MAREK'S DISEASE AND NEW APPROACHES TO ITS CONTROL

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Summary. – Marek's disease (MD) is a lymphoproliferative disorder induced by a herpesvirus. Several factors, including those virus-encoded and host-dependent, affect the course of the disease. Existing vaccination program is based on the use of attenuated strains of MD virus (MDV) serotype 1 and on strains of non-oncogenic serotype 2 (MDV2) and serotype 3 (herpesvirus of turkeys – HVT) viruses. Failures resulting in disease progress have been reported and indicate need for production of new, more effective vaccines. It is likely that future development of MD vaccines will rely on recombinant molecules technology.

Marek's disease

MD is a lymphoproliferative disease that occurs worldwide and causes significant economic losses in commercial chicken flocks. The disease is named after J.Marek, the Hungarian veterinary pathologist born in Horná Streda (now in Slovak Republic) who first described it. Its pathology is rather more complex and embraces disorders of (a) the lymphoid system, as revealed by lymphoma development and immunosuppression, (b) the nervous system, as expressed by the peripheral and more rarely central neuropathies, (c) the cardiovascular system, epitomized by atherosclerotic lesions; and (d) epithelial lesions, as revealed in the feather follicles (Payne, 1985).

Etiological cause of MD was discovered in late sixties by *in vitro* propagation and subsequent isolation of a herpesvirus from MD tumour cells (Churchill and Biggs, 1967). The isolation of MDV and its characterization as an herpesvirus also ended the dispute on the etiological relationship between MD and avian lymphoid leukosis, which is caused by retroviruses. Together with Epstein-Barr virus (EBV), MDV is thus one of a few herpesviruses capable of inducing tumours in their natural hosts. The discovery of a herpesvirus as the causative agent of neoplastic transformation has been one of the hallmarks of cancer research because MDV-infected birds provide an excellent model to study biological, genetic and immunological aspects of lymphoma development. Another significant breakthrough in this area of research was the introduction of efficient vaccines that were based on non-oncogenic strains of closely related herpesvirus of turkeys (HVT) or on attenuated and non-oncogenic strains of MDV. These findings have been the first example of prevention of cancer induced by tumour virus.

Despite the introduction of vaccines, precise mechanisms of MDV oncogenicity remains unclear. Furthermore, new, highly oncogenic strains of MDV were isolated that are capable to induce disease in HVT-vaccinated chickens. In addition to pathogenesis and oncogenicity of MDV strains, there are also other factors that can affect tumour development (e.g. host age, sex, genotype, haplotype etc.). These questions have stimulated further studies of MDV oncogenicity, pathogenesis and immunology and have demanded preparation of new, more effective vaccines against MD.

Related strains of MDV and HVT

First oncogenic MDV strains were isolated in late sixties (Churchill and Biggs, 1967; Solomon *et al.*, 1968). Shortly, an antigenically related herpesvirus (HVT) was isolated from healthy turkeys (Kawamura *et al.*, 1969; Witter *et al.*, 1970). The existence of a third related group of viruses was demonstrated by Biggs and Milne (1972). It is now generally accepted that MDV and HVT isolates can be divided into three serotypes (Schat, 1985). All oncogenic MDV form serotype 1, while nononcogenic viruses belong to serotype 2. HVT isolates are classified as serotype 3 (Ta-

ble 1). This classification was first proposed by von Bülow and Biggs (1975) according to immunofluorescence assays and was further confirmed by other immunochemical methods [agar gel precipitation and indirect fluorescent antibody assays (Schat and Calnek, 1978); virus-neutralization tests (King *et al.*, 1981); two-dimensional gel patterns of virus-specific polypeptides (van Zaane *et al.*, 1982*a*,*b*)]. During the last decade differences between serotypes were further confirmed by modern immunological methods using monoclonal antibodies (e.g. Lee *et al.*, 1983) and by methods of molecular biology.

