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Cloned bovine cytolytic T cells recognize bovine herpes virus-1 in a genetically restricted, antigen-specific manner

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SUMMARY

The inability to demonstrate bovine herpes virus-1 (BHV-1)-specific lymphocyte responses from BHV-1-infected cattle has been a major difficulty in confirming the importance of cellular effector mechanisms during BHV-1 infection. We have examined the capacity of bovine cytolytic T-cell clones to lyse BHV-1-infected, concanavalin A-stimulated blast cells. Cytolysis was as high as 58% at an effector to target (E:T) ratio of 1:1. All cytolytic T-cell clones produced were genetically restricted in killing cells of the autologous genotype. Cytotoxic T-lymphocyte (CTL) clones were specific for BHV-1 but not related herpes viruses, i.e. BHV-2 and pseudorabies virus. These results provide evidence that cytolytic T lymphocytes have an antigen-specific role in the immune response of cattle against BHV-1, and that CTL may serve as effector cells in the identification of glycoproteins useful in recombinant vaccine preparation since only determinants of BHV-1 were recognized.

INTRODUCTION

Bovine herpes virus-1 (BHV-1) is the cause of infectious bovine rhinotracheitis, an acute and occassionally fatal respiratory infection of cattle, associated with the respiratory disease complex, often termed shipping fever. This disease is the most serious single cause of cattle deaths in the U.S.A. (Yates, 1982). Similar to the herpes simplex infection in humans, antibody offers little protection against the cell-associated virus (Hyland, Easterday & Pawlisch, 1975). Current protection is afforded by killed and modified live vaccines; however, virulent infections occur in vaccinated animals and distinguishing between vaccinated and infected animals is impossible (Hyland *et al.*, 1975). Although a variety of cellular mechanisms have been reported to function against herpes virus-infected cells, cytotoxic T cells are suspected as being of major importance (Sethi, Stroehmann & Brandis, 1980; Nahmias & Roizman, 1973; Oakes, 1975; Pfizenmaier *et al.*, 1977). In BHV-1-infected cattle, others have been unable to demonstrate genetically restricted killing of BHV-1-infected target cells, even though cytotoxicity was evident against BHV-1 (Rouse & Babiuk, 1977). Generation of proliferative populations against the virus (Splitter & Eskra, 1986; Miller-Edge & Splitter, 1986a) and limiting-dilution frequency analysis (Miller-Edge & Splitter, 1986b) have been demonstrated. Importantly, a suppressor cell population has been shown as being predominate in some immunized animals (Miller-Edge & Splitter, 1986b). Identification of the BHV-1 glycoproteins recognized by cytotoxic T lymphocytes has been hampered by the inability to establish virus-specific, genetically restricted CTL. Therefore, production of efficient recombinant vaccines against shipping fever caused by BHV-1 requires the identification of specific viral components that induce CTL production. As a first step in this process, we have generated bovine cytolytic T-cell clones with specificity for BHV-1.

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MATERIALS AND METHODS

Cattle

Normal healthy Guernsey cattle (*Bos taurus*), 3–6 years old, were reared and maintained at the Department of Dairy Science, University of Wisconsin-Madison. Cattle were immunized annually with killed BHV-1 vaccine (Fort Dodge, Fort Dodge, IA) intramuscularly and were sero-positive for BHV-1. Blood was taken for cloning purposes without reference to immunization; however, all animals were vaccinated at least 3 months prior to experiments.

BoLA typing

Bovine lymphocyte typing (BoLA) was performed as described elsewhere (Terasaki & Park, 1976), using a panel of alloantisera produced in our laboratory, and specificity substantiated using a panel of alloantisera provided by Dr Domenico Bernoco, University of California–Davis. All antisera identifying BoLA-A locus-encoded phenotypes have been confirmed by international BoLA typing workshops (Anon., 1982).

Viruses

BHV-1 (Cooper strain) and BHV-2 (New York strain) were obtained from ATCC (Rockville, MD) no. VR-864 (York,

Schwartz & Estela, 1957) and ATCC no. VR-845 (Weaver, Dellers & Dardiri, 1972), respectively. The pseudorabies virus, Sullivan/p3/4 was provided by Sandy McGregor *et al.* (1985).

Cloning peripheral blood lymphocytes with specificity for BHV-1

Peripheral blood mononuclear cells from immunized animals were cultured with UV-irradiated BHV-1, and clones were established from one of the animals by methods reported for human CTL (Yasukawa & Zarling, 1984a). Briefly, peripheral blood mononuclear cells were isolated from Ficoll–Hypaque density gradients centrifuged at 1000 *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 plaque-forming units (pfu)/ml. On 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 (Taswell, 1981) where 76% of the wells were negative for cell growth. Clones were selected 3 weeks later, split into two wells and fed at 7–10-day intervals with 5% MLA-144, 5% TCGF-containing supernatant, 1×10^5 irradiated feeder cells and 1×10^5 pfu UV-irradiated BHV-1.

