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Marek's Disease Virus Down-Regulates Surface Expression of MHC (B Complex) Class I (BF) Glycoproteins during Active but not Latent Infection of Chicken Cells

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Infection of chicken cells with three Marek's disease virus (MDV) serotypes interferes with expression of the major histocompatibility complex (MHC or B complex) class I (BF) glycoproteins. BF surface expression is blocked after infection of OU2 cells with MDV serotypes 1, 2, and 3. MDV-induced T-cell tumors suffer a nearly complete loss of cell surface BF upon virus reactivation with 5-bromo-2′-deoxyuridine (BUdR). The recombinant virus (RB1BUS2gfpΔ) transforming the MDCC-UA04 cell line expresses green fluorescent protein (GFP) during the immediate early phase of viral gene expression. Of the UA04 cells induced to express the immediate early GFP, approximately 60% have reduced levels of BF expression. All of the reactivated UA04 and MSB1 tumor cells expressing the major early viral protein pp38 display reduced levels of BF. Thus, BF down-regulation begins in the immediate early phase and is complete by the early phase of viral gene expression. The intracellular pool of BF is not appreciably affected, indicating that the likely mechanism is a block in BF transport and not the result of transcriptional or translational regulation.

INTRODUCTION

The evasion of acquired cellular immunity is critical for infection and lifelong persistence of herpesviruses in their hosts. The importance of cellular immunity is emphasized by the considerable number of viral proteins herpesviruses use to alter the antigen-presenting machinery of the host cell. This interference with the major histocompatibility complex (MHC) class I antigen-presenting pathway is a common mechanism used by all mammalian herpesviruses to evade immune detection (Johnson and Hill, 1998). The herpes simplex viruses (HSV-1 and -2) are α-herpesviruses that alter the transport of viral antigens into the endoplasmic reticulum (ER) (Hill *et al.,* 1994; Hinkley *et al.,* 1998; Jugovic *et al.,* 1998). HSV-1 encodes the immediate early protein ICP47 (US12) which binds to the transporter proteins associated with antigen presentation (TAP) (Galocha *et al.,* 1997; Neumann *et al.,* 1997). The binding of ICP47 to the TAP1/TAP2 complex inhibits the translocation of peptides from the cytosol to the lumen of the ER. In the absence of peptides in the ER, the nascent class I proteins fail to mature into the trimolecular complex composed of peptide, heavy chain, and β2-microglobulin. These naked class I proteins are degraded instead of being transported through the Golgi complex to the cell surface. Varicella zoster virus (VZV), pseudorabies virus (PRV), and bovine herpesvirus (BHV) are other α-herpesviruses that reduce the cell surface expression of MHC class I in infected cells (Cohen, 1998; Hariharan *et al.,* 1993; Mellencamp *et al.,* 1991; Nataraj *et al.,* 1997). VZV, PRV, and BHV do not contain ICP47 homologues and the viral proteins and mechanisms altering antigen presentation have yet to be identified. Although the viral proteins altering MHC expression are unknown, BHV was recently shown to inhibit the peptide transport activity in bovine cells (Hinkley *et al.,* 1998). Thus, the peptide transport mechanism appears to be a favorite target of the α-herpesvirus family for altering antigen presentation and evading cell-mediated immunity.

The Marek's disease virus (MDV) of chickens is the only known oncogenic α-herpesvirus inducing T-cell tumors. Serotype I MDV is homologous both antigenically and molecularly to the nononcogenic serotype 2 and 3 viruses. All three serotypes are highly cell-associated and spread through cell-to-cell contact (Biggs, 1975). The cell-associated nature of MDV suggests that the cellular immune response (CMI) is critical in controlling infection and tumor cell development (Omar and Schat, 1996; Pratt *et al.,* 1992) but conclusive *in vivo* evidence is lacking (Schat, 1991). MDV is recognized by virus-specific cytotoxic T lymphocytes (CTL) (Omar *et al.,* 1998; Pratt *et al.,* 1992); however, this response is weak compared with the

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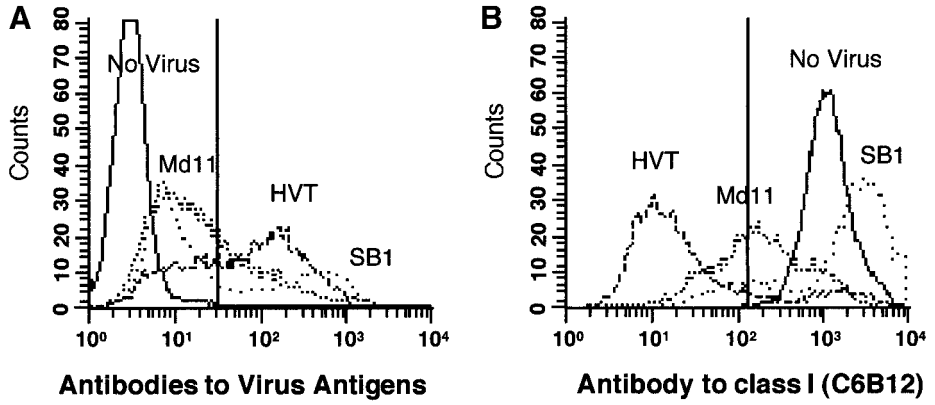

FIG. 1. Down-regulation of cell surface class I on OU2 cells infected with serotype 1, 2, and 3 MD viruses. (A) Flow cytometry analysis of OU2 cells infected with Md11, SB1, and HVT. Infected and control cells were analyzed for expression of viral proteins. (B) Infected cells were analyzed for the surface expression of MHC class I glycoproteins. The vertical bars indicate the gate (fluorescent channel) used to determine the percentage infected versus-uninfected (A) or down-regulated class I (B) presented in Table 1. (——) Uninfected; (– • –) Md11; (· · ·) SB1; (– –) HVT.