Table 1. Strains of related herpesviruses from chickens and turkeys

Virus type	Serotype	Oncogenicity[a]	Representative isolates
MDV-1	1	Very high	RB-1B, Ala-8, Md/5, Md11
		High	GA, HPRS-16, JM
		Moderate	HPRS-17, Conn-A
		Low	Cu-2, CVI988
MDV-2	2	None	HPRS-24, SB-1, HN-1
HVT	3	None	FC126, WTHV-1, HPRS-26

MDV – Marek's disease virus; HVT – herpesvirus of turkeys.
[a]Very high: causes Marek's disease in vaccinated, genetically susceptible and non-vaccinated, resistant chickens; High: causes high incidence in susceptible, but low or none incidence in resistant chickens; Moderate: causes moderate or low incidence in genetically susceptible birds; Low: causes minimal lesions, often only in very susceptible chickens.

It is not known what is the cause of different oncogenic potential of various serotype 1 MDV strains. Two-dimensional gel pattern analysis of the virus-induced polypeptides did not identify any differences between GA-5, K, Cu-2 and CVI 988 strains (van Zaane *et al.*, 1982*a*). A repeated passage of serotype 1 viruses in cell culture lead to an attenuation resulting in loss of oncogenicity (Churchill *et al.*, 1969*a*; Nazerian, 1970). The attenuation has also been described for HVT, where it resulted in inability to replicate *in vivo* (Witter and Offenbecker, 1979).

Molecular biology of MDV and HVT

Genetic structure of MDV and HVT

Genomes of both MDV and HVT consist of linear double-stranded DNA. MDV DNA is approx. 175 kbp and that of the HVT is 150 kbp long (Cebrian *et al.*, 1982; Lee *et al.*, 1971). By isopycnic sedimentation analysis it was predicted that MDV DNA consists from 46% guanine plus cytosine residues (Lee *et al.*, 1971). Based on their biological properties, especially on their lymphotropism, MDV and HVT are classified as gammaherpesviruses (Roizman, 1992). However, electron microscopy studies revealed that genomes of MDV and HVT contain inverted repeat nucleotide sequences similarly as in case of alphaherpesviruses (Cebrian *et al.*, 1982). These observations were further confirmed by restriction endonuclease mapping of MDV (Fukuchi *et al.*, 1984) and HVT (Igarashi *et al.*, 1987) DNAs. Collinear parts of MDV and HVT genomes share extensive homology (70-80%) as demonstrated by low-stringency hybridizations (Gibbs *et al.*, 1984; Igarashi *et al.*, 1987).

Recent sequencing data of some MDV and HVT genes further confirmed that MDV- and HVT-encoded proteins display higher homology to those encoded by alphaherpesviruses, such as herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV), than to gammaherpesvirus proteins. Buckmaster *et al.*(1988) sequenced small, randomly obtained parts of MDV and HVT genomes. In this way they identified and localized 35 MDV and 24 HVT genes that encode proteins with evident homology to VZV proteins. In addition, identified genes were found to be collinear with their VZV homologues. Fully sequenced genes include those encoding proteins involved in nucleic acids metabolism [e.g. HVT and MDV thymidine kinase (Scott *et al.*, 1989)], proteins regulating gene expression [US1 homologues in MDV and HVT (Brunovskis and Velicer, 1992; Zelník *et al.*, 1993); MDV homologues of ICP4 (Anderson *et al.*, 1992), VP16 (Yanagida *et al.*, 1993; Koptidesová *et al.*, in press), and ICP27 (Ren *et al.*, 1994)], and homologues of HSV-1 structural proteins: MDV and HVT glycoproteins gB (Ross *et al.*, 1989; Yoshida *et al.*, 1994*a*; gC (Coussens and Velicer, 1988; Binns and Ross, 1989; Bandyopadhyay, 1989), gD (Ross *et al.*, 1991, Zelník *et al.*, 1993), gH (Scott *et al.*, 1993), gE and gI (Brunovskis and Velicer, 1992; Zelník *et al.*, 1993), MDV gK (Ren *et al.*, 1994) and gL (Yoshida *et al.*, 1994*b*). In addition there were sequenced and identified open reading frames (ORF) functions of which are unclear. Some of them have homologous counterparts within alphaherpesvirus family [e.g. US2, US3-encoded protein kinase, US10, UL49 (Brunovskis and Velicer , 1992; Sakaguchi *et al.*, 1992; Zelník *et al.*, 1993; Yanagida *et al.*, 1993)]. On the other hand, there were also identified genes encoding proteins that do not display significant homology with proteins contained in recent databases [e.g. meq protein (Jones *et al.*, 1992); pp38 gene (Cui *et al.*, 1990, Ross *et al.*, 1993*a*); SORF3, (Sakaguchi *et al.*, 1992; Zelník *et al.*, 1993)]. Furthermore, there were sequenced other parts of MDV DNA that do not contain evident ORFs, but have important functions during virus replication [e.g. origin of replication (Camp *et al.*, 1991)

