IDENTIFICATION AND CHARACTERIZATION OF GLYCOPROTEIN H OF MDV-1 GA STRAIN

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Summary. – A 2439 bp open reading frame (ORF) was identified from the DNA sequence of *Bam*HI-F and -K2 fragments of Marek's disease virus of serotype 1 (MDV-1) GA strain, which predicts an 813 amino acid polypeptide. This peptide is homologous to HSV-1 gH, and has typical glycoprotein features. There are nine potential N-linked glycosylation sites within the extracellular domain. A fragment of the gH ORF was cloned into pGEX vector in frame with glutathione S-transferase (GST) to produce a GST-gH fusion protein in *Escherichia coli*. The GST-gH fusion protein was used to develop gH monoclonal and polyclonal antibodies. Expression of gH was detected in duck embryo fibroblasts (DEFs) infected with MDV-1 GA strain by immunofluorescence assay (IFA) with these antibodies. Virus neutralization and plaque-forming inhibition analyses were conducted with the gH antiserum. There were no neutralization and plaque-forming inhibition activities of gH antiserum. Comparison of the DNA sequence of gH gene between GA and RB1B strains of MDV-1 revealed major difference in the upstream control elements of gH ORF.

Key words: MDV; HSV; glycoprotein H; DNA; protein sequence

Introduction

It is presumed that enveloped virions of cell-free MDV enter susceptible cells by conventional absorption and penetration, whereas the cell-associated virus infection is initiated by cell-to-cell fusion, or direct contact with infected cells (Hlozanek, 1970). Due to the highly cell-associated nature of MDV, the cell-to-cell transfer is normally accomplished through the formation of intracellular bridges (Kaleta and Neumann, 1977), and is presumed to be the principal mode of virus spread both *in vitro* and *in vivo*. The principal events in the interaction of a virion with cellular membrane are through attachment and penetration. Attachment of virus to a cell surface activates a cellular process mediated by viral surface glycoproteins that lead to the fusion of the viral envelope with the cellular plasma membrane. In HSV, multiple viral glycoproteins are required for these processes, including gB, gC, gD, and gH-L complex. The gB, gC, gD, and gH-L complex act individually or in combination to trigger pH-independent fusion of the viral envelope with the host cell plasma membrane (Spear, 1993).

gH is conserved structurally and functionally throughout the *Herpesviridae* family. Like gB, gH homologues of herpesviruses can induce neutralizing antibodies, affect cell free virion entry into host cells, and also affect virus cell-to-cell transfer (Buckmaster *et al.*, 1984). The mature gH is always expressed on the cell surface, and the surface expression of gH is important for gH function (Buckmaster *et al.*, 1984). In MDV, the DNA sequences of gH homologues of RB1B strain (MDV-1) and HVT (MDV-3) have been reported (Scott *et al.*, 1993), and MDV-2 gH sequence data were also described recently (Shimojima *et al.*, 1997). No detailed study on the expression and characterization of the protein in MDV-infected cells has been reported since 1993. The major problem is that the gH antibodies are not available. In the present report, the gH DNA sequence of MDV GA strain was determined and the antibodies against gH peptide were developed. Serologic features of these antibodies were investigated, and the potential biologic functions of MDV gH are also discussed.

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Materials and Methods

Cells and virus. DEFs and CEFs were grown in Leibovitz-McCoy's medium (GIBCO Laboratories, Grand Island, NY), supplemented with 4% calf serum (growth medium) or 1% calf serum (maintenance medium). MDV-1 GA strain (Eidson and Schmittle, 1968) underwent 25 passages in DEFs or CEFs. Cell-free preparation of GA strain made from feather follicles was a kindly provided by Dr. R.L. Witter (USDA-ARS Avian Disease and Oncology Laboratory, East Lansing, MI).

DNA sequencing was performed on a double-stranded plasmid by the dideoxy chain termination method using [α-^{35}S]dATP (New England Nuclear, Life Science Products, Boston, MA) and the TAQuence version 2.0 DNA Sequencing Kits (United States Biochemical Corporation, Cleveland, Ohio) as suggested by the manufacturer. Both strands of the DNA of *Bam*HI-F and -K2 fragments of MDV GA strain (Fukuchi *et al.*, 1984) were partially sequenced. The sequence data were analyzed with various computer programs (see computer analysis in this section). The junction of *Bam*HI-F and -K2 fragments was confirmed by PCR amplification, and the product was sequenced with an automated sequencer (373A DNA Sequencer, Applied Biosystems, Foster City, CA) by dideoxy sequencing method (Prism, Applied Biosystems, Foster City, CA).

