Detection of the *meq* gene in the T cell subsets from chickens infected with Marek's disease virus serotype 1

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(Accepted for publication : June 15, 2005)

Abstract

The *meq* gene was thought to be only detected in Marek's disease virus serotype 1 (MDV1) including a very virulent strain, Md5, while L-*meq*, in which a 180-bp sequence is inserted into the *meq* open reading frame, is found in other strains of MDV1, such as CVI988/R6. However, both *meq* and L-*meq* were previously detected by PCR in chickens infected with MDV1, suggesting that MDV1 may consists of at least two subpopulations, one with *meq*, the other with L-*meq*. To further analyze these subpopulations, we analyzed the time course changes in distribution of these subpopulations among T cell subsets from chickens infected with MDV1. Both *meq* and L-*meq* were detected in CD4\(^+\) and CD8\(^+\) T cells infected with strain Md5 or CVI988/R6. The shift in MDV subpopulations from one displaying *meq* to the other displaying L-*meq* and/or the conversion from *meq* to L-*meq* occurred mainly in the CD8\(^+\) T cell subset from Md5-infected chickens. PCR products corresponding to L-*meq* rather than *meq* were frequently amplified from the CD8\(^+\) T cell subset from CVI988/R6-infected chickens. These results suggest that a dominant subpopulation of MDV1 changes depending on the T cell subsets, and that L-*meq* is dominantly present in the CD8\(^+\) T cells which play a role in the clearance of pathogenic agents.

KEY WORDS : Marek's disease virus, *meq*, L-*meq*, CD4\(^+\) T cell, CD8\(^+\) T cell

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**Introduction**

Marek’s disease virus (MDV) is the causative agent of Marek’s disease (MD), which is characterized by CD$^+$ T cell lymphoma and nerve enlargement caused by infiltration of lymphocytes and/or lymphoma cells$^{1,6}$. The pathogenesis of MD can be chronologically divided into three phases: early cytolysis; latent infection; and secondary cytolysis with immunosuppression and tumor development$^{1,14,15}$. Early cytolysis occurs mainly in B cells, and latently infected lymphocytes are mainly activated T cells$^{19}$. In addition, target cells for transformation by MDV are mainly CD$^+$ T cells, suggesting that latent infection in this T cell subset could be intimately related to the subsequent transformation by MDV$^{20}$.

MDV strains are classified into three serotypes based on their pathogenicity, and only strains of MDV serotype 1 (MDV 1) are oncogenic. An MDV 1-specific gene, *meq*, has been identified, and its gene product, Meq, a basic leucine zipper (bZIP) oncoprotein, has been detected in all tumor samples and established MDV-transformed T-cell lines examined thus far, suggesting that Meq protein plays an important role in the transformation by MDV$^{19,10,15,16}$. However, the role of the Meq protein in MDV transformation remains to be determined. In the genome of strains of vvMDV 1, the *meq* open reading frame (ORF) encoding a 339-amino-acid bZIP protein, is present,

