MOLECULAR CHARACTERISTICS OF VERY VIRULENT EUROPEAN MDV ISOLATES

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Summary. – Marek's disease virus (MDV) strains with increasing virulence have been reported from many parts of the world. Many of these recent MDV isolates produce an acute early cytolytic disease with high mortality and severe atrophy of the lymphoid organs, thymus and the bursa of Fabricius. Although the degree of the atrophic changes and the virulence of the virus are correlated, the molecular basis of the increased virulence is not known. We examined the characteristics of the disease induced by 3 such MDV isolates, C12/130, MR36 and MR48, isolated from Europe. All the three viruses produce high early mortality and atrophy of the lymphoid organs. As a first step in understanding the determinants of the increased virulence of these isolates, we have compared the sequences of MEQ and the ICP4 genes of these three viruses with that of the published sequences. Some of the amino acid changes seen within the Meq and ICP4 proteins were conserved in all the three isolates and could account for the increased virulence characteristics.

Key words: MDV; pathotypes; MEQ; ICP4

Introduction

Despite of the widespread use of Marek's disease (MD) vaccines, often comprising one, two or sometimes even three MDV serotypes, MD still poses great threat to poultry industry because of the emergence of new MDV pathotypes. In terms of the relative virulence, these new pathotypes represented a continuum of viruses with increasing virulence. Based on the data from extensive pathogenicity studies,

Witter (1997) classified these isolates as m (mild), v (virulent), vv (very virulent) and vv+ (very virulent+) MDV pathotypes. In addition to the increased oncogenic potential, many of these isolates produced acute cytolytic disease with heavy mortality and massive atrophy of lymphoid organs. Using selected isolates representing different pathotype groups, Calnek *et al.* (1998) observed that the degree of bursal and thymic atrophy, measured by both relative organ weights and histopathology, was directly related to the virulence of the pathotype.

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Recently, we have reported an isolate of MDV from the United Kingdom, designated C12/130, showing high virulence based on severe early cytolytic disease, high early mortality and marked persistent atrophy of the lymphoid organs (Venugopal *et al.*, 1996). Two other MDV isolates, designated MR36 and MR48, producing a very similar disease with high early mortality with massive destruction of lymphoid tissue have also been isolated from Europe (Kross *et al.*, 1998). The severe destruction of the lymphoid organs during infection with these viruses resulted in overwhelming immunosuppression and could account for the high levels of early mortality. The molecular basis of the increased virulence or cytolytic nature of the disease induced by these viruses is not clear. During our preliminary studies on C12/130 virus (Venugopal *et al.*, 1996), we have demonstrated by *in situ* hybridization that two MDV genes, ICP4 and MEQ, are strongly expressed both in the thymus and bursa undergoing cytolytic infection. In this paper, we compared the characteristics of the disease induced by three European isolates of MDV, C12/130, MR36 and MR48, with that of HPRS-16. We also compared the sequences of ICP4 and MEQ genes of these three isolates with that of the published sequences to examine whether these gene sequences could be associated with the cytolytic nature of the disease.

Materials and Methods

Viruses. Plaque-purified stocks of MDV strains C12/130 and HPRS-16 were prepared on chick kidney cells (CKC) or chick embryo fibroblasts (CEF) using standard methods (Churchill and Biggs, 1968). Virus stocks were also prepared from strains MR36 and MR48 (Kross *et al.*, 1998). All the virus stocks were tested negative for chicken infectious anemia and avian leukosis viruses by PCR.

Experimental design. One-day-old specific-pathogen-free HPRS-RIR birds maintained at the Institute for Animal Health, Compton were used in the study. The birds were randomly divided into groups I, II, III, IV and V and were injected intraperitoneally with 10^3 PFU of HPRS-16, C12/130, MR36, MR48 and PBS, respectively, and maintained in separate rooms. Three birds per group were killed at day 7 and 14, and whole body weights as well as weights of spleen, bursa and thymus were determined and the relative organ weights, expressed as percent of the whole body weight, were calculated. Atrophic changes in the tissues revealed by histological examination were scored as (–) (no changes), (+) (10%), (++) (up to 50%) and (+++) (above 50%).

Polymerase chain reaction (PCR), cloning and sequencing. Total DNA was isolated from CEF or CKC infected with the C12/130, MR36 and MR48 strains of MDV following proteinase K digestion using standard methods (Sambrook *et al.*, 1989). MEQ genes of the three viruses were amplified from each of the DNA preparation by PCR using primers derived from the published sequences in the database (Jones *et al.*, 1992). Similarly, overlapping clones of the ICP4 gene were also amplified from each of the DNA preparation using primers derived from published sequences (Anderson *et al.*, 1992). The PCR products were gel purified and cloned into pGEM-T vector (Promega) following protocols supplied by the manufacturers. The sequences were determined with vector-specific and internal oligonucleotide primers using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase FS (Perkin-Elmer) on an ABI 373 DNA sequencer. Sequence analysis was performed using the Genetics Computer Group software package.