Construction of expression plasmid for GST-gH fusion protein. In order to over-express gH gene, the *Bgl*II-*Eco*RV fragment (0.8 kbp) of gH gene was cloned into plasmid pGEX-5X-3 (Pharmacia Biotech AB, Uppsala, Sweden) at *Bam*HI and *Sma*I sites. An adapter (5'-**GGATCC**GAGCTCG**AGATCT**-3') was obtained by cloning the gH *Bgl*II-*Eco*RV fragment into pBlueBac4 (Invitrogen Corporation, Carlsbad, CA) at *Eco*RV-*Bam*HI and cutting out with *Bgl*II-*Eco*RV. This adaptor allows that the gH fragment fills in GST ORF to generate GST-gH fusion protein expression vector pGST-gH. The construction was confirmed by restriction patterns and sequence analysis.

GST-gH fusion protein and anti-gH antibodies. The expression and purification of GST-gH fusion protein were according to the manufacturer's procedure (Pharmacia Biotech AB, Uppsala, Sweden). Female mice were inoculated subcutaneously with 100 μg/mouse (50 μl in PBS) of purified GST-gH fusion protein emulsified in equal volume of Titlemax research adjuvant #R-1 (CytRx Corporation, Norcross, GA) at 2 sites in the base of tail. Each mouse was boosted with 100 μg of same protein intraperitoneally 3 weeks post-inoculation. Four days later, the spleen cells were isolated and fused with myeloma cells line, NS-1 cells. The hybridomas were screened by ELISA against GST-gH and GST proteins. The rabbit antiserum was produced by inoculating a New Zealand white rabbit with about 200 μg (100 μl in PBS) of GST-gH fusion protein emulsified in Titlemax research adjuvant #R-1. The rabbit was given two boosts at 4 and 8 weeks, subcutaneously with 200 μg GST-gH emulsified in #R-1 at first boost, and intravenously with 200 μg GST-gH at second boost. The rabbit was bled 10 days after the last booster.

Indirect immunofluorescence assay (IFA). CEFs were grown in a monolayer on glass coverslips. The monolayer was infected with MDV GA strain at about 200 PFU per 60 mm tissue culture dish. When lytic plaques were observed, the coverslips were harvested by washing once in PBS, fixed for 5 mins in ice-cold acetone, and air-dried. Fixed coverslips were stored at -20°C for use. IFA was performed as previously published (Wu *et al.*, 1997). The samples were observed under confocal microscope (Carl Zeiss, Inc, New York, NY) with 40x oil lane, 488 argon laser line with green (BP521-650) barrier filter, fluorescence operation and confocal modes.

Virus neutralization and plaque-forming inhibition assays. Secondary DEF monolayers on 35-mm tissue culture dishes were used for the virus neutralization and the plaque-forming inhibition assays. The rabbit antiserum and the pre-bleeding negative control serum were inactivated at 56°C for 30 mins. For neutralization, 10-fold diluted sera (10^{-1}–10^{-3}) in SPGA-EDTA (218 mmol/l sucrose, 3.8 mmol/l M KH_2PO_4, 7.2 mmol/l K_2HPO_4, 4.9 mmol/l monosodium glutamate, 1% BSA, and 0.2% EDTA in water) were incubated with known amount of cell-free virus (GA strain, 50 PFU/dish) for 30 mins on ice. The serum-virus mixtures (100 ml per plate) were inoculated into secondary DEF monolayers. The virus was allowed to absorb for 30 mins at 37°C, and 2 ml of maintenance medium was added. The plaques were counted 7 days post-inoculation. For plaque-forming inhibition assay, about 50 PFU of cell-free virus GA strain was inoculated into secondary DEF monolayers, and allowed to absorb for 30 mins at 37°C; then the maintenance medium with 10-fold diluted sera (10^{-2} and 10^{-3}) was added. The plaques were counted 7 days post-inoculation.