and *a*-repeat like sequences (Kishi *et al.*, 1991)]. Structure of MDV and HVT DNAs and sequenced genes mapping and collinearity with homologous HSV-1 genes is presented in Fig 1.

Another approach of sequencing MDV-encoded fragments has been the identification of MDV-specific cDNA clones in libraries prepared from tumour cell lines. It has been noted that transcription of MDV DNA in transformed cells is restricted mainly to inverted repeat regions (TR_L/IR_L and TR_S/IR_S, respectively) (Schat *et al.*, 1989; Peng *et al.*, 1992; Li *et al.*, 1994; Ohashi *et al.*, 1994*a*,*b*; Cantello *et al.*, 1994). Because some of these regions are also clearly affected by attenuation (Bradley *et al.*,1989; Fukuchi *et al.*, 1985; Ross *et al.*, 1993*a*; Wilson and Coussens, 1991), it has been speculated that they are tumour-associated. However, there are also other regions of MDV genome affected by attenuation, e.g. significantly reduced expression of MDV gC (Wilson *et al.*, 1994). However, some of these transcripts, especially those that are antisense in respect to MDV ICP4 transcript, might be rather latency-associated (Li *et al.*, 1994, Cantello *et al.*, 1994).

Although the information on primary structure of MDV and HVT genomes is widening rapidly and several differences between oncogenic MDV and nonpathogenic HVT were described, the evidence for virus-encoded factors that are responsible for MDV oncogenic potential has not been confirmed and is missing. Similarly, the relationship between oncogenic transformation and latency is also unclear, though it is believed to be synergistic.

Proteins encoded by MDV and HVT

Early studies on MDV and HVT structural proteins were difficult due to cell-associated nature of both viruses. This means that it is impossible to obtain a sufficient quantity of purified enveloped viral particles for biochemical analysis. With the availability of