CTL response to other avian viruses (Fulton *et al.,* 1995; Thacker *et al.,* 1995; Uni *et al.,* 1994). Interference with the antigen-presenting machinery is one potential mechanism incapacitating the cell-mediated immune response to MDV. Here we report on the ability of MDV to down-regulate the surface expression of class I glycoproteins upon initial infection of chicken fibroblasts. A similar down-regulation of cell surface class I is observed after reactivation of latent MDV in T-cell tumor lines. This down-regulation of class I is complete by the time the major early viral protein pp38 (Chen *et al.,* 1992; Cui *et al.,* 1990; Ui *et al.,* 1998) is expressed. The intracellular pool of class I heavy-chain proteins is not grossly affected, suggesting that the peptide transport mechanism is a potential target.

RESULTS

Correlation of MDV infection and loss of cell surface class I

To investigate interactions between antigen presentation and MDV infection we used the OU2 chicken fibroblast cell line, which expresses high levels of BF glycoproteins. The cells were infected with tissue culture adapted stocks of serotypes 1, 2, and 3 to achieve reasonable levels of infection. The IAN86 monoclonal antibody recognizes the gB glycoproteins of serotypes 1 and 3 and was used to determine the percentage of OU2 cells infected with the Md11 (serotype 1) and HVT (serotype 3) viruses. The Y5 monoclonal antibody recognizes a serotype 2 specific epitope (Silva and Lee, 1984) and was used to determine the percentage of OU2 cells infected in the SB1 (serotype 2) cultures. The results are shown in Fig. 1A and quantitated in Table 1. Uninfected OU2 cells have little or no background fluorescence when labeled with the virus-specific monoclonal antibodies. As expected, the three viral serotypes infect at much different levels. Serotype 3 (HVT) established an active infection in approximately 84% of the cells, while serotype 2 (SB1) was detected in only 30% of the cells. The percentage of cells infected by the Md11 virus was 63% and is uncharacteristically high for a serotype 1 virus. The Md11 stock used for this analysis was passed in culture more than 100 times, which may account for this high level of viral infection.

The class I expression of infected versus uninfected cells was analyzed with the C6B12 monoclonal antibody as shown in Fig. 1B. The uninfected OU2 cells express high levels of class I glycoproteins on their cell surface. Infection with SB1, and to a lesser extent with the other two serotypes, produces two subpopulations of cells, one with reduced expression of cell surface class I glycoproteins and a second with equal or enhanced

TABLE 1

Quantification of the Class I Down-Regulation in OU2 Cells Infected with Serotypes 1, 2, and 3 MD Virus

Infecting virus	% virus-specific mAb positive[a]	% with decreased surface class I[a]
No virus (control)	1	2
HVT (serotype 3)	84	88
SB1 (serotype 2)	30	28
Md11 (serotype 1)	63	68

[a] The percentage of cells positive for virus antigens and the percentage of cells displaying class I down-regulation were calculated based on the gates shown in Figs. 1A and 1B, respectively. The monoclonal antibodies to virus-specific antigens were Y5 (serotype 2) and IAN86 (serotypes 1 and 3, gB). The percentage of control (no virus) cells falling in the positive range using the virus-specific antibodies was 1% or less. Less than 2% of the control (no virus) cells were down-regulated for surface protein expression using the C6B12 antibody to chicken class I.

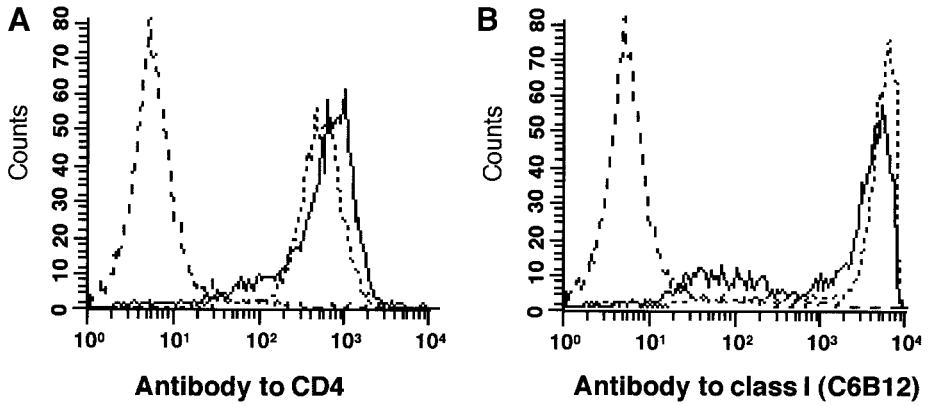

FIG. 2. Expression of CD4 and class I glycoproteins on the surface of BUdR-treated MSB1 cells. Flow cytometry analysis of MD virus reactivation by BUdR in MSB1 cells. Quantification of cell surface CD4 (A) and class I (B) were analyzed in cultures containing 0 and 25 μg/ml of BUdR for 48 h. The dashed line represents a negative control antibody, the dotted line represents cells cultured in 0 μg/ml of BUdR, and the solid line represents cells cultured in 25 μg/ml of BUdR.

levels of class I compared with the uninfected control. The mean channel fluorescence of the enhanced subpopulation in the SB1-infected cells is 4500 compared to 1200 mean channels of fluorescence in the uninfected control cells. This represents a 3200 mean channel increase of fluorescence in the SB1-infected cells compared to the uninfected control cells. The reduced class I subpopulation in the SB1 culture represents about 30% of the total cells and is reduced about 40-fold compared to the enhanced subpopulation. The HVT culture produces the most dramatic down-regulation of surface class I with greater than 80% of the cells (see Table 1) displaying 100- to 200-fold less class I than in the enhanced subpopulation. The enhanced subpopulation in the serotype 1 Md11 cultures was not as dramatic as in the other two serotypes. Approximately 70% of the cells in the Md11 cultures displayed reduced levels of class I expression. Compared with SB1, this represents a higher percentage; however, the intensity of the class I expression is similar to its serotype 2 counterpart and not as complete as in the HVT cultures. As shown in Table 1, the percentages of cells expressing viral proteins correlates with the percentage of cells displaying reduced levels of class I glycoproteins.