The culture medium used throughout was RPMI-1640 with 25mM HEPES (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 2 mM 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 (Miller-Edge & Splitter, 1984). TCGE-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 Chemical Co., St Louis, MO). The supernatant was treated with α-methyl-D-mannoside (Sigma Chemical Co.) at a concentration of 0·1 M and filtered through a 0·22-μm filter.

Cell-mediated cytotoxicity

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 ^{51}Cr added, followed by a 1·5-hr incubation at 37° and two washes. Infected and labelled 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:

$$\% \text{ specific lysis} = \frac{\text{c.p.m. experimental} - \text{c.p.m. spontaneous release}}{\text{c.p.m. total release} - \text{c.p.m. spontaneous release}} \times 100.$$

Kinetics of viral glycoprotein expression on target cells was determined by flow cytometry. 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 (Marshall, Rodriguez & Letchworth, 1986), or a swine anti-pseudorabies virus (PRV) antiserum (provided by Sandy McGregor). Fluorescein-labelled rabbit anti-mouse (Jackson Immuno Res. Lab., Avondale, PA.) or rabbit anti-swine antibody was added for 30 min at 37°, washed, and the percentage fluorescence determined by flow cytometry.

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

	% cytolysis of Con A-stimulated blast cells			
	Animal 686		Animal 686 + virus	
Clone no.	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* 14·9±4·1†
D5	7·6±0·8	3·0±1·0	40·0±6·2	43·0±4·1 3·1±0·3
C10	3·7±1·4	ND	57·7+9·4	ND
F7	4·2±3·3	−1·0±0·5	32·8±3·2	21·2±2·9 7·8±0·8
G10	4·7±0·2	−2·0±1·2	25·1±4·3	13·3±4·0 3·4±0·3
D2	0·3±2·6	ND	32·8±0·6	ND
E2	2·4±2·1	0·6±4·1	25·9±1·1	10·7±0·8 2·3±1·6
E3	2·7±2·5	−0·5±2·2	26·2±4·0	8·0±3·0 1·6±0·5
E7	5·5±1·6	−2·4±1·1	19·8±3·0	5·0±1·4 0·6±1·8
E4	2·4±2·9	ND	−2·5±2·3	ND
D7	4·1±1·8	0·0±0·7	7·7±2·4	18·4±1·0 4·3±1·9
F2	0·8±1·8	ND	3·0±1·3	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.

RESULTS

Genetically restricted killing of BHV-infected cells

In mice (Pfizenmaier *et al.*, 1977) and humans (Yasukawa & Zarling, 1984a), it has been concluded that CTL activity against herpes simplex virus (HSV) is not only antigen specific but also genetically restricted. In contrast early experiments in cattle, examining CTL activity against bovine herpes virus-1, concluded that CTL activity functions in a genetically non-restricted manner (Rouse & Babiuk, 1977). Using cloning methodology now currently available, it was important to discern whether cattle possessed MHC-restricted killing of BHV-1 or whether MHC non-restricted cytolytic killing occurred, as reported in other species with herpes viruses (Yasukawa & Zarling, 1983). 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. Twelve of the 22 clones that were produced were characterized in this study, as shown in Table 1. Nine of 12 clones possessed cytolytic activity against

Table 2. Genetic restriction of cytolytic T-cell clones

	% cytolysis 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.

BHV-1-infected cells at an E:T ratio of 1:1. Clones were cytolytic for virally infected cells but not non-infected cells, with lysis ranging from 20% to 58%. Three clones had either low or no cytolytic activity. Two clones (E3 and E7) had cytotoxic activity in Experiment 1 but had lost activity 1 month later when Experiment two was performed. Originally, clone D7 had low cytotoxicity; later, in Experiment two, D7 was cytotoxic for virally infected cells. Specificity was maintained in some of the clones for as long as 6 months. 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 1 do not demonstrate whether the cytolytic cells are genetically restricted or function as lymphokine-activated killer cells capable of destroying cells infected with other viruses. To address this question, six clones were assayed on autologous as well as allogeneic BHV-1-infected Con A-stimulated blast cells. Results in Table 2 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 the clones tested. These findings support the concept that bovine CTL function in a genetically restricted manner in killing herpes virus-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 3). Con A-stimulated blast cells from two different animals infected with virus produced maximal glycoprotein expression between 8 hr 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 PRV expressed PRV glycoproteins in a similar time period, as indicated in Table 3.