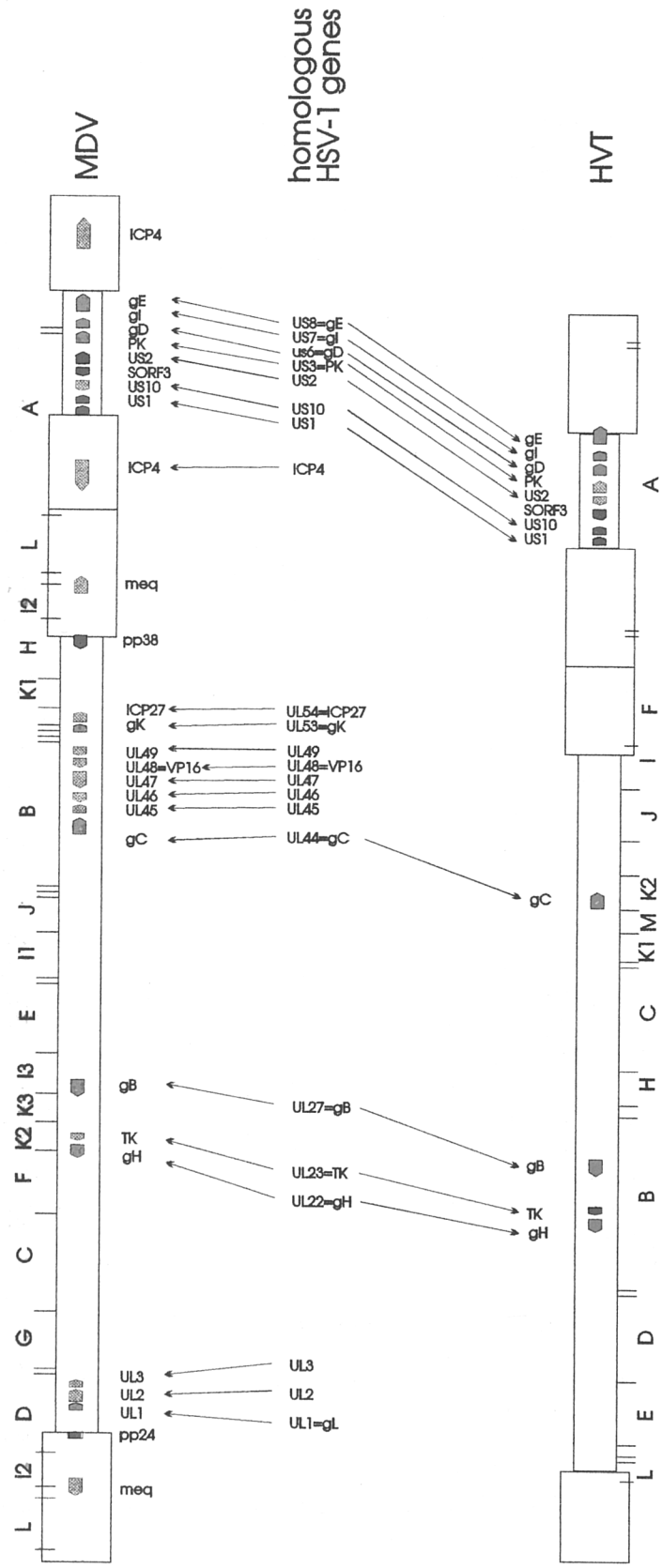

Fig. 1

Genetic structure and mapping of MDV and HVT genes

Genes encoding HSV-1 homologous proteins are indicated by arrows. Nomenclature of HSV-1 genes refers to McGeoch *et al.* (1988). Small vertical lines above MDV and below HVT structure outlines characterize BamHI restriction sites and letters specify restriction fragments as described for MDV by Fukuchi *et al.*(1984) and for HVT by Igarashi *et al.*(1987). Genes encoding experimentally identified glycoproteins are shown in green boxes. Phosphoproteins in blue and red coloured boxes identify genes that are dispensable for virus growth.

antibodies directed against virus-specific polypeptides and recent sequencing data, a number of MDV- and HVT-encoded proteins has been characterized.

Two viral enzymes involved in DNA metabolism have been characterized. It was demonstrated that MDV induces increased levels of DNA polymerase and thymidine kinase (TK) activities in infected cells. The virus-induced DNA polymerase was partially purified and characterized (Boezi *et al.*, 1974). There are data on properties (Kit *et al.*, 1973) and DNA sequence (Scott *et al.*, 1989) of both MDV- and HVT-encoded thymidine kinases.