Virus reactivation in MDV tumor cells induces class I down-regulation

Treatment of the MDV-transformed MSB1 cells with BUdR results in viral gene expression and virus production presumably by reactivating the virus from the latent state (Dunn and Nazerian, 1977). Figure 2 shows that the treatment of MSB1 cells with BUdR specifically reduced the surface expression of class I without grossly affecting the expression of CD4 on the surface of MSB1 cells. In this experiment, 25.3% of the cells treated with 25 μg/ml BUdR had reduced levels of surface class I. In the absence of BUdR the figure was 5.2% with reduced levels of class I, indicating that 20.1% of the cells lost class I upon culture with BUdR. In the same cultures only 6.5% of the cells had slightly reduced levels of the CD4 surface antigen. The histogram profiles in Fig. 2 clearly show a distinct subpopulation of cells that have lost class I expression that is not observed in the CD4 profiles.

Down-regulation of class I by MDV occurs in the immediate early phase of gene expression

To determine the temporal nature of the class I down-regulation three different cell lines were analyzed by two-color flow cytometry for virus protein expression and class I down-regulation after treatment with BUdR (Fig. 3). The Marek's virus in the RP1 cell line is a nonproducer or latent virus since treatment of the cells with BUdR does not reactivate the transforming virus (Nazerian *et al.,* 1977). In this analysis the RP1 cells serve as a negative control for the virus antigen-specific antibodies used for the two-color labeling procedure and to show that BUdR treatment alone does not grossly alter class I surface expression. The GFP recombinant virus RB1BUS2gfpΔ (serotype 1) was used to transform the UA04 chicken T-cell line. The GFP in the UA04 cell line is regulated as an immediate early gene by the recombinant virus since the cytomegalovirus immediate early promoter (CMVp) is used to drive GFP expression (Dienglewicz and Parcells, 1999). The expression of GFP in the UA03 cells after virus reactivation with BUdR and is used here as a marker for immediate early gene expression. As noted above, the MSB1 cell line expresses the pp38 protein upon virus reactivation. The pp38 protein is used as a marker for the early phase of virus gene expression (Ui *et al.,* 1998).

As expected, the virus in the RP1 cell line was not

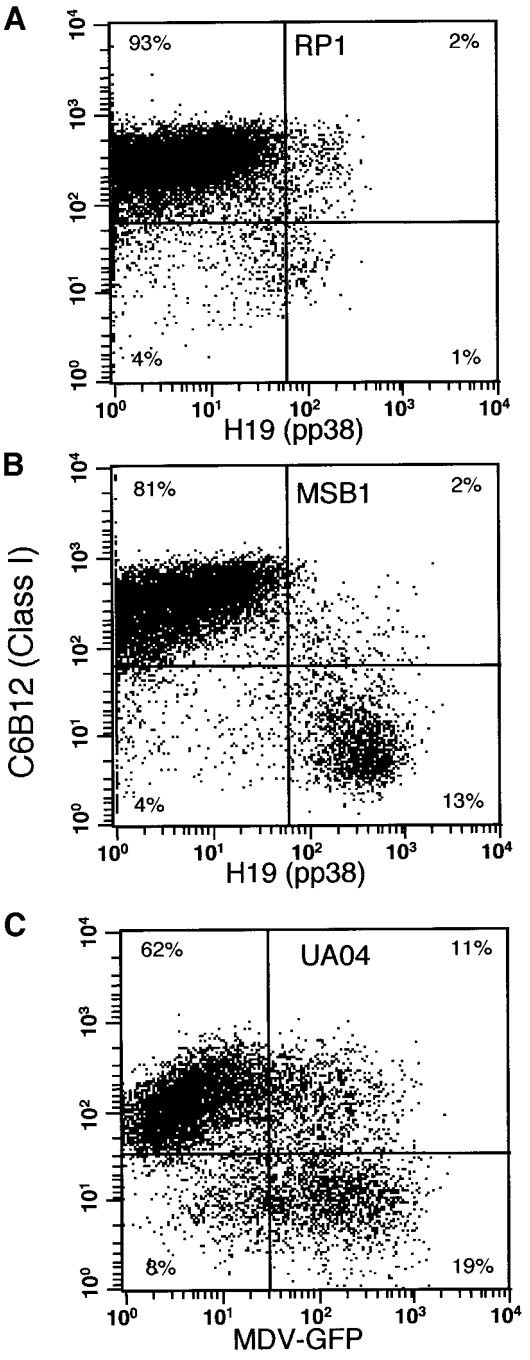

FIG. 3. Two-color analysis of expressed virus proteins (pp38 and GFP) and cell surface class I glycoproteins. RP1 (A), MSB1 (B), and UA04 (C) were induced for 48 h with 10 μg/ml BUdR to reactivate the transforming MD virus.

induced to express the pp38 viral protein and the surface expression of class I was not down-regulated (Fig. 3A). Reactivation of the virus in MSB1 cells resulted in the expression of pp38 and, as shown in Fig. 3B, nearly all of the MSB1 cells expressing pp38 have down-regulated their surface class I glycoproteins. In contrast, there is a heterogeneity between the expression of GFP and cell surface class I in the BUdR-treated UA04 cells (Fig. 3C). Although 19% of the cells expressing the GFP have down-regulated their class I glycoproteins, a significant number of cells (11%) express both the GFP and normal levels of cell surface class I (Table 2).