Computer analysis. Several computer programs were used for different purposes. DNASTAR package (DNASTAR Inc. Madison, WI) was used for sequence data input, multiple sequence analysis, and phylogenic tree generation. MacVector Program (Scientific Image systems, New Haven, CT) was used to find the ORF from the DNA data, to translate ORF to polypeptide, and to generate protein profiles. GCG package (Genetics Computer Group, Madison, WI) was used for pairwise comparison (GAP Program) and homologous searches (FASTA Program). Oligo Program (National Biosciences, Inc., Plymouth, MN) was used to design the oligonucleotide primers for PCR and sequencing. Blast Program at National Center of Biotechnology Information was used for searching homologues, and retrieving the sequence data from Genbank and Swiss-Port databases.

Results

Analysis of the nucleotide and amino acid sequences of gH gene of MDV-1 GA strain

The nucleotide sequence of *Bam*HI-F and -K2 fragments (Fukuchi *et al.*, 1984) of MDV-1 GA strain was partially determined in order to obtain complete ORF of gH gene. An overall map of the corresponding genomic region is shown in Fig. 1. Computer analysis of the partial sequence revealed 3 ORFs corresponding to HSV-1 UL21, UL22 (gH gene) and UL23 (TK gene, partial) (McGeoch *et al.*, 1988), respectively. The gH ORF has a size of 2,439 bp with an average base composition of 30.4 % A, 19.1 % G, 19.7 % C and 30.8 % T. Its ORF is

leftward, and located downstream of TK ORF (Fig. 1). The DNA sequence of gH gene was compared with that of gH gene of MDV-1 RB1B strain (Scott *et al.*, 1993). The results indicated that only three nucleotide substitutions were present within the ORFs at nt 1414 (T to C), 2377 (T to G), and 2378 (T to G). These substitutions resulted in two substitutions at aa 472 (valine to alanine) and 793 (isoleucine to arginine) between GA and RB1B gH peptides. The major difference in the DNA sequence of the gH genes between GA and RB1B strains was located in the upstream at nt –55 to –129 (Fig. 2). We found a potential „TATA" box at nt –113 and a „CAT" box at nt –127 in the DNA sequence of GA but not in that of the RB1B. The polypeptide predicted from the nucleotide sequence comprises 813 amino acids with a calculated M_r 90.9 kDa. There are four hydrophobic helices in the gH precursor, located at aa 1-18, 619-641, 660-682, and 770-792 (Table 1) according to the results predicted by SOSUI system (Mitaku Laboratory, 1996). The N-terminal helix (aa 1-18) appears to be the signal sequence. The C-terminal helix (aa 770-792) may be the transmembrane domain. The other two helices may interact with or span the membrane. There is a very short cytoplasmic domain located in the C-terminus at aa 793-813. There are 9 potential N-linked glycosylation sites located in proposed extracellular domain.

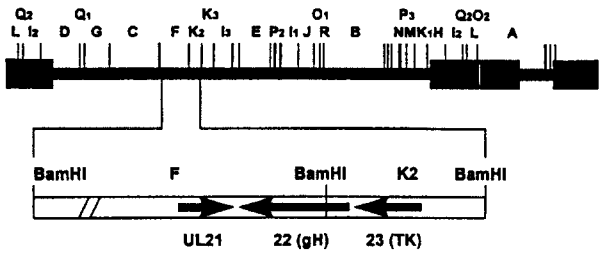

Fig. 1
The location of gH and the adjacent genes on the genome of MDV-1 GA strain
The upper part of the schematic map shows the *Bam*HI restriction map. The *Bam*HI-F and -K2 fragments are enlarged in the lower part of this map. Three potential ORFs are indicated with arrows and labeled. The directions of the arrows represent the direction of transcription. The gH ORF is extended from the *Bam*HI-K2 to -F fragments. The U_L21 ORF is located at 294 bp downstream of the gH stop codon.

Comparison of MDV gH with homologues of other herpesviruses

Total of 16 gH sequences were obtained from Genbank, and Swiss-Prot databases. The source for each gH and the database accession number are listed in Table 2. The multiple alignment analysis was done by the cluster method of Multiple Sequence Alignment Program in DNASTAR package. The rooted phylogenic tree was generated within same program (Fig. 3). This tree shows three clusters of the gH proteins which agree basically with the classification of α-, β-, and γ-herpesviruses. The gHs from β- and γ- herpesviruses are more closely related to each other. MDV gHs are related to, but not absolutely within the cluster of α-herpesviruses.