Results and Discussion

Compared to the uninfected control and HPRS-16 MDV-infected birds, most of the birds infected with the recent isolates C12/130, MR36 and MR48 were noticeably ill at about 8–9 days post-infection (PI) and subsequently died or had to be killed *in extremis*. The mortality rates in the birds infected with these isolates increased rapidly and at day 12 PI, the cumulative mortality rates reached 60% (MR36 and MR48) and above 40% (C12/130) compared to about 15% in HPRS-16 infected birds at this period (Fig. 1). Similar high early mortality rates have also been observed in previous studies with these viruses (Kross *et al.*, 1998; Venugopal *et al.*, 1996). There are also reports from other countries showing that many of the recent MDV isolates induce a severe cytolytic disease with high early mortality

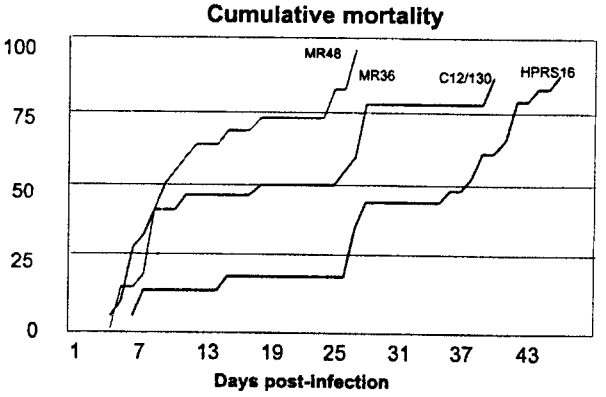

Fig. 1
Mortality rates in the birds experimentally infected with different strains of MDV

rates (Imai *et al.*, 1992; Calnek *et al.*, 1998) confirming the apparent increase in virulence of the recent MDV isolates (Witter, 1997).

There was extensive damage to the bursa and thymus in early infection (day 7 PI) with the 3 isolates compared to the uninfected and HPRS-16 virus-infected birds while the spleen showed increase in relative weight (Fig. 2). Damage to the thymus and bursa in birds infected with these viruses were also observed in previous studies (Venugopal *et al.*, 1996; Kross *et al.*, 1998). Similar observations were also made by other workers in infections with many recent MDV isolates (Imai *et al.*, 1992) suggesting that the ability to induce thymic and bursal damage may be one of the virulence characteristics of these virus isolates. Furthermore, direct correlation between virulence of the pathotypes and the degree of thymic and bursal damage has been demonstrated (Calnek *et al.*, 1998). Histologically, the thymus and bursa from birds infected with C12/130, MR36 and MR48 viruses showed massive depletion of lymphocytes (Table 1) with necrosis and destruction of the architecture at day 7 PI. Compared to the birds infected with less virulent vMDV strains, where the lymphoid organs recover from the early atrophic changes during 8–14 days PI, the recovery is minimal in birds infected with vvMDV and vv+MDV pathotypes (Calnek *et al.*, 1998). Previously with C12/130 virus we have shown that, unlike HPRS-16 infection, the destruction of these organs was permanent with little signs of recovery (Venugopal *et al.*, 1996). The experimental infection in the present study was conducted in RIR birds that are very susceptible to MD. However, the early atrophic changes in the lymphoid organs induced by the virulent pathotypes do occur both in genetically resistant and susceptible chickens (Calnek *et al.*, 1998; Venugopal *et al.*, 1996), and the host genetic resistance factors may not be

