CONSTRUCTION AND CHARACTERIZATION OF A H19 EPITOPE POINT MUTANT OF MDV CVI988/RISPENS STRAIN

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Summary. – A recombinant virus, CVI/rpp38, was developed from the Marek's disease virus (MDV) CVI988/Rispens vaccine strain. This recombinant was obtained by transfection of CVI988/Rispens-infected chich embryo fibroblasts (CEFs) with plasmid pHA25 DNA containing pp38 gene from GA strain of MDV. Monoclonal antibody (MAb) H19 which reacts with pp38 from GA but not with that from CVI988 was used to screen for recombinant viruses in transfected cell culture plates by immunofluorescent assay (IFA). A positive plaque was isolated, propagated, and purified from cell-free virus particles after sonication of infected CEFs. The mutant CVI/rpp38 was not only reactive with MAb H19 in IFA but also in immunoprecipitation. A 38 kDa protein was immunoprecipitated from the CVI/rpp38 mutant virus but not from parental CVI988 virus. DNA sequence of the mutant virus showed a substitution of G at position 320 by A resulting in a change of an amino acid residue from arginine to glutamine. Comparison of nucleotide sequence of pp38 from strains GA, Md5 and Md11/75c/R2 and CVI988 revealed change to glutamine in this position. The result of this study provides a direct evidence for the location of the identified H19 epitope in pp38. This mutant is potentially useful to further explore the biological function of pp38 and its H19 epitope.

Key words: MDV; pp38; monoclonal antibody H19; point mutant

Introduction

The 38 kDa phosphorylated protein (pp38) of MDV was among the first MDV genes identified and characterized. It is unique for MDV and no homologs exist in other herpesviruses reported to date. pp38 is a 290-amino-acid protein, rich in acidic residues, and translated from an unspliced message. The gene is located in the *Bam*HI-H fragment of MDV genome and encompasses a significant portion of the long inverted repeated region. Also located in this region are 132 bp repeats which undergo amplification during prolonged passages of MDV which correlates with the attenuation of viral oncogenicity (Silva and Witter, 1985). The promoter-enhancer region of pp38 gene overlaps that of the transformation-related 1.8 kbp gene family which is also located in *Bam*HI-H fragment (Bradley *et al.*, 1989; Cui *et al.*, 1991). The pp38 gene and the 1.8 kb transcript apparently form a district transcription unit and are thus likely to be coordinately regulated.

The pp38 has been considered a transformed cell-associated antigen of MDV (Ikuta *et al.*, 1985). Only recently, Xie *et al.* (1996) reported that induction of pp38 antisense RNA in MSB cell line had a singificant effect on the colony formation by MSB1 cells in soft agar. This result suggests that pp38 may be involved in the maintenance of transformation of MSB1 cells.

We have been interested in the biological function of pp38 in MDV tumors and cell lines. We attempted to generate sufficient amount of pp38 protein in baculovirus in order to study the effect, if any, of pp38 administration in chicks. Our recent finding showed that the baculovirus recombinant pp38 expressed in insect cells depressed immune responses of chicks to mouse red blood cells (Cui *et al.*, 1996). This experimental system, however, does not allow us to consider directly the biological role of pp38 in MDV pathogenesis. Deletion or substitution mutants would be more useful for this study.

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MDV CVI988/Rispens strain is widely used as an effective vaccine strain for protection against Marek's disease. This is the only strain of MDV-1 in which the pp38 protein is not recognized by MAb H19 (Lee *et al.*, 1983). Recently, we sequenced CVI988/Rispens strain and compared it with four other MDV-1 strains, GA, Md11/75c/R2, Md5 and CVI988 (Cui *et al.*, 1991; Chen *et al.*, 1992; Edoh *et al.*, 1994; Iwata *et al.*, 1992). We found that the H19-recognizing epitope was located between aa 27 and 180 of the pp38 polypeptide corresponding to nt 81 and 541 of pp38 open reading frame (ORF) (Cui *et al.*, 1990, 1991). Detailed comparison of this region showed that the substitution of A by G at position 320 in CVI988 may be responsible for not having the H19 epitope shared by other MDV-1 strains. The present study was to construct a H19 epitope as a point mutant of CVI988/Rispens strain and to characterize the mutant virus for use in determining the biological activities of its pp38.

Materials and Methods

Virus. MDV CVI988/Rispens strain was a stock (about passage 45) maintained in the Avian Disease and Oncology Laboratory (Witter *et al.*, 1995). To avoid the possibility that the stock may be contaminated with MAb H19-reactive ($H19^+$) viruses, CEF monolayers infected with the same stock in six 60 mm dishes were examined by IFA test with MAb H19.