Anti-gH antibodies are gH-specific and react with MDV-infected DEF cells

The antibodies were developed according to the procedures described in the Materials and Methods; a total of 9 MAbs and one rabbit serum were obtained. The specificity of these antibodies was confirmed by ELISA with GST-gH fusion protein and GST protein. All these antibodies were positive for GST-gH fusion protein and negative for GST protein, except MAb 90, which was also positive for GST protein. MAb 32 was used to detect the expression of gH from MDV GA-infected DEF cells. A representative plaque (with some single cells and some multinuclear syncytial cells) is shown in Fig. 4. The fluorescence was mainly present in the cytoplasm, and absent from the nucleus.

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        -169
GA      tatcgcgctt ctataattag cttgcccaca tcacaatgat gcggcaatat tgacttatat
RB1B    ---------- ---------- ---------- ---------- aacttattat tggtccatgc

        -109
GA      taagatagta atttggcgtc cttagatcca ataaatatcc atgatttagt aagtgtgttc
RB1B    tagaatagtc atacgctacg atctgttgct atatatgacc atcgccaaac      -----

        -49                                                  -1+1
GA      atacggatcg tagcacttgc aagttgcatt ggatggctac atatccaacA TGggtcttcc
RB1B    ---------- ---------- ---------- ---------- ---------- ----------
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Fig. 2
Comparison of the upstream DNA sequences of gH gene between GA and RB1B strains of MDV-1
The position of a nucleotide is referenced to the start codon of gH ORF, counting as +1 at the A of ATG, which is underlined and bolded. The first nucleotide left of the A is numbered as –1. The matched nucleotide in RB1B is represented by dash (–). Different nucleotides are aligned and bolded in both GA and RB1B. At least, a „TATA" box at nt –113 and a „CAT" box at nt –127 (underlined) were absent from RB1B sequence.

Table 1. Locations and amino acid sequences of 4 hydrophobic helices of MDV gH precursor predicted by SOSUI system

Helix No.	N-terminal (aa)	aa sequence	C-terminal (aa)	Type	Length
1	1	MGLPGSIVFLIMIHAFCA	18	Primary	18
2	619	ADILEATALLVLPISGLGSYVVT	641	Primary	23
3	660	NQLYITYVRLPCTTTAGNIVPMV	682	Secondary	23
4	770	TYVATATAGASIAISIAIITVRM	792	Primary	23

Table 2. List of database resources of gH homologues of herpesviruses

No.	Viruses	Subfamilies	Accession Nos.	References
1	HSV-1	α	P06477	(McGeoch and Davison, 1986)
2	Bovine herpesvirus type 1.1 (BHV-1.1)	α	P27599	Direct submission
3	PRV	α	P27416	(Klupp and Mettenleiter, 1991)
4	Feline herpesvirus type 1 (FHV-1)	α	S64566	(Maeda *et al.*, 1993)
5	EHV-1	α	P09101	(Telford *et al.*, 1992)
6	VZV	α	P09260	(Davison and Scott, 1986)
7	HVT	α	P36337	(Scott *et al.*, 1993)
8	MDV-1 GA strain	α		
9	MDV-1 RB1B strain	α	P36336	(Scott *et al.*, 1993)
10	Herpes simplex virus type 7 (HSV-7)	β	P52353	Direct submission
11	HCMV	β	P12824	(Cranage *et al.*, 1988)
12	EBV	γ	P03231	(Baer *et al.*, 1984)
13	Equine herpesvirus 2 (EHV-2)	γ	S55616	(Telford *et al.*, 1995)
14	Kaposi's sarcoma-associated herpesvirus (KSAH)	γ	U75698	(Chang *et al.*, 1994)
15	Bovine herpesvirus-4 (BHV-4)	γ	Z79633	(Lomonte *et al.*, 1997)
16	herpesvirus saimiri (SHV)	γ	P16492	(Gompels *et al.*, 1988)