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**MDV genome**

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<td>Leucine Zipper</td>
<td>Transactivation Domain</td>
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<tr>
<td>VS-Meq</td>
<td>1</td>
<td>78</td>
<td>120</td>
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*Fig. 1. Structures of the meq ORF in MDV1. The meq ORF of oncogenic MDV 1, Md5, encodes a 339 amino-acid protein consisting of an N-terminal proline-glutamine-rich region (Pro/Gln), the basic region, and the leucine zipper, as well as the transactivation domain. In the case of L-meq found in the MDV tumor cell lines, MSB 1 and MTB 1, and JM attenuated by serial passage, 60 amino acids (a 180-bp sequence) are inserted into the transactivation domain. In the case of S-Meq and VS-Meq, 92 amino acids (a 276-bp sequence) and 41 (a 123-bp sequence) are deleted in the transactivation domain of the Meq protein, respectively. The proline-rich repeat region is composed of 6 copies of the repeats in Meq, 9 copies in L-Meq, 4 copies in S-Meq, and 2 copies in VS-Meq.*
while a slightly longer meq ORF, termed L-meq, in which a 180-bp sequence is inserted into the meq ORF, is found in other strains of MDV, such as JM and CVI988/R6. Furthermore, short meq (S-meq) and very short meq (VS-meq), in which 123 and 275-bp sequences are deleted in the meq ORF, have been also detected, respectively. This diversity in meq is due to the difference in the copy number of the proline-rich repeat region (Fig. 1)\(^\textsuperscript{5,7}\). When the meq gene was monitored in chickens infected with vvMDV, both the meq and L-meq genes were detected, suggesting that MDV consists of some subpopulations though the possibility of conversion from meq to L-meq can not be ruled out\(^\textsuperscript{6}\). Since an MDV subpopulation displaying the L-meq gene is detected at the latent phase, the L-meq and its gene product, if any, might contribute to the maintenance of the MDV latency\(^\textsuperscript{2}\). Though the shift in subpopulations and/or conversion of the meq genes have been observed in chickens infected with MDV 1, these changes in the T cell subsets have not been elucidated. Thus, in this study, to characterize the shift in subpopulations and/or conversion of the meq genes in T cell subsets, especially CD4\(^+\) T cells, targets for transformation by MDV 1, we have analyzed the time course changes in the meq genes detected in T cell subsets from chickens infected with MDV 1. In addition, we have also compared this shifts/conversion in the T cell subsets from both MDV-sensitive and -resistant lines of SPF chickens.

Materials and methods

Chickens

Fertilized eggs of commercial chickens were purchased from Hokuren Co. Ltd. (Sapporo, Japan) and two lines of SPF chickens (MHC B\(^\textsuperscript{18}\) B\(^\textsuperscript{19}\) sensitive to MD: B19 and B\(^\textsuperscript{21}\) B\(^\textsuperscript{22}\) resistant to MD: B21)\(^\textsuperscript{23}\) were obtained from Nisseiken Co. Ltd. (Oume, Japan). These eggs were hatched in our laboratory, and chickens were raised in isolators.

Viruses and cells

A strain of very virulent MDV 1, Md5,\(^\textsuperscript{20}\) was obtained from chicken kidney cell culture from experimentally infected chickens. A vaccine strain of MDV 1, CVI 988/R6, was purchased from Gehn Corp. Inc. (Gifu, Japan). These viruses were propagated in chicken embryo fibroblasts (CEF). When cytopathic effects were confluent, the infected cells were harvested, and virus titers were measured by plaque assay. These infected CEFs were used for viral inoculation of chickens.

Inoculation of MDVs and sample collection from infected chickens

Groups of 3-day-old chickens were inoculated intraperitoneally with 2,000 plaque forming unit (PFU)/0.2ml of Md5, or 10,000 PFU/0.2ml of CVI988/R6. We used B19 and B21 chickens for the inoculation of Md5 or CVI988/R6. The spleens were collected from 4 chickens from each group infected with MDV every week pi. for 10 weeks.

Isolation of CD4\(^+\) and CD8\(^+\) T cells

Peripheral blood mononuclear cells (PBMC) were obtained by homogenizing the spleens between two frostended glass slides and subsequent centrifugation over Ficoll-Conray gradients to remove dead cells and red blood cells. PBMC (5 × 10\(^7\)) were incubated with 1 ml of monoclonal antibodies (mAbs), anti-chicken CD4\(^+\) mAb (CT4\(^+\))\(^\textsuperscript{21}\) or anti-chicken CD8\(^+\) mAb (11-39)\(^\textsuperscript{22}\) (culture supernatant), for 30min at 4 °C. After incubation, cells were washed twice with DMEM/F\(_2\)12 (GIBCO BRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS: Filtron, Brooklyn, Australia), 5 × 10\(^{-5}\)
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M 2-mercaptoethanol (2-ME), 100 IU/ml penicillin, and 100μg/ml streptomycin (complete DMEM/F-12). These cells were then resuspended in 500μl of complete DMEM/F-12 containing 2 × 10^7 of sheep anti-mouse IgG magnetic beads (Dynabeads M-450, Dynal Inc., Oslo, Norway), and incubated at 4°C for 45 min with gentle rotation. After incubation, cells attached to the beads were trapped by a magnetic field. To separate the cells from the beads, the cells binding to the beads were cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY) containing 10% heat-inactivated FCS, 5 × 10^-5 M 2-ME, 100IU/ml penicillin, and 100μg/ml streptomycin, at 40°C for 3.5 hours in a humidified atmosphere containing 5% CO₂. Flowcytometric analysis revealed that the purity of each T cell subset separated by this procedure ranged from 92 to 98%. The isolated CD4⁺ and CD8⁺ T cells were used for the extraction of total cellular DNA.