Construction and screening of H19 epitope point mutant in pp38 of CVI988/Rispens strain. CVI988/Rispens-infected CEF monolayers in 60-mm dishes were transfected with plasmid pHA25 DNA containing an intact pp38 gene from nt –580 to nt +1910 (Cui *et al.*, 1991) by using LipofectAMINE Reagent (Life Technologies, Gibco-BRL) at 12 hrs after infection. The procedure of transfection was according to the manufacturer's instructions. Several trials were made by using different doses of LipofectAmine, various concentrations of plasmid DNA, and various transfection exposure times. The primary transfected CEF monolayers were maintained in culture media for 3 days, resuspended by trypsinization and the cell suspension from a 60 mm dish were plated into a 96-well plate. These plates were cultured for additional 2–3 days. When plaques were visible, 50 µl of trypsin solution was added to each well and incubated at 37°C for 3–5 mins. Calf serum (25 µl) was added to these cultures to inactivate the trypsin. By using an 8-channel pipet, CEFs in each well were suspended, then 20 µl of suspension from each well was transferred into a coresponding well of duplicate new 96-well plates with fresh CEF monolayers. The newly infected CEF cultures were incubated for about 3 days, and then one of each paired duplicate plates was kept in the incubator and the other was fixed with acetone : alcohol (2:1) and stained with MAb H19 and goat anti-mouse FITC conjugates. The viruses in the duplicate wells with IFA-positive plaque(s) were further passed into fresh CEFs and rescreened.

Cloning of the $H19^+$ mutant virus. First, the plaques positive in IFA with MAb H19 were purified twice by limited dilutions through several passages. Only the plaque, from which all plaques of the next generation were positive in IFA, was recognized as a purified H19+ mutant plaque. The mutant virus from the plaque was further amplified through several passages in CEFs until sufficient amount of virus to seed 150-mm plates was obtained. At this time, the virus from 150-mm plates was resuspended in SPGA buffer and sonicated to obtain cell-free virus preparations (Calnek *et al.*, 1970). The sonicated cell lysates were centrifuged at 10,000 rpm for 10 mins and the supernatant was filtered through a 0.45 µ filter. The filtered cell-free virus suspension was serially diluted 1:5 and 10 ml of each dilution was inoculated into a 96-well plate with fresh CEF monolayers. All plates were incubated for 7–8 days. The wells with only one plaque were used for further screening and cloning. The cell-free virus cloning procedure was repeated once.

^{35}S-methionine labeling and immunoprecipitation. CEF monolayers infected with CVI988 and the mutant viruses were labeled with ^{35}S-methionine and immunopreciptitated (Wu *et al.*, 1997) with MAb H19 (Lee *et al.*, 1983) and BA4 specific to gB1 (Cui *et al.*, 1996).

DNA sequencing of CVI/rpp38 mutants. DNA was extracted from CEFs infected with CVI/rpp38 mutant viruses and used as template for PCR reaction. The forward primer 5'-AGGGACCGGTGGAGA TTC (nt +190 to nt +207 of pp38 ORF) and the reverse primer 5'-CTGCCGTTCTACCGACTAAC (nt +751 to nt +732) were used in the amplification. The PCR product was cloned into pCR2.1 TA cloning vector (Invitrogen) and sequenced by using an automated sequencer (373A DNA sequencer, Applied Biosystems) and the dideoxy sequencing method (Prism, Applied Biosystems).

Results

Identification and screening of $H19^+$ mutants of CVI988/Rispens strain

Seven different transfections were carried out and the $H19^+$ mutant virus plaques were consistently detected by IFA with MAb H19. The recombination ratios varied in the range of 0.1% to 0.003% depending on different transfection conditions, doses of LipofectAMINE, plasmid DNA concentration, and transfection exposure time. In some experiments, H19 FA^+ plaque(s) was detected directly in the original transfected CVI988-CEF monolayers. In most cases, we found positive plaques after one amplification through a blind passage into 96-well plates (Fig. 1).