Specificity of the T-cell clones for viral determinants of BHV-1 but not BHV-2 is shown in Table 4. Ten of the 11 clones killed only BHV-1-infected autologous target cells. Cytolysis ranged from 10% to 35% at an E:T 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 herpes virus, pseudorabies virus (Table 5). Lysis of BHV-1-infected autologous target cells ranged from 4% to 20% at E:T 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 frequent epitopes recognized in common with other bovine herpes viruses.

DISCUSSION

These results indicate two important findings. First, antigen-specific CTL can be derived from the peripheral blood of BHV-1-immunized cattle. This is important because a commonly held conclusion, without documented evidence, is that T-cell mediated defence 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 herpes viruses 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 (Rouse & Babiuk, 1977), 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 (Rouse & Horohov, 1984). No other studies have adequately addressed bovine cytotoxic T-cell effector mechanisms specific for BHV-infected cells. Recent attempts by others to detect cytotoxic T cells against BHV-1 have not been successful (Brigham & Rossi, 1986). 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 recrudescent infection in cattle has not 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 (Larsen, Russell & Rouse, 1983; Larsen *et al.*, 1984; Nash, Phelan & Wildy, 1981; Sethi, Omata & Schneweis, 1983).

Table 3. Expression of BHV-1 and pseudorabies virus glycoproteins on Con A-stimulated blast cells*

	% fluorescence			
Total hr infected	Anti-BHV-1 Rabbit anti-mouse	Anti-BHV-1 +rabbit anti-mouse	Anti-PRV Rabbit anti-swine	Anti-PRV +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 4. Specificity of CTL clones for BHV-1 but not BHV-2*

	% cytolysis of target cells			
Clone	Animal 686	Animal 686 +BHV-1	Animal 686 +BHV-2	E:T ratio
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.

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 (Zarling *et al.*, 1986). It is important, therefore, to determine if CTL generated to BHV-1 can recognize determinants in common with other herpes viruses which might infect cattle. Data from the work presented here indicate that bovine CTL clones do not recognize determinants shared by other herpes viruses 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 herpes viruses. One clone, E2, in the present study was able to lyse PRV-infected cells (Table 5), suggesting that PRV may share determinants with BHV-1. Antibodies recognize shared determinants among HSV-1, BHV-2 and PRV by agar gel immunodiffusion (Killington *et al.*, 1977), and HSV-1 glycoproteins B and D are antigenically similar to BHV-2 (Snowden *et al.*, 1985). Therefore, considerable conservation of peptides exist between human and certain bovine herpes viruses. However, antisera from BHV-1 sero-positive animals fail to identify determinants on BHV-2 and vice versa (Osorio *et al.*, 1985).

T-cell recognition of common viral determinants between BHV-1 and HSV has been demonstrated using human peripheral blood mononuclear cells (Zair, Leary & Levin, 1978). Cells from HSV sero-positive but BHV-1 sero-negative individuals could proliferate to BHV-1. Therefore, antibody and T-cell recognition of determinants in common among different herpes viruses suggests that bovine T-cell clones might be found that recognize several herpes viruses. Such was not the case in this study. Bovine T-cell clones recognize determinants specific for BHV-1 even when assayed against other herpes viruses 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. Previously, it was concluded that HSV glycoprotein(s) serve as recognition structures for mouse (Lawman *et al*, 1980; Carter, Schaffer & Tevethia, 1981) and human (Yasukawa & Zarling, 1984b) 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.

Table 5. Absence of cytolytic activity of T-cell clones for pseudorabies virus*

Clones	% cytolysis 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.

Findings in the present study illustrate that genetic restriction by bovine CTL occurs in cattle, as has been demonstrated in humans for HSV-1 (Zarling *et al.*, 1986). 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 leucocyte reactivity, data not presented. In humans, MHC class II molecule recognition of HSV-1 and -2 has been reported (Yasukawa & Zarling, 1984b; Zarling *et al.*, 1986). 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 (Pfizenmaier *et al.*, 1977). 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 (Braun *et al.*, 1984) and that the predominance of class II-restricted CTL may serve as an important defence against infection (Yasukawa & Zarling, 1984a). 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 (McKercher, 1973). The virus binds in high amounts to lectin-stimulated lymphocytes, suggesting a possible *in vivo* role of proliferating lymphocytes in viral pathogenesis (Splitter & Eskra, 1986). 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 not clear at present 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 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.

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