**Extraction of DNA from T cell subsets**

Total cellular DNA was extracted from CD4⁺ and CD8⁺ T cell subsets using SepaGene (Sankoujinya Co., Japan). The DNA was treated with RNase and re-extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1), precipitated with ethanol, and dissolved in distilled water.

**Nested polymerase chain reaction (PCR)**

Total cellular DNA samples extracted from each of T cell subsets of infected chickens were used as a template for nested PCR amplification to detect the *meq* gene. The first round of PCR was performed with a primer set, M-S and M-AS, to amplify the 1.0-kb *meq* or 1.2-kb L-*meq* gene fragments as described by Lee *et al*⁸. After the amplification, 1 μl of the reaction was used for the second round of PCR. The second round of PCR was performed in the 20μl reaction mixture containing 1.5mM MgCl₂, and a primer set, 10pM of MR-S (5'-AGTTGGCTTGTCATGAGCCAG-3') and MR-AS (5'-TGTTCCGGATCCTCGGTAA GA-3'), to amplify a 0.6-kb *meq* or 0.8-kb L-*meq* fragment. Amplification was carried out over 35 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1.5 min. The amplified fragments were separated on an agarose gel (1.5%) and visualized by staining with ethidium bromide.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total cellular RNAs were extracted from CD4⁺ and CD8⁺ T cell subsets using the Trizol reagent (Invitrogen, CA). Lymphocytes separated by immuno-magnetic beads were resuspended in 1 ml of Trizol reagent, and 200μl of chloroform was added. After centrifugation at 12,000 × g for 10 min at 4°C, 500μl of isopropanol was added to the resultant supernatants. Total cellular RNA was precipitated by centrifugation at 12,000μg for 10 min at 4°C after incubation at room temperature for 10 min. The resultant RNA was dissolved in diethylpyrocarbonate-treated water, and used as template to synthesize cDNA by using RAV 2 reverse transcriptase (Takara, Japan). The cDNA samples were used to amplify the *meq* transcripts by PCR using the M-S and M-AS primer set and MR-S and MR-AS primer set as described above.

**Results**

**Detection of the *meq* gene in the T cell subsets from chickens infected with vvMDV 1, Md5**

When the *meq* gene was monitored in the T cell subsets from chickens infected with Md5, the *meq* gene was detected in CD4⁺ and CD8⁺ T cells from the infected chickens throughout the 10 weeks though the intensities of the bands corresponding to the *meq* gene detected were variable (Figs. 2-4). The
L-meq gene was also detected in both the CD4+ and CD8+ T cells after 3 weeks pi. Interestingly, the frequency of the chickens from which the L-meq gene was transiently increased in the CD4+ T cell from Md5-infected chickens at 3 to 6 weeks pi, presumably the latent phase of the MDV infection, and then reduced after 7 weeks pi (Figs. 3-4). However, this frequency was lower in the case of CD8+ T cells at 3 to 6 weeks pi, while higher after 7 weeks pi. In addition, 0.5kb-short meq (S-meq) was occasionally detected at 2 to 3 and 6 to 7 weeks pi. These changes were observed both in the B19 and B21 chickens, but the de-
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When the meq genes were monitored in chickens infected with CVI 988/R 6 in which the L-meq gene was already identified (11, 18), the L-meq gene was consistently detected in both CD4+ and CD8+ T cells from the infected chickens throughout the experimental period. The meq gene was also detected in the CD4+ and CD8+ T cells, though the frequency of the chickens from which the meq gene was detected was lower than that from which the L-meq gene was detected (Figs. 2-4). These frequencies were gradually decreased after 7 weeks pi. In addition, 0.5kb-S-meq was detected temporarily at 2 to 3 or 6 to 8 weeks pi. These changes were observed both in B19 and in B21 chickens. The detection frequency of the meq genes was lower in B21 than B19 chickens throughout the experimental period (Figs. 3-4).