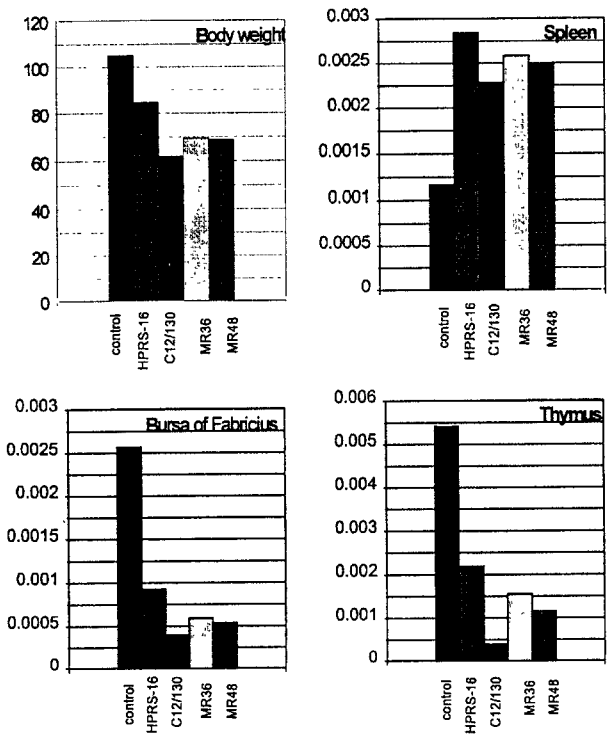

Fig. 2
Body weight and the relative weights of spleen, bursa and thymus at day 7 PI

Table 1. Histopathological changes in the thymus and bursa at days 7 and 14 PI in birds infected with MDV

	Thymus		Bursa of Fabricius	
	Day 7	Day 14	Day 7	Day 14
Control	–	–	–	–
HPRS-16	+	+	+	+
C12/130	++	+++	+++	+++
MR36	+++	+++	+++	+++
MR48	+++	+++	+++	+++

operational in the early infections probably due to the severe immunosuppressive effects.

In order to see whether these isolates replicated faster thereby infecting more lymphocytes, we determined number of infected peripheral blood cells from birds infected with different viruses. There was no significant difference in the numbers of infected cells in the peripheral blood between any groups including those infected with HPRS-16 (data not shown). Calnek *et al.* (1988) noted that, compared to vMDV infection, birds infected with vv+MDV and vvMDV pathotypes fail to control the cytolytic infection and hence do not enter the latency. It is not clear whether the sequence changes in the viral genes of the recent isolates are responsible for their increased virulence. By using *in situ* hybridization, we showed that two MDV genes, MEQ and ICP4, are highly expressed in the thymus and the bursa during early cytolytic infection with C12/130 (Venugopal *et al.*, 1996). In order to understand the molecular determinants of increased virulence, we decided initially to compare the sequence of these two genes from the three viruses with the published sequences to identify any conserved sequence changes among the three isolates.

MEQ gene from all the three isolates encoded the 339-amino-acid Meq protein with conserved features such as the N-terminal basic region, the leucine zipper motif and a C-terminal proline-rich region. The C-terminal 33 amino acid residues shown to be critical for the transactivation function (Qian *et al.*, 1996) were fully conserved in all the three viruses. Alignment of the sequences showed two types of sequence changes: those that were seen only in the individual viral sequences and those that were conserved among all the three isolates. Two substitutions, K>E (position 77) and D>Y (position 80), seen in all the three isolates were located close to the BR2 region (Liu *et al.*, 1997). The basic region of Meq protein is divided into BR1 and BR2, each containing stretch of at least 5 Arg/Lys residues. Mutational studies have shown that BR2 is the major nuclear localization signal (NLS) and the sole nucleolar localization signal (NoLS), while BR1 serves an auxiliary function in the nuclear translocation of Meq protein (Liu *et al.*, 1997). BR2 domain is further divided into BR2N and BR2C containing 5 and 6 consecutive Arg/Lys residues, respectively. Although either one of these residues was sufficient for its NLS function, the entire BR2 region was required for the NoLS function (Liu *et al.*, 1997). It is not known whether the nucleolar translocation function of the Meq protein could be affected by the loss of one Lys residue due to the K>E mutation at position 77 and by the D>Y mutation in the flanking region of the Meq protein of these isolates.

ICP4 gene of MDV, like that of other herpesviruses, functions as transactivator and regulates the expression of several viral genes. On the basis of homology among different herpesviruses, the ICP4 protein is subdivided into 5 domains. The overall domain structure of the ICP4 from the 3 viruses was very similar to the published sequences (Anderson *et al.*, 1992), with the putative autoregulatory sequence and polyadenylation signals conserved. The Ser tract, the hallmark of the domain 1 is present in all the three virus sequences, although MR48 sequence shows a S>C mutation at position 198. C12/130 and MR36 sequences also show a G>S change at position 207. However, these changes in the Ser tract are thought to be less significant as HSV mutants

without the Ser stretch were still viable (Patterson and Everett, 1990). Three amino acid substitutions, C>L (position 529), S>L (position 585) and N>H (position 600) were present in the sequence of all the three isolates. Although MDV ICP4 shares the domain structure with other herpesviruses, whether the domain functions are conserved has not been tested. Hence the significance of these mutations is hard to predict. The functional significance of these mutations can be understood only by further mutational studies of the MDV genome.

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