$H19^+$ mutant and $H19^-$ native CVI988/Rispens clones

To purify the $H19^+$ mutant virus, the positive plaques were screened by IFA with MAb H19 from wells with only one plaque by limited dilutions in 96-well plates and the same procedure was repeated once to further purify the cell-associated virus. To clone the $H19^+$ mutant virus, CEFs infected with the purified plaque were sonicated, and the cell-free virus was screened by IFA selecting only wells with one plaque. From the first screening of the cell-free $H19^+$ mutant virus, 7 of 8 plaques were positive with MAb H19 by IFA. After the

second screening of cell-free virus plaques, all 16 tested plaques were positive in IFA. The $H19^+$ mutant virus clone was designated as CVI/rpp38. During the second plaque purification, an IFA-negative plaque was also amplified and the infected CEFs were sonicated. One $H19^-$ plaque was chosen from the cell-free virus stock by the same procedure as for H19-positive clone and designated as CVI988/4.

Comparison of DNA sequences in the H19 epitope region of pp38 genes between the mutant CVI/rpp38 and its parent CVI988

From the DNA sequence comparison, the only difference in pp38 ORF between native CVI988 and the mutant CVI/rpp38 was the substitution of G by A at position 320 in the mutant. As a consequence, the arginine at position 107 of pp38 was substitued by glutamine in the mutant. This substitution is the basis for mutant CVI/rpp38 virus gaining the MAb H19-recognized epitope and differring from its parent CVI988/Rispens strain which was not recognized by MAb H19.

Immunoprecipitation the mutant CVI/rpp38 pp38

Virus-infected CEF monolayers were labeled with ^{35}S-methionine and immunopreciptitated with Mab H19 (pp38) and MAb BA4. As shown in Fig. 2, MAb H19 did not immunoprecipitate pp38 from the native CVI988-infected CEFs (lane D) but immunoprecipitated pp38 and pp24 from the mutant CVI/rpp38-infected CEFs (lane C). As expected, MAb H19 also immunoprecipitated pp38 and pp24 from two other pathogenic MDV-infected CEFs (GA, lane A and Md5, lane B). MAb BA4 specific for glycoprotein gB1 of MDV was used as control to ensure sufficent antigen labeling in MDV-infected cells. Fig. 2 (right panel) shows that three bands typical for gB were present in all lanes. Both native and recombinant CVI988 had similar gB bands.

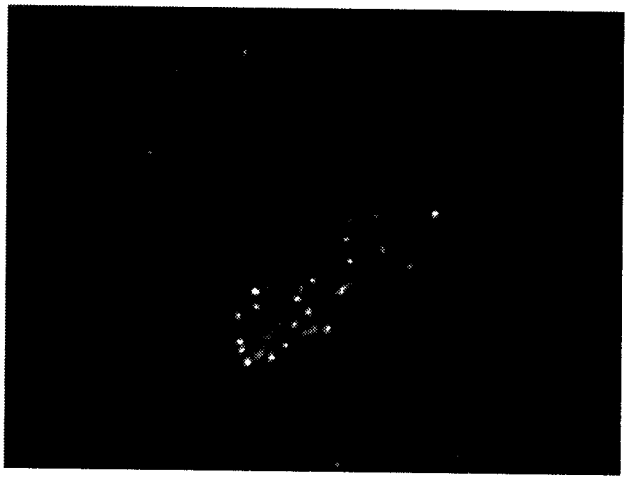

Fig. 1
MAb H19-positive plaques in IFA from the second passage recombinant CVI988/Rispens-infected CEFs

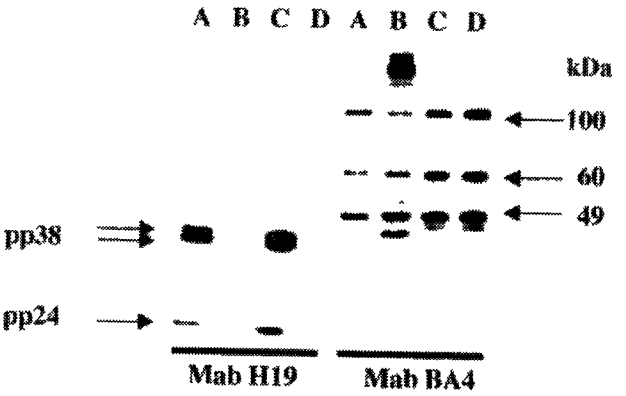

Fig. 2
Immunoprecipitation of ^{35}S-methionine labeled CEFs
Cells infected with GA (A), Md5 (B), the mutant CVI/rpp38 (C) and CVI988/4 (D) precipitated with MAb H19 and MAb BA4. MAb H19 is specific for pp38 and Mab BA4 is specific for gB1.