By agar immunoprecipitation assay Churchill *et al.* (1969*a*) identified three major MDV antigens (A, B, C). A antigen is a heterogenous glycoprotein with isoforms of Mr 52 K to 72 K (van Zaane *et al.*, 1982*b*). It is an abundant antigen which can be found in sera of infected birds, infected cell culture fluids and also associated with the plasma membrane of infected cells (Isfort *et al.*, 1986). Interestingly, its expression is significantly reduced after 30 – 50 passages *in vitro* (Churchill *et al.*, 1969*a*; Wilson *et al.*, 1994). Gene encoding the MDV A antigen was sequenced and it was demonstrated that its amino acid (aa) sequence displays homology to HSV-1 gC (Coussens and Velicer, 1988; Binns and Ross, 1989). HVT gC homologue was also sequenced (Bandyopadhyay, 1989; Coussens *et al.*, 1990) and was demonstrated to cross-react strongly with MDV gC (A antigen). MDV and HVT B antigens also contain a number of cross-reacting determinants. The first sequenced and characterized as HSV-1 gB homologue was MDV B antigen (Ross *et al.*, 1989; Chen and Velicer, 1992; Niikura *et al.*, 1992). Monoclonal antibodies (MoAbs) directed against MDV gB identified 3 glycosylated forms in infected cell lysates: gp100, gp60 and gp49 (Niikura *et al.*, 1992; Yanagida *et al.*, 1992); the latter two glycoproteins are believed to be the cleavage products of gp100 (Sithole *et al.*, 1988). MDV gB glycoprotein is an important immunogen as judged by protection against MD of chickens immunized with recombinant fowlpox virus expressing the MDV gB (Nazerian *et al.*, 1992). MDV serotype 2 gB shares 83% and HVT (serotype 3) gB 82% identity on aa level with MDV serotype 1 gB (Yoshida *et al.*, 1994*a*). The size differences between MDV gB and HVT gB complexes were demonstrated to be due to different carbohydrate modifications (Yoshida *et al.*, 1994*a*).

Among other characterized MDV polypeptides are the pp38/24 complex and some proteins encoded within MDV and HVT U_S regions. The pp38/24 complex was thought to be MDV-specific and tumour-associated. As the pp38 gene is located within BamHI-H fragment at junction of U_L and IR_L (Cui *et al.*, 1991; Ross *et al.*, 1993*a*), the same N-terminal encoding sequences can also be found in BamHI-D. These N-terminal identical aa give rise to U_L/TR_L junction-encoded pp24 component that, of course, does not share C-terminal identical aa sequences with pp38 (Zhu *et al.*, 1994). The function of pp38/24 complex is unknown and its role in oncogenic transformation has not been confirmed. Recently, a pp38 homologue was identified in MDV serotype 2 (Ono *et al.*, 1994). Interestingly, similarly as in case of HVT pp38 homologue (Smith, G.D. personal communication), MDV serotype 2 pp38 shares homology with MDV serotype 1 pp38 at its C-terminus, whilst N-terminal parts do not display any homology with each other.

By immunoprecipitation with polyclonal sera directed against individual *E.coli*-expressed polypeptides Brunovskis and Velicer (1992) identified and characterized some of the MDV U_S-encoded proteins. In this way they identified MDV homologues of HSV-1 US1, US10, US2, protein kinase (PK), and gI and gE glycoproteins. Sequence of three other MDV-specific ORFs was reported (SORF1, SORF2 and SORF3), but their products have not yet been identified in infected cells. PK encoded within MDV U_S was expressed in baculovirus, and MDV PK purified either from MDV-infected cells or obtained from recombinant MDV PK-carrying baculovirus was demonstrated to phosphorylate protamine, similarly as it was reported for PKs encoded by HSV-1 and pseudorabies virus (PRV) (Sakaguchi *et al.*, 1993). Furthermore, in this study a PK-deletion mutant was isolated and it was demonstrated that MDV PK is non-essential for virus growth *in vitro*. An alternative approach to the *in vitro* expression (the *in vitro* transcription followed by the *in vitro* translation) and characterization of HVT U_S-encoded proteins was described by Zelnik *et al.* (1994). MDV and HVT homologues of glycoprotein gD genes that are in other alphaherpesviruses one of the major targets of neutralizing antibodies and have an essential role during virus penetration, were also sequenced (Ross *et al.*, 1991; Zelnik *et al.*, 1993) and characterized by expression *in vitro* (Zelnik *et al.*, 1994). However, product of MDV gD gene has not been identified in infected cells (Brunovskis *et al.*, 1992). In addition, gD-deletion MDV recombinant virus was isolated and demonstrated to have similar *in vitro* and *in vivo* growth characteristics as the parental MDV (Parcells *et al.*, 1994), sustaining thus hypothesis that low or none expression of MDV gD might be the cause of cell-associated nature of MDV.