Intracellular class I protein is abundant in OU2 cells with down-regulated surface class I

Serotype 3 (HVT) produces the most dramatic effects on cell surface class I expression, presumably due to its aggressive replication *in vitro* compared with serotypes 1 and 2. Therefore, lysates representing equal numbers of uninfected and serotype 3 (HVT)-infected OU2 cells were analyzed by immunoprecipitation for total class I production. Approximately 80% of the infected cells used for this analysis displayed down-regulated cell surface class I as determined by flow cytometry. Figure 4 shows the intracellular levels of the 42-kDa BF glycoproteins determined by autoradiography (Plachy *et al.,* 1992). Figure 4A indicates a 4-h exposure and densitometry of the class I protein bands shows a 15% decrease (density = 7576 for uninfected versus 6471 for infected cells for a 1.2-fold reduction) in the infected versus the uninfected cells. Figure 4B indicates a 72-h exposure, showing the background bands as a measure of total protein synthesis in the infected and uninfected cells. As indicated by densitometry of the background bands there is a 10% average decrease (range 4 to 18%) in total protein production in the infected versus uninfected cells. The 10% decrease in total protein production in infected cells does not account for the 100-fold (99%) reduction of surface class I observed in the serotype 3-infected OU2 cells (Fig. 1B) and suggests a specific virus-induced inhibition of class I expression.

DISCUSSION

We have shown that class I glycoproteins are severely down-regulated on the surface of MDV-infected OU2

TABLE 2

Quantification of the Down-Regulation of Class I Glycoproteins on the Surface of BUdR-Treated MSB1 Cells

BUdR (μg/ml)	24 h		48 h	
	CD4	Class I	CD4	Class I
0	0.0	2.9	1.0	5.2
10	0.1	5.5	5.8	21.0
25	0.1	6.9	7.5	25.3

Note. The effect of 24- and 48-h BUdR treatment on the levels of CD4 and class I expression in MSB1 cells. MSB1 cells were cultured in media containing 0, 10, or 25 μg/ml of BUdR and analyzed for cell-surface CD4 or class I glycoproteins. The percentage of cells with reduced levels of each surface glycoprotein is presented based on the histograms presented in Fig. 2.

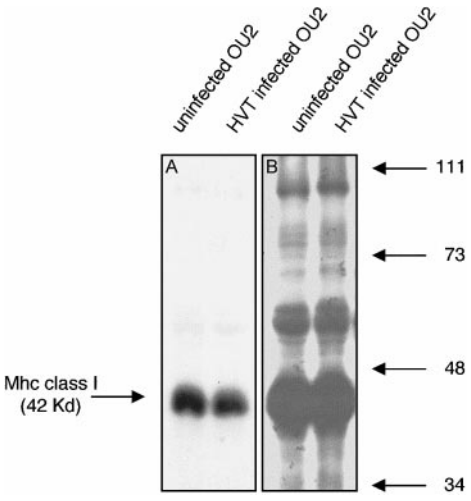

FIG. 4. Immunoprecipitation of MHC class I from uninfected and serotype 3-infected OU2 cells. Infected and uninfected cells (10^5 cell equivalents of each) were analyzed by immunoprecipitation using the C6B12 monoclonal antibody to MHC class I. The total amount of class I protein is similar in infected and uninfected cells. (A) 4-h exposure showing the density of the class I protein band in the infected and uninfected cells (density = 7576 for uninfected, 6471 for infected). (B) 72-h exposure showing a slight decrease in total protein synthesis as indicated by the background bands in the infected versus uninfected cells (10% average decrease with a range of 4 to 18%). The arrows on the right indicate the position of the protein size markers (kDa).

cells and in transformed tumor cells undergoing reactivation of latent virus. When uninfected, the OU2 fibroblast cells express large quantities of class I on their cell surface, displaying over 1000 channels of fluorescence using the C6B12 monoclonal antibody. In contrast, cell surface expression of class I on serotype 3-infected OU2 cells is reduced 100-fold to 10 channels of fluorescence. Our results indicate that this effect is not due to an overall shutdown of host protein synthesis since cell surface CD4 is nearly normal after virus reactivation in MDV-induced tumor cells. Further, the total (intracellular and cell surface) levels of class I glycoprotein are only slightly lower (~15% or 1.2-fold reduction) in the HVT-infected versus control OU2 cells. This suggests that neither transcriptional nor translational inhibition is the primary factor in this down-regulation of cell surface class I.

In HSV, the ICP47 (US12) protein is instrumental in class I down-regulation. The ICP47 protein interacts with the transporter proteins and prevents cytosolic peptides from binding to the TAP1/TAP2 complex (Jugovic *et al.,* 1998). The genomic structure of MDV is similar to HSV; however, the unique short (US) region of serotype I MDV does not encode a HSV US12 (ICP47) homologue (Brunovskis and Velicer, 1995). The unique short region of human cytomegalovirus (HCMV) contains at least four genes (US2, US3, US6, and US11) that are instrumental in down regulating peptide transport and cell surface expression of class I (Ahn *et al.,* 1997; Machold *et al.,* 1997; Wiertz *et al.,* 1996). Early in infection, US3 retains class I in the ER while US2 and US11 reroute class I heavy chains to the cytosol for degradation. The US6 glycoprotein inhibits translocation of peptides by binding TAP in the lumen of the ER (Ahn *et al.,* 1997). As with ICP47, homologues of the HCMV US regions are not present in the US region of serotype I MDV. Thus, the MDV gene product or products responsible for class I down-regulation are likely to be unique.