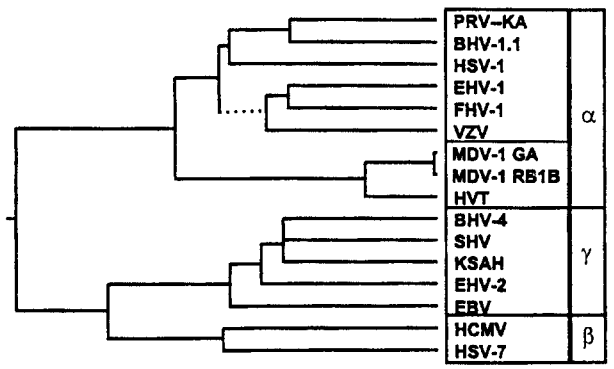

Fig. 3
Phylogenic tree of gH homologues of herpesviruses
Total of 16 gH homologues of herpesviruses were analyzed. The multiple alignment analysis was done by the cluster method of Multiple Sequence Alignment Programs in DNASTAR package. The rooted phylogenic tree was generated within same program. The group of the 16 gHs was exactly matched with the classification of herpesviruses. Within α-herpesviruses, MDV gHs were first separated from others. The sequence resources were listed in Table 3.

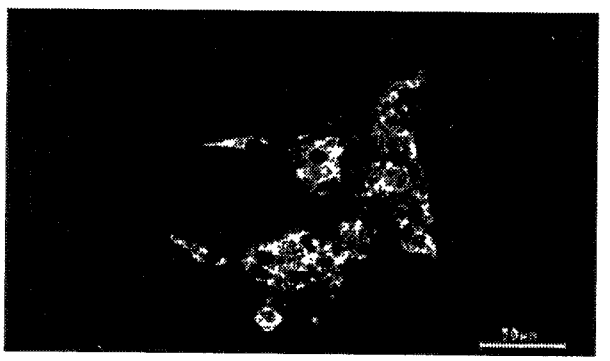

Fig. 4
Microphotograph of gH in DEFs infected with MDV GA strain
MAb 32 (1:100) was used to detect gH expression in MDV-infected DEFs. One plaque was examined under laser scanner confocal microscope. The fluorescence is mainly present in the cytoplasm, leaving a black hole in the nuclear area. Bar = 50 mm.

The product of BglII-EcoRV fragment of gH gene does not induce neutralization nor plaque-forming inhibition antibodies

Both the serum neutralization and plaque-forming inhibition tests were conducted with anti-GST-gH rabbit serum. The *Bgl*II-*Eco*RV fragment of gH is the main antigenic region of gH molecule according to the antigenecity profile generated by MacVector Program. The result of virus neutralization test is shown in Fig. 5. The plaque-forming inhibition assay was composed of two trials, one trial with 4 repeats, and the other with 8 repeats. The results are sum-

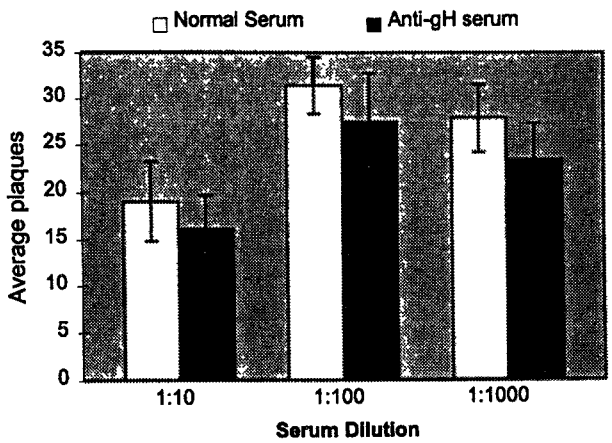

Fig. 5
Results of neutralization tests
Average numbers of plaques were obtained from 8 single observations. Columns represent average numbers of plaques. Vertical bars represent standard deviations.

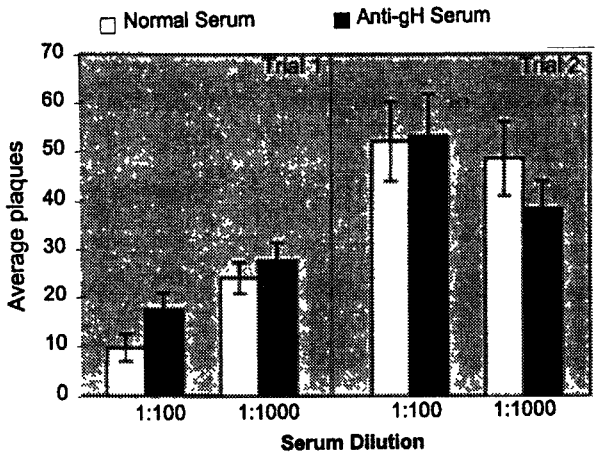

Fig. 6
Plaque-forming inhibition tests
Average numbers of plaques (columns) were obtained from 8 single observations in trial 1, and from 4 single observations in trial 2. Vertical bars represent standard deviations.

marized in Fig. 6. In both assays, there were no significant differences between the normal serum and the anti-gH serum.

