virus or gI, gIII and gIV viral proteins is a key step in determining the contribution of each cell population in the immune response to the components of the virus (Objective 3). Identification of the phenotype and function of responding lymphocytes will suggest how the immune system may recognize the importance of each protein for host defense. This grant has provided a systematic approach to address the importance of viral proteins and cells of the immune system that participate in the response to the virus. Information from this work provides the basis for exploring in greater detail the individual proteins of the virus that stimulate a cellular immune response. This knowledge will have a major guiding influence on the construction of alternative BHV-1 vaccines.

List of publications.

1. O'Reilly K.L. and G.A. Splitter. Biochemical and functional characterization of 8-10C5, a non-clustered molecule present on bovine T lymphocytes, comparison with CD2 and CD5. Manuscript accepted by Immunology.
2. Splitter, G.A., Eskra, L. and Abruzzini, A.F. 1988. Cloned bovine cytolytic T cells recognize bovine herpes virus-1 in a genetically restricted, antigen-specific manner. Immunology. 63:145-150.

3. Cook, C.G. and Splitter, G.A. 1989. Characterization of bovine mononuclear cell populations with natural cytolytic activity against bovine herpesvirus 1-infected cells. *Cellular Immunology* 120:240-249.
4. Carol G. Cook, Letchworth III, G.J. and G.A. Splitter. 1989. Bovine naturally cytolytic cell activation against bovine herpes virus type 1-infected cells does not require late viral glycoproteins. *Immunology* 66:565-569.
5. Cook, C.G. and G.A. Splitter. Comparison of bovine mononuclear cells with other species for cytolytic activity against virally-infected cells. *Vet. Immunol. Immunopath.* 20:239-261, 1989.
6. Palmer, L.D. and G.A. Splitter. Bovine natural killer-like cell responses against cell lines expressing recombinant bovine herpesvirus type 1 glycoproteins. Manuscript accepted by *Journal of Immunology* with revision.
7. O'Reilly, K.L. and G.A. Splitter. CD5^{dim} peripheral blood lymphocytes proliferate to exogenous IL-2 in the absence of antigen and kill in an NK-like manner. Manuscript submitted.
8. Leary, T.P. and G.A. Splitter. Recombinant herpesviral proteins produced by cell-free translation provide a novel approach for the mapping of T lymphocyte epitopes. Manuscript submitted.

Patent disclosure.

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Dear Margaret,

This letter is intended to provide a brief overview of recent concepts that we discussed regarding identification of T cell epitopes that may be important in peptide vaccine development. This process has potential application to a wide spectrum of pathogens.

The reason to identify T cell epitopes. T cells are important in an immune response for two basic reasons. First, subpopulations of these cells are necessary to provide soluble cytokines to B cells for the production of antibodies by the B cells. Second, other subpopulations of T cells are important in the direct destruction of virally infected host cells. Identification of the epitopes on proteins that are recognized by T cells would be the first step in the construction of potential protective peptide vaccines. Peptide vaccine construction would contain the appropriate T cell epitopes in combination with B cell epitopes important to protective immunity.

Current progress. Presently, we are interested in identifying the T cell epitopes of bovine herpesvirus type 1 (BHV-1) that would be important in a protective immune response. In this work my laboratory has been using the pE5 vector system developed by Ann Palmenberg. Tom Leary, a graduate student, has been the sole worker on this project. We have inserted three BHV-1 genes individually 3' to the 5' noncoding region of encephalomyocarditis present in the vector. Transcription of the vector followed by translation of the mRNA in a rabbit reticulocyte lysate system results in the protein of interest. These proteins of BHV-1 have been added to immune bovine lymphocytes and antigen specific proliferation of the lymphocytes has occurred. Further, we have truncated the inserted BHV-1 gIV gene specifically and have rapidly produced a series of truncated protein peptides of increasing lengths. When these peptides were tested on a bovine lymphocyte cell line, the cells proliferated to a series of peptides containing the epitope but not to peptides which lacked the epitope. Using this method we will be able to characterize all of the T cell epitopes present on this BHV-1 glycoprotein which is important for viral attachment to cells. This information will be useful in a potential peptide vaccine construct that will stimulate an immune response to the BHV-1 gp IV protein. (Manuscript appended).

Future studies. Application of this vector system will be useful for identifying T cell epitopes in a broad spectrum of pathogens. Presently, I have submitted a proposal to NIH that would examine the T cell epitopes of an entire virus, Mengo virus. This work would have direct implication not only for Mengo virus but to all picornaviruses. Other examples of picornaviruses are Poliovirus, Foot and Mouth Disease virus, and Rhinovirus (the cause of the

common cold). These other viruses hold the potential for improvement of present vaccines and the possibility for understanding construction of new vaccines. Further, this methodology has application for virtually any pathogen of interest where its genes are available.

Prior Art.

1. Zarling, J. et al. 1986. Herpes Simplex virus (HSV)-specific human T-cell clones recognize HSV glycoprotein D expressed by a recombinant vaccinia virus. *J. Virology* 59:506.
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4. Lasky, L. et al. 1984. Protection of mice from lethal herpes simplex virus infection by vaccination with the secreted form of cloned glycoprotein D. *Bio/technology*. June:527.
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7. van Drunen Littel-van den Hurk, S. et al. 1989. Synthesis, cellular location, and immunogenicity of bovine herpesvirus 1 glycoproteins gI and gIII expressed by recombinant vaccinia virus. *J. Virology* 63:2159.
8. Fitzpatrick, D.R. et al. 1988. Expression of bovine herpesvirus 1 glycoproteins gI and gIII in transfected murine cells. *J. Virology* 62:4239.

Looking forward to your comments on this work.

Best Regards,

Gary Splitter
Professor