Although the MDV gene products down-regulating class I appear unique, the general mechanisms are likely to resemble HSV. Like ICP47, the MDV proteins affecting class I expression appear to be expressed in the immediate early phase of viral replication, the amount of intracellular class I protein does not appear to be grossly affected, and enhanced degradation of the heavy chain is not observed in the cell lines used for this analysis. Therefore peptide transport or retention of class I in the ER is a probable target altering class I surface expression in active MDV infections (Jugovic *et al.,* 1998).

The ability of MDV to alter antigen presentation is likely to play a role in the pathogenesis of the virus *in vivo* and helps explain various *in vitro* observations. All three serotypes, including vaccine strains (HVT and SB1), have the capacity to down-regulate cell surface class I and evade cellular immunity. Evasion of cellular immunity is likely to play a key role in the lifelong persistence demonstrated by all three serotypes. The expression of pp38, the major virus early antigen, is consistently observed *in vivo* but recognized only weakly by the host's cellular immune system (Baigent *et al.,* 1998, 1996; Omar and Schat, 1996; Omar *et al.,* 1998). Down-regulation of cell surface class I before pp38-expression may help to explain how pp38 positive tumor cells escape immune detection and the weak nature of the CTL activity to this major virus antigen. The loss of cell surface class I is a signal to activate killing by NK cells and may contribute to the robust natural killer activity observed after MDV infection (Lessard *et al.,* 1996; Quere and Dambrine, 1988; Sharma, 1981).

Infection with all three serotypes induces a subpopulation of presumably uninfected OU2 cells to express increased levels of surface class I. This is may be due to the induction of interferon or other cytokines that then up-regulate class I expression on the uninfected population. Importantly, there is heterogeneity among the serotypes used in this study with regard to this activity. The SB1 virus, a common strain used to complement HVT in bivalent vaccines, induces a high level of class I expression in this subpopulation. Induction of cytokines like interferon may be important since they are known to partially overcome loss of antigen presentation in herpesvirus-infected tumor cells (Rowe *et al.,* 1995). Testing

and optimizing this activity may be important for future vaccine design.

We are attempting to identify the viral gene(s) encoding the protein(s) involved in down-regulating the cell surface class I. In HSV and CMV, the US regions contain many of the viral genes affecting antigen presentation. The series of recombinant RB1B Marek's viruses with deletions in MDV specific open reading frames (SORF) 1, 2, 3 and US1, 2, 3, 6, and 10 will be helpful in identifying genetic regions of importance in this regard (Parcells *et al.,* 1994a, b, 1995). Once identified, recombinants lacking these viral genes can be produced and their biologic function tested *in vitro* and *in vivo.* Recombinant viruses lacking the ability to down-regulate class I may have decreased virulence and improved vaccine efficacy.

MATERIALS AND METHODS

Cells and viruses

Virus stocks of HVT (serotype 3) (Okazaki *et al.,* 1970), and SB1 (serotype 2) (Schat and Calnek, 1978) were propagated in chick embryo fibroblasts (CEFs) (Witter *et al.,* 1970). The Md11 (serotype 1) was propagated on duck embryo fibroblasts (DEFs) as described (Witter, 1982). The viruses used were highly tissue culture adapted with over 50 passages *in vitro.*

CHCC-OU2 (OU2) cells are a chemically transformed, virus-free chick embryo fibroblast isolated from chickens of unknown B haplotype (Ogura and Fujiwara, 1987). Flow cytometric analysis of this cell line identified the B13 haplotype and an unknown haplotype similar, but not identical, to B21 (data not shown, for details see Fulton *et al.,* 1996). The MDCC-MSB1 (MSB1) (Akiyama and Kato, 1974), MDCC-UA04 (Dienglewicz and Parcells, 1999), and RP1 (Nazerian *et al.,* 1977) cells are MDV-transformed T-cell lines. The MSB1 (B haplotype unknown) and RP1 (B2/15) are T-cell lines transformed with the BC-1 and JM strains of MDV, respectively (Nazerian *et al.,* 1977; Nazerian and Witter, 1975). UA04 (B haplotype unknown) is a T-cell line transformed by a green fluorescent protein (GFP) recombinant of the RB1B strain of serotype 1 MDV (RB1BUS2gfpΔ) (Dienglewicz and Parcells, 1999). The latent virus in MSB1 and UA04 can be reactivated upon treatment with BUdR (Dunn and Nazerian, 1977). Conversely, the JM virus transforming the RP1 cells is refractory to reactivation and is considered a nonproducer cell line (Nazerian *et al.,* 1977). All cells were grown in Leibovitz-McCoy medium supplemented with antibiotics, 5% fetal bovine serum (FBS), and 12% tryptose phosphate broth (GIBCO, Grand Island, NY).

To induce virus reactivation, the MDV tumor cells were cultured in 10 or 25 μg/ml BUdR (Sigma, St. Louis, MO) for 24 and 48 h. Fresh media containing BUdR was used to maintain cell densities at approximately $1–10 \times 10^6$ cells per milliliter for the 48-h culture period.

The OU2 cells were initially infected with approximately 500 plaque-forming units of each virus stock. All serotypes of MDV are highly cell-associated, spread by cell-to-cell contact, and produce little or no cell-free virus. Initial infection ranges from approximately 20% with serotype 3 to 5% with serotypes 1 and 2. To achieve a high percentage of cells infected with each serotype, the infected OU2 cells were passaged two to four times on subconfluent cultures of OU2 cells until the plaques for each serotype culture were greater than approximately 10 per microscope field (25× magnification). The OU2 cells were then harvested using 0.5% trypsin (GIBCO) in PBS/EDTA (0.5 mM EDTA), washed with PBS, and subsequently analyzed for class I and viral protein expression.