Detection of the meq gene in the T cell subsets from chickens infected with CVI 988/R 6

To determine whether the meq genes present in T cell subsets were transcribed, RT-PCR analysis was performed. As shown in Fig. 5, when RNA samples were analyzed from infected chickens at 6 weeks pi, the meq gene was abundantly transcribed in the CD4+ T cells from Md5-infected chickens from which both the meq and L-meq genes were detected. On the other hand, both the meq and L-meq genes were transcribed in the CD8+ T cells from these chickens. Only the meq transcript was detected in CD4+ and CD8+ T cells from chickens from which the meq gene was detected (data not shown).

In the case of CVI 988/R 6-infected chickens, the L-meq gene was transcribed in both the CD4+ and CD8+ T cells though the meq transcript was also detected in the CD4+ T cells. Only the L-meq transcript was detected in the CD8+ T cells.
in CD4+ and CD8+ T cells from chickens from which the L-meq gene was detected (data not shown).

**Discussion**

There is evidence that the meq gene and its gene product, Meq, play important roles in transformation of CD4+ T cells by MDV. The Meq protein is consistently expressed in the vast majority of MD-transformed cell lines and CD4+ T cells obtained from lymphomas. MDV 1 mutants lacking a functional meq failed to cause tumors in chickens. Polymorphism of the meq genes in the attenuated MDV 1, MDV-tumor cell lines and chickens infected with MDV 1 has been observed. This polymorphism suggested that MDV 1 consists of at least 3 subpopulations, virus displaying the meq, L-meq and S-meq genes, respectively. During the MDV 1 infection, a dynamic shift from one subpopulation of MDV 1 to another may occur due to some selective pressures by host chickens. In addition, the L-meq gene product suppressed the transactivation activity of the Meq protein of oncogenic MDV 1, and replication of vvMDV 1 carrying the meq gene was partially suppressed in cells transfected with the L-meq gene.

In this study, we monitored the meq gene in the T cell subsets from chickens infected with strains of oncogenic and nononcogenic MDV 1 for 10 weeks pi. Surprisingly, both the meq and L-meq genes were detected in the T cell subsets from infected chickens during experimental periods. In addition, S-meq has also been detected temporarily though biological significance of the S-meq gene and its gene product remains unknown. It is of interest that, in chickens infected with vvMDV 1, Md5, the L-meq gene was detected during the
latent phase (3–6 weeks pi.) in CD4+ T cells which are the target cells for latent infection and subsequent transformation by oncogenic MDV 1, though the meq gene was consistently detected throughout the experimental period. In the case of chickens infected with CVI 988/R6, the L-meq but not meq gene was consistently detected in both the CD4+ and CD8+ T cells. The detection frequency of the meq genes in the chickens was decreased more rapidly in the CD8+ than in the CD4+ T cells. These results suggest that a major subpopulation displaying the meq gene in the CD4+ T cells would be important for the transformation by Md5, and that the presence of a subpopulation displaying the L-meq gene may contribute to the maintenance of MDV latency. Furthermore, the presence of the major subpopulation with the L-meq gene in the CD8+ T cells, as detected in CVI 988/R6 (vaccine)-inoculated chickens, suggest that the L-meq gene product recognized by CD8+ T cells could play a role in the cell-mediated immunity against MDV infection. It should be noted that the L-meq rather than meq gene is dominantly detected in MD tumor cell lines.

The shift in subpopulations and/or conversion of the meq genes mentioned above was observed in both the B19 chickens sensitive to MD, and the B21 chickens resistant to MD. Interestingly, detection frequency of the meq gene was decreased faster in both the CD4+ and CD8+ T cells from B21 chickens than from B19 chickens. The reason for this observation remains unknown, but these differences observed between two lines of chickens may be intimately related to the resistance to tumor formation by MDV 1.

Thus, MDV 1 could potentially express not only the meq but also L-meq gene in each of the T cell subsets from the infected chickens dependent upon the phase of MDV 1 infection. In the future, it will be necessary to analyze the roles of the L-meq and S-meq gene and/or their gene products in viral transcription and transformation and immune responses to MDV infection.

References