Discussion

The MDV DNA is a linear double-stranded molecule, composed of 166–184 kbp (Lee *et al.*, 1971). The *Bam*HI restriction map has been constructed from MDV-1 GA strain (Fukuchi *et al.*, 1984), which became a basis for most MDV gene identification and localization. The gH gene of MDV-1 RB1B strain has been mapped to *Bam*HI-K2 (one quarter) and -F (three quarters) fragments (Scott *et al.*, 1993). About same time, the *Bam*HI-K2 and -F fragments of GA strain (Fukuchi *et al.*, 1984) were also sequenced to determine the location of gH (Lee, unpublished data). Pairwise comparison of GA and RB1B gH DNA and deduced amino acid sequences revealed that the gH coding region is 99.7% conserved. The major difference between GA and RB1B gH gene is not within the coding region, but in the upstream control elements of the gH ORFs. These differences have resulted in some changes in the potential transcription elements. The biological effects of these changes on the gH expression or virus infectivity remains to be studied.

The deduced gH amino acid sequence is also compared with the homologues of other herpesviruses, including α-, β-, and γ- herpesviruses. A phylogenic tree was generated from the multiple sequence alignment. All of the 16 entries (including 3 MDV gH homologues) are grouped into 3 main clusters, which basically agree with the classification of α-, β-, and γ-herpesviruses. Although closely related to α-herpesvirus, the MDV subgroup is not absolutely located within the α-herpesvirus cluster. This separation may reflect the features of MDV in both biological properties (closer to γ-herpesviruses) and genomic structure and gene organization (closer to α-herpesviruses).

In some herpesviruses, such as equine herpesvirus 1 (EHV-1) (Robertson *et al.*, 1991), EHV-4 (Nicolson *et al.*, 1990), PRV (Klupp and Mettenleiter, 1991), and infectious laryngotracheitis virus (ILTV, another avian herpesvirus) (Ziemann *et al.*, 1998), the sequences downstream of gH ORF appear very active. Some direct repeat elements and putative replication origin (Ori) sequences have been found in this area. In PRV and ILTV, this region even becomes the junction for the internal reversion within the U_L region (Ziemann *et al.*, 1998). Whereas in MDV, just like HSV-1 and varicella-zoster virus (VZV), the homologous or equivalent region of the direct repeat elements and Ori sequence are not present downstream of gH ORF.

A number of distinct hydrophobic regions were identified from MDV gH precursor. Two primary hydrophobic stretches were noted at both the N- and C-termini, which are likely to represent signal and anchor (transmembrane domain) sequences. The homologues of these two stretches can be found in the HSV-1 gH molecule. However, compared with HSV-1 gH protein, MDV gH has third primary hydrophobic stretch which is located at aa 619-641, and does not have homology to HSV-1 gH. This fragment may also function to anchor the protein to the cell membranes. A secondary hydrophobic stretch is closer to the third primary hydrophobic stretch, and is unlikely to be membrane spanning, but may be associated with cell membrane. Therefore, from aa 619-792, the MDV gH possesses a large hy-

drophobic area in the C-terminus which may tightly interact with cell membranes. The above described feature may reflect on the highly cell-associated properties of MDV.

The biological role of gH appears to be in virus entry into host cells (penetration) and its subsequent cell-to-cell spread. Strong neutralization antibodies have been raised against the gH proteins of HSV-1 (Showalter *et al.*, 1981), VZV (Keller *et al.*, 1984), human cytomegalovirus (HCMV) (Rasmussen *et al.*, 1984), and EBV (Strnad *et al.*, 1982). The plaque-forming inhibition activities were also present with these neutralization antibodies (Desai *et al.*, 1988). Similar activities are not found with our rabbit serum against the MDV GST-gH fusion protein. Since our GST-gH fusion protein was made from a small portion of gH gene (*Bgl*II-*Eco*RV fragment, 0.8 kbp), it is likely that this fragment does not contain the neutralization epitope. Therefore, the failure in detecting virus neutralization and plaque-forming inhibition with our anti-gH serum may not be a representation of real biological features of the entire gH molecule.

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