Antibodies and reagents

The chicken class I glycoproteins (BF) were detected by flow cytometry and immunoprecipitation using the C6B12 monoclonal antibody (Shamansky *et al.,* 1988). The MDV antigens were detected using the H19 (serotype 1, anti-pp38), Y5 (serotype 2), and IAN86 (serotypes 1 and 3, gB) monoclonal antibodies (Cui *et al.,* 1990; Silva and Lee, 1984). Chicken CD4 was detected using the CT-4 monoclonal antibody (Chan *et al.,* 1988) (Southern Biotechnology Associates, Birmingham, AL). The secondary reagents used to detect the monoclonal antibodies include fluorescein-conjugated goat anti-mouse, biotin-conjugated goat anti-mouse, PE-CY5-conjugated avidin, and horseradish peroxidase-conjugated goat anti-mouse. All of the secondary reagents were purchased from Southern Biotechnology Associates or Kirkegaard Perry Laboratory, Inc. (Gaithersburg, MD), and used as described by the manufacturer.

Flow cytometry

Cells were washed and resuspended in cold flow cytometry media (FCM) consisting of phosphate-buffered saline (PBS) supplemented with 2% FBS and 0.1% NaN_3. Single-color surface labeling of cells for the BF, CD4, and gB antigens was done by incubating $2–5 \times 10^6$ cells with the appropriate dilution (determined by titration) of primary monoclonal antibody for 20 min on ice. The excess primary antibody was removed by three successive washes with ice-cold FCM followed by incubation of the cells for an additional 20 min with the secondary antibody reagent. The secondary antibody consisted of either fluorescein- or biotin-conjugated goat anti-mouse antibody. The excess secondary antibody was removed by three additional washes. To label the biotin-conjugated antibodies, the cells were incubated for 10 min with PE-CY5-conjugated avidin (1:50). The cells were then washed as above and 1×10^4 cells were analyzed by flow cytometry using a Becton–Dickinson FACSort,

(Becton–Dickinson Immunocytometry Division, San Jose, CA).

For dual-color analysis of class I and internal viral proteins, the cells were first labeled for BF using the single-color fluorescein procedure described above. The cells were then fixed with 4% (w/v) paraformaldehyde (Sigma) in PBS (pH 7.4–7.6) for 20 min, washed in FCM buffer, and permeabilized with 0.1% (w/v) saponin (Sigma) in FCM (permeabilization buffer). The succeeding steps, including antibody dilutions, were carried out in permeabilization buffer. To label intracellular pp38, the cells were incubated with the H19 mAb for 20 min, washed, and incubated for an additional 20 min with biotin-conjugated goat anti-mouse. The cells were again washed and incubated for 10 min with PE-CY5-conjugated avidin. The cells were then washed extensively and analyzed by flow cytometry. Controls included cells incubated with equal concentrations (as determined by OD280) of an isotype control mAb (M1, Kodak, Rochester, NY) and stained as described for pp38. To adjust the FACSort for two-color acquisition fixed but unlabeled, surface label only and intracellular label only was used in addition to the controls listed above.

The UA04 cells express GFP as a marker for viral protein expression and dual labeling simply consisted of developing the C6B12 (class I) antibody with the biotin-conjugated goat anti-mouse and PE-CY5-conjugated avidin using viable, unfixed cells. Controls to adjust the FACSort consisted of UA04 and UA04 plus an isotype control (M1 see above) labeled with the biotin-conjugated goat anti-mouse/PE-CY5-conjugated avidin system.

Immunoprecipitation

Uninfected and HVT-infected OU2 cells were radiolabeled in methionine-free medium (MEM plus 1% dialyzed FBS) containing 50 μCi of [^{35}S]methionine/ml for 4 h at 37°C. Radiolabeled cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.4], 1% DOC, 0.1% SDS, and 1% Triton X-100) and the cell lysates preadsorbed to protein A sepharose (Pharmacia, Uppsala, Sweden) for 30 min at 4°C. Preadsorbed cell lysates were then incubated with monoclonal antibody to MHC Class I (C6B12) for 1 h at 4°C and then with protein A sepharose for 1 h at 4°C. Immune complexes were then washed three times with RIPA buffer, separated on a 9% SDS–PAGE gel (Laemmli, 1975), and proteins visualized by autoradiography. The density of the bands in the autoradiographs were analyzed using the program NIH Image (Version 1.61) according to the program instructions for gel analysis (http://rsb.info.nih.gov/nih-image).

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REFERENCES

Ahn, K., Gruhler, A., Galocha, B., Jones, T. R., Wiertz, E. J., Ploegh, H. L., Peterson, P. A., Yang, Y., and Fruh, K. (1997). The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity* **6**(5), 613–621.

Akiyama, Y., and Kato, S. (1974). Two lymphoblastoid cell lines from Marek's disease. *Biken J.* **17,** 105–106.

Baigent, S. J., Ross, L. J., and Davison, T. F. (1998). Differential susceptibility to Marek's disease is associated with differences in number, but not phenotype or location, of pp38+ lymphocytes. *J. Gen. Virol.* **79**(Pt. 11), 2795–2802.

Baigent, S. J., Ross, L. J. N., and Davison, T. F. (1996). A flow cytometric method for identifying Marek's disease virus pp38 expression in lymphocyte subpopulations. *Avian Pathol.* **25,** 255–267.

Biggs, P. M. (1975). Marek's disease—The disease and its prevention by vaccination. *Br. J. Cancer* **31**(Suppl. 2), 152–155.