Discussion

It is well known that all pathogenic MDV strains cause immunodepression in infected chickens but vaccine strain CVI988/Rispens of serotype 1 does not (Witter *et al.*, 1995). The mechanism responsible for the difference in immunodepressive ability of the MDV is not clear. We have tested all the known serotype 1 MDVs and have found that they share a MAb H19 epitope within their pp38 except for CVI988 or CVI988/Rispens. In addition, our previous study showed that the recombinant pp38 from insect cells immunodepressed the humoral responses of chicks to mouse red blood cells (Cui *et al.*, 1996). Therefore, it would be of interest to see if there is a relationship between the MDV-induced immunodepression and H19-epitope in pp38. Since we have previously determined that CVI988/Rispens does not have H19 epitope, we thought a point mutation of this virus would clear our speculations.

From analysis and comparison of our pp38 sequence data (Cui *et al.*, 1990, 1991) with those of the published data (Chen *et al.*, 1991; Edoh *et al.*, 1994; Iwata *et al.*, 1992), it was clear that all serotype 1 strains have identical nucleotides in the region except positions 320 and 326 which varied between G and A from strain to strain regardless whether they reacted with MAb H19 or not. However, all 3 MAb H19-reactive strains GA, Md5 and Md11/75c/R2 had A at nt 320. The only H19-negative strain CVI988 had G at the same position and this change resulted in an amino acid substitution from glutamine

Table 1. Comparison of H19 epitope sequences of pp38 from various MDV strains

Strains	H19 epitope	Nucleotide sequences						
		313		320[a]		326[b]		333
Md11/75c/R2	+	CTG	TCA	CAG	TGG	GAG	GAG	CTA
Md5	+	CTG	TCA	CAG	TGG	GAG	GAG	CTA
GA	+	CTG	TCA	CAG	TGG	GGG	GAG	CTA
CVI988	–	CTG	TCA	CGG	TGG	GGG	GAG	CTA
CVI/rpp38	+	CTG	TCA	CAG	TGG	GGG	GAG	CTA
		Amino acids						
Strains	H19 epitope	105	106	107[a]	108	109[b]	110	111
Md11/75c/R2	+	L	S	Q	W	E	E	L
Md5	+	L	S	Q	W	E	E	L
GA	+	L	S	Q	W	G	E	L
CVI988	–	L	S	G	W	G	E	L
CVI/rpp38	+	L	S	Q	W	G	E	L

[a]The position directly related to H19 epitope.
[b]The position where changes do not affect H19 epitope.

to arginine (Table 1). This finding suggests that the substitution of G by A at nt 320 may be the basis for CVI988 lacking the H19 epitope. The recombinant CVI/rpp38 provides a direct evidence for the location of the identified H19-epitope.

To compare the biological characteristics of both native CVI988 and its mutant CVI/rpp38, some preliminary experiments in chickens were carried out. The antibody responses to the recombinant CVI/rpp38 ($H19^+$) were delayed and antibody titers were significantly lower than those of CVI988 ($H19^-$) in line 15 x 7 chickens (data not shown). This result seems to correlate with our published data related to immunodepressive effect of pp38 of GA strain of MDV (Cui *et al.*, 1996).

The major difficulty in developing MDV-1 mutants resides in the cell-associated nature of MDV. Morgan *et al.* (1990) have been successful in developing a deletion mutant of MDV by cotransfection of CEFs with MDV genomic DNA and plasmid DNA carrying the gene of interest. In this study, we developed a point mutant of CVI988 by transfection of infected cells directly with plasmid DNA containing pp38. The recombination ratio was estimated in the range of 0.003% to 0.1% in 7 experiments with different transfection conditions. To avoid the possibility of H19+ viruses presence in the original CVI988/Rispens stock, we screened six 60 mm dishes with CVI98/Rispens-infected CEFs and none was found to contain H19 IFA-positive plaques. To further confirm the recombinant CVI/rpp38 is not a contaminant of GA, we performed PCR to verify the pattern of 132 bp repeats region of MDV-1 (Silva, 1992). The patterns of 2–7 repeat ladders were the same among CVI988/Rispens, CVI988/4 and the recombinant CVI/rpp38 viruses but different in the pathogenic MDV strains (data not shown). In addition, we have repeated the same procedure by transfection of cloned CVI988/4 and obtained another recombinant designated as CVI/rpp38A (data not shown). The procedure used in this study offers an alternative strategy for developing MDV mutants.

Four MDV genes are reported to be involved in the maintenance of transformation (Xie *et al.*, 1996), pp38 is one of them. These authors also speculated that no single viral gene is sufficient for the induction and maintenance of transformation, but rather expression of several viral genes is necessary. The study reported here could serve as a model to explore MDV gene or genes involved in such phenomenon as immunodepression or other functions related to epitopes.

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