Recently identified MDV meq gene that resembles fos/jun oncogene and encodes basic leucine-zipper protein is the best candidate of tumour-associated antigens among up to date analyzed MDV-specific proteins. This theory is supported by the fact that meq protein is specifically expressed in transformed cells but not in lytically infected chick embryo fibroblast (Jones *et al.*, 1992). Characteristics of proteins encoded by MDV and HVT genes are summarized in Table 2.

Although a number of MDV- and HVT-encoded proteins were characterized, sequenced and identified in infected cells, information on their activities and functions during infection is progressing more slowly. In case of polypeptides that are homologous to alphaherpesvirus proteins of known function, there can be made some preliminary deductions on their role during viral infection. However, as it was discussed above for gD homologues, function of homologous proteins might differ between viruses.

Recent MDV vaccines

Most of recently used and commercially available MDV vaccines are based on attenuated strains of serotype 1 and on non-pathogenic strains of serotype 2 and 3 viruses. Protective immunity against MD was first obtained by vaccination of chickens with attenuated MDV (Churchill *et al.*, 1969*b*). However, mere introduction of HVT as a live vaccine (Okazaki *et al.*, 1970) resulted in significant reduction of MD incidence. Apathogenic isolates of serotype 1 (Rispens *et al.*, 1972) and serotype 2 of MDV (Schat and Calnek, 1978) were later used, mainly in combination with former vaccines. All these vaccines utilize cross-reactivity of their polypeptides with oncogenic MDV immunoreactive determinants (Ikuta *et al.*, 1983), their homologous genomic structure (Gibbs *et al.*, 1984; Buckmaster *et al.*, 1988; Ono *et al.*, 1992) and similar tropism in infected chickens (Calnek *et al.*, 1970; Witter *et al.*, 1972). It was noted that the use of two (or three) vaccine strains provides better protection against MDV challenge when combined as a polyvalent vaccine than when used alone. This effect,designated protective synergism, can be affected by serotype and virus strain of vaccines (Witter, 1992). There are also other factors influencing effectiveness of MD vaccine. As mentioned in previous paragraphs, different oncogenic MDV strains can have different tumorigenic potential, but reasons for these differences are unknown. Among various host factors (genotype, sex, age etc.) the main one seems to be the B-haplotype where B^2 and B^{13} lines of 15.B-congenic chickens develop less protection against MD than B^{15} chicken line (Bacon and Witter, 1993). Interestingly, it is possible to vaccinate chick embryos before hatching (*in ovo* vaccination; Sharma and Burmester, 1984) with protection similar to that obtained by conventional vaccination of one day-old chickens (Miles *et al.*, 1992).

Temporary B-lymphocyte dysfunction and reduced resistance to infection in chickens was noted for monovalent and more for polyvalent live vaccines (Friedman *et al.*, 1992). Another disadvantage is the cell-associated nature of MDV and HVT. Since it is impossible to produce sufficient amounts of cell-free vaccine virus for immunization, infected cells or their lysates are used for production of vaccines. Such preparation are unstable and must be stored and transported at very low temperatures. Little is known about the status of vaccine virus in infected birds. There are indications that HVT DNA persists in CD4+ and CD8+ cells of some lymphoid tissues (spleen and thymus) in latent form, where the gene expression (namely gB gene) is restricted (Holland *et al.*, 1992).

The only, recently available way to control MD incidence is the vaccination. Taking in account all above mentioned factors, it is clear that the induction of protection against MD in chickens is rather more complex process involving several factors. There are fears that new more virulent oncogenic MDV strains might emerge and that they would be able to cause disease even in genetically resistant vaccinated birds. It is therefore important to understand not only the basis of MDV oncogenicity but also the mechanism of protection against it.