Brunovskis, P., and Velicer, L. F. (1995). The Marek's disease virus (MDV) unique short region: Alphaherpesvirus-homologous, fowlpox virus-homologous, and MDV-specific genes. *Virology* **206**(1), 324–338.

Chan, M. M., Chen, C. L., Ager, L. L., and Cooper, M. D. (1988). Identification of the avian homologues of mammalian CD4 and CD8 antigens. *J. Immunol.* **140**(7), 2133–2138.

Chen, X. B., Sondermeijer, P. J., and Velicer, L. F. (1992). Identification of a unique Marek's disease virus gene which encodes a 38-kilodalton phosphoprotein and is expressed in both lytically infected cells and latently infected lymphoblastoid tumor cells. *J. Virol.* **66**(1), 85–94.

Cohen, J. I. (1998). Infection of cells with varicella-zoster virus down-regulates surface expression of class I major histocompatibility complex antigens. *J. Infect. Dis.* **177**(5), 1390–1393.

Cui, Z. Z., Yan, D., and Lee, L. F. (1990). Marek's disease virus gene clones encoding virus-specific phosphorylated polypeptides and serological characterization of fusion proteins. *Virus Genes* **3**(4), 309–322.

Dienglewicz, R. L., and Parcells, M. S. (1999). Establishment of a lymphoblastoid cell line using a mutant MDV containing a green fluorescent protein expression cassette. *Acta Virologica* **43,** 106–112.

Dunn, K., and Nazerian, K. (1977). Induction of Marek's disease virus antigens by IdUrd in a chicken lymphoblastoid cell line. *J. Gen. Virol.* **34**(3), 413–419.

Fulton, J. E., Thacker, E. L., Bacon, L. D., and Hunt, H. D. (1995). Functional analysis of avian class I (BFIV) glycoproteins by epitope tagging and mutagenesis in vitro. *Eur. J. Immunol.* **25**(7), 2069–2076.

Fulton, J. E., Young, E. E., and Bacon, L. D. (1996). Chicken MHC alloantiserum cross-reactivity analysis by hemagglutination and flow cytometry. *Immunogenetics* **43**(5), 277–288.

Galocha, B., Hill, A., Barnett, B. C., Dolan, A., Raimondi, A., Cook, R. F., Brunner, J., McGeoch, D. J., and Ploegh, H. L. (1997). The active site of ICP47, a herpes simplex virus-encoded inhibitor of the major histocompatibility complex (MHC)-encoded peptide transporter associated with antigen processing (TAP), maps to the NH2-terminal 35 residues. *J. Exp. Med.* **185**(9), 1565–1572.

Hariharan, M. J., Nataraj, C., and Srikumaran, S. (1993). Down regulation of murine MHC class I expression by bovine herpesvirus 1. *Viral Immunol.* **6**(4), 273–284.

Hill, A. B., Barnett, B. C., McMichael, A. J., and McGeoch, D. J. (1994). HLA class I molecules are not transported to the cell surface in cells infected with herpes simplex virus types 1 and 2. *J. Immunol.* **152**(6), 2736–2741.

Hinkley, S., Hill, A. B., and Srikumaran, S. (1998). Bovine herpesvirus-1 infection affects the peptide transport activity in bovine cells. *Virus Res.* **53**(1), 91–96.

Hsue, S. M., Raine, L., and Fanger, H. (1981). A comparative study of the peroxidase–antiperoxidase method and an avidin-biotin–peroxidase

complex method for studying polypepetide hormones with radioimmunoassay antibodies. *Am. J. Clin. Pathol.* **75,** 734–738.

Johnson, D. C., and Hill, A. B. (1998). Herpesvirus evasion of the immune system. *Curr. Top. Microbiol. Immunol.* **232,** 149–177.

Jugovic, P., Hill, A. M., Tomazin, R., Ploegh, H., and Johnson, D. C. (1998). Inhibition of major histocompatibility complex class I antigen presentation in pig and primate cells by herpes simplex virus type 1 and 2 ICP47. *J. Virol.* **72**(6), 5076–5084.

Laemmli, U. K. (1975). Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine. *Proc. Natl. Acad. Sci. USA* **72**(11), 4288–4292.

Lessard, M., Hutchings, D. L., Spencer, J. L., Lillehoj, H. S., and Gavora, J. S. (1996). Influence of Marek's disease virus strain AC-1 on cellular immunity in birds carrying endogenous viral genes. *Avian Dis.* **40**(3), 645–653.

Machold, R. P., Wiertz, E. J., Jones, T. R., and Ploegh, H. L. (1997). The HCMV gene products US11 and US2 differ in their ability to attack allelic forms of murine major histocompatibility complex (MHC) class I heavy chains. *J. Exp. Med.* **185**(2), 363–366.

Mellencamp, M. W., O'Brien, P. C., and Stevenson, J. R. (1991). Pseudorabies virus-induced suppression of major histocompatibility complex class I antigen expression. *J. Virol.* **65**(6), 3365–3368.

Nataraj, C., Eidmann, S., Hariharan, M. J., Sur, J. H., Perry, G. A., and Srikumaran, S. (1997). Bovine herpesvirus 1 downregulates the expression of bovine MHC class I molecules. *Viral Immunol.* **10**(1), 21–34.

Nazerian, K., Stephens, E. A., Sharma, J. M., Lee, L. F., Gailitis, M., and Witter, R. L. (1977). A nonproducer T lymphoblastoid cell line from Marek's disease transplantable tumor (JMV). *Avian Dis.* **21**(1), 69–76.

Nazerian, K., and Witter, R. L. (1975). Properties of a chicken lymphoblastoid cell line from Marek's disease tumor. *J. Natl. Cancer Inst.* **54**(2), 453–458.