Novel approaches to MD vaccines

Recombinant molecules technology might represent another approach to preparation of MD vaccines. There are two general strategies: (a) attenuation of an oncogenic MDV strain by gene deletion(s), and (b) live virus vectors expressing immunogenically important MDV genes. As there was not yet identified a MDV genomic region that is clearly and significantly associated with oncogenicity, attempts to attenuate MDV by gene deletions might lead to lower virulence but not to complete loss of oncogenicity. Furthermore, viruses with reduced replication *in vivo* might not reach the same level of protection that can be achieved with recently used vaccines. Recently, two MD viruses were described with deletions in their genomes. Cantello *et al.* (1991) isolated MDV with *E.coli* LacZ gene expression cassette replacing the MDV US2 gene. Insertional mutagenesis with LacZ gene expression cassette allows visual selection of recombinant viruses (blue/white plaques after staining). A similar strategy was used by Sakaguchi *et al.* (1993) to produce deletion in neighbouring protein kinase gene (US3 homologue in alphaherpesviruses). Both viruses have been described to grow well in cell culture, however, information about their growth characteristics *in vivo* and about the attenuation of their oncogenic potential is missing. Isolation of MDV gD-deletion mutant virus was also reported (Parcells *et al.*, 1994), but as the insertion of the LacZ gene was carried out at the gD locus of attenuated, highly passaged (presumably nonpathogenic) GAatt85 strain of MDV, it is impossible to make any conclusions about effect of the gD deletion on pathogenesis of wild type MDV.

There are three factors that are important for the preparation of subgenomic vaccines based on live viral vectors

Table 2. Properties of some characterized proteins encoded by MDV and HVT

Protein	HSV-1 homologue[a]	Properties[b] M_r(o)	M_r(p)	other	Reference
MDV gB	UL27, gB	100 K 60 K 49 K	98 K	gp	Ross *et al.* (1989) Chen & Velicer (1992)
HVT gB	UL27, gB	100 K 60 K 49 K	98 K	gp	Yoshida *et al.* (1994)
MDV gC	UL44, gC	57 K 65 K	56 K	gp	Binns & Ross (1989) Coussens *et al.* (1988)
HVT gC	UL44, gC		42 K	gp	Bandyopadhyay (1989)
MDV gD	US6, gD	56 K	45 K	gp ND	Ross *et al.* (1991) Zelník *et al.* (1994)
HVT gD	US6, gD	47 K	44 K	gp	Zelník *et al.* (1993) Zelník *et al.* (1994)
MDV gE	US8, gE	62 K 72 K	54 K	gp complex with gI	Brunovskis *et al.* (1992) Velicer (1992)
HVT gE	US8, gE	64 K	55 K	gp	Zelník *et al.* (1993) Zelník *et al.* (1994)
MDV gH	UL22, gH		91 K	gp	Scott *et al.* (1993)
HVT gH	UL22, gH		91 K	gp	Scott *et al.* (1993)
MDV gI	US7, gI	45 K	38 K	gp complex with gE	Brunovskis *et al.* (1992)
HVT gI	US7, gI	47 K 44 K	40 K	gp	Zelník *et al.* (1993) Zelník *et al.* (1994)
MDV gK	UL53, gK		40 K	gp	Ren *et al.* (1994)
MDV gL	UL1, gL	25 K	18 K	gp	Yoshida *et al.* (1994*b*)
MDV ICP4	IE175, ICP4		155 K	IE	Anderson *et al.* (1992)
MDV ICP27	UL54, ICP27	55 K	55 K	IE	Ren *et al.* (1994)
MDV PK	US3, PK	53 K 45 K	45 K	NEVG	Ross *et al.* (1991) Sakaguchi *et al.* (1993)
HVT PK	US3, PK	53 K	44 K		Zelník *et al.* (1993) Zelník *et al.* (1994)
MDV TK	UL23, TK		39 K		Scott *et al.* (1989)
HVT TK	UL23, TK		40 K	NEVG	Scott *et al.* (1989) Ross *et al.* (1993*b*)
MDV UL2	UL2				Yoshida *et al.* (1994*b*)
MDV UL3	UL3				Yoshida *et al.* (1994*b*)
MDV UL45	UL45		24 K		Yanagida *et al.* (1993)
MDV UL46	UL46		64 K		Yanagida *et al.* (1993)
MDV UL47	UL47		92 K		Yanagida *et al.* (1993)
MDV UL49.5	UL49.5		10 K		Yanagida *et al.* (1993)
MDV UL49	UL49	32 K	28 K		Yanagida *et al.* (1993) Koptidesová *et al.*
MDV US1	US1	24 K 27 K	20 K	IE	Brunovskis & Velicer (1992)
HVT US1	US1	23 K	19 K		Zelník *et al.* (1993) Zelník *et al.* (1994)
MDV US2	US2	30 K	30 K	NEVG	Brunovskis & Velicer (1992) Cantello *et al.* (1992)
HVT US2	US2	31 K	31 K		Zelník *et al.* (1993) Zelník *et al.* (1994)
MDV US10	US10	24 K	24 K	pp	Brunovskis & Velicer (1992)
HVT US10	US10	24 K	24 K	NEVG	Morgan *et al.* (1992) Zelník *et al.* (1993) Zelník *et al.* (1994)
MDV VP16	UL48, VP16	55 K 49 K	49 K		Yanagida *et al.* (1993) Koptidesová *et al.*
MDV meq	NH	40 K	40 K		Jones *et al.* (1992)
MDV pp38	NH	41 K 38 K	32 K	IE	Cui *et al.* (1990) Ross *et al.* (1993*a*)
MDV SORF3	NH		41 K		Brunovskis & Velicer (1992)
HVT SORF3	NH	41 K	41 K		Zelník *et al.* (1993) Zelník *et al.* (1994)