Neumann, L., Kraas, W., Uebel, S., Jung, G., and Tampe, R. (1997). The active domain of the herpes simplex virus protein ICP47: A potent inhibitor of the transporter associated with antigen processing. *J. Mol. Biol.* **272**(4), 484–492.

Ogura, H., and Fujiwara, T. (1987). Establishment and characterization of a virus-free chick cell line. *Acta Med. Okayama* **41**(3), 141–143.

Okazaki, W., Purchase, H. G., and Burmester, B. R. (1970). Protection against Marek's disease by vaccination with a herpesvirus of turkeys. *Avian Dis.* **14,** 413–429.

Omar, A. R., and Schat, K. A. (1996). Syngeneic Marek's disease virus (MDV)-specific cell-mediated immune responses against immediate early, late, and unique MDV proteins. *Virology* **222**(1), 87–99.

Omar, A. R., Schat, K. A., Lee, L. F., and Hunt, H. D. (1998). Cytotoxic T lymphocyte response in chickens immunized with a recombinant fowlpox virus expressing Marek's disease herpesvirus glycoprotein B. *Vet. Immunol. Immunopathol.* **62**(1), 73–82.

Parcells, M. S., Anderson, A. S., Cantello, J. L., and Morgan, R. W. (1994a). Characterization of Marek's disease virus insertion and deletion mutants that lack US1 (ICP22 homolog), US10, and/or US2 and neighboring short-component open reading frames. *J. Virol.* **68**(12), 8239–8253.

Parcells, M. S., Anderson, A. S., and Morgan, R. W. (1994b). Characterization of a Marek's disease virus mutant containing a lacZ insertion in the US6 (gD) homologue gene. *Virus Genes* **9**(1), 5–13.

Parcells, M. S., Anderson, A. S., and Morgan, T. W. (1995). Retention of oncogenicity by a Marek's disease virus mutant lacking six unique short region genes. *J. Virol.* **69**(12), 7888–7898.

Plachy, J., Pink, J. R., and Hala, K. (1992). Biology of the chicken MHC (B complex). *Crit. Rev. Immunol.* **12**(1, 2), 47–79.

Pratt, W. D., Morgan, R., and Schat, K. A. (1992). Cell-mediated cytolysis of lymphoblastoid cells expressing Marek's disease virus-specific phosphorylated polypeptides. *Vet. Microbiol.* **33**(1–4), 93–99.

Quere, P., and Dambrine, G. (1988). Development of anti-tumoral cell-mediated cytotoxicity during the course of Marek's disease in chickens. *Ann. Rech. Vet.* **19**(3), 193–201.

Rowe, M., Khanna, R., Jacob, C. A., Argaet, V., Kelly, A., Powis, S., Belich, M., Croom-Carter, D., Lee, S., Burrows, S. R., *et al.* (1995). Restoration of endogenous antigen processing in Burkitt's lymphoma cells by Epstein–Barr virus latent membrane protein-1: Coordinate up-regulation of peptide transporters and HLA-class I antigen expression. *Eur. J. Immunol.* **25**(5), 1374–1384.

Schat, K. A. (1991). Importance of cell-mediated immunity in Marek's disease and other viral tumor diseases. *Poult. Sci.* **70**(5), 1165–1175.

Schat, K. A., and Calnek, B. W. (1978). Characterization of an apparently nononcogenic Marek's disease virus. *J. Natl. Cancer Inst.* **60**(5), 1075–1082.

Shamansky, L., Long, X., and Miller, M. M. (1988). Isolation and characterization of reticulendotheliosis virus transformed bone marrow cells from congenic lines of chickens differing at the MHC. *FASEB J.* **2,** A482.

Sharma, J. M. (1981). Natural killer cell activity in chickens exposed to Marek's disease virus: Inhibition of activity in susceptible chickens and enhancement of activity in resistant and vaccinated chickens. *Avian Dis.* **25**(4), 882–893.

Silva, R. F., and Lee, L. F. (1984). Monoclonal antibody-mediated immunoprecipitation of proteins from cells infected with Marek's disease virus or turkey herpesvirus. *Virology* **136**(2), 307–320.

Thacker, E. L., Fulton, J. E., and Hunt, H. D. (1995). In vitro analysis of a primary, major histocompatibility complex (MHC)-restricted, cytotoxic T-lymphocyte response to avian leukosis virus (ALV), using target cells expressing MHC class I cDNA inserted into a recombinant ALV vector. *J. Virol.* **69**(10), 6439–6444.

Ui, M., Endoh, D., Cho, K. O., Kon, Y., Iwata, A., Maki, Y., Sato, F., and Kuwabara, M. (1998). Transcriptional analysis of Marek's disease virus (MDV) genes in MDV-transformed lymphoblastoid cell lines without MDV-activated cells. *J. Vet. Med. Sci.* **60**(7), 823–829.

Uni, Z., Pratt, W. D., Miller, M. M., O'Connell, P. H., and Schat, K. A. (1994). Syngeneic lysis of reticuloendotheliosis virus-transformed cell lines transfected with Marek's disease virus genes by virus-specific cytotoxic T cells. *Vet. Immunol. Immunopathol.* **44**(1), 57–69.

Wiertz, E. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J., and Ploegh, H. L. (1996). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* **84**(5), 769–779.

Witter, R. L. (1982). Protection by attenuated and polyvalent vaccines against highly virulent strains of Marek's disease virus. *Avian Pathol.* **11,** 49–62.

Witter, R. L., Nazerian, K., Purchase, H. G., and Burgoyne, G. H. (1970). Isolation from turkeys of a cell associated herpesvirus antigenically related to Marek/s disease virus. *Am. J. Vet. Res.* **31,** 525–538.