[a]Nomenclature of HSV-1 encoded proteins as proposed by McGeoch *et al.* (1988); NH identifies MDV- and HVT-encoded proteins sequences of which do not display significant homology to known herpesvirus proteins.
[b]Abbreviations: gp – glycoprotein, pp – phosphoprotein, IE – immediate early protein, M_r(o) – relative molecular mass observed in protein analysis, M_r(p) – relative molecular mass predicted from nucleotide sequence, ND – protein not detected in lysates of productively infected cells, NEVG – gene not essential for virus growth.

expressing heterologous antigens: (a) the recombinant virus must replicate well *in vivo*, (b) the choice of expressed antigen should ensure an efficient immune response, and (c) the protection against challenge with relevant pathogen and the expression of this antigen should be suitably controlled (choice of promoters, enhancers etc.). The best candidate for induction of the protective immunity against MDV is glycoprotein gB, because it is the main antigen that induces neutralizing antibodies (Ikuta *et al.*, 1984; Niikura *et al.*, 1992). Recombinant fowlpox viruses expressing either MDV gB or pp38 genes were constructed (Yanagida *et al.*, 1992) and used in protection experiments. Whereas fowlpox virus expressing pp38 gene failed to elicit neutralizing antibodies and to protect against MDV challenge, fowlpox recombinant virus expressing gB gene protected chickens of cross lines $15I_5$ and 7 against challenge with GA, Md5 and RB1B strains similarly as did HVT (Nazerian *et al.*, 1992). However, when different lines of chickens were vaccinated with the gB-fowlpox recombinant virus and the experiment was prolonged, the incidence of MD increased to 50 – 60%, whilst protection with the HVT remained at the same level (Ross and Zelník, 1993). Together with the fact that MDV and HVT gB aa sequences share 82% identity (Yoshida *et al.*, 1994a), this experiment indicates that expression of single gB antigen is not sufficient to induce a protective immunity in all chicken lines and that there are other factors involved in induction of a protection against MD.

Recent progress in recombinant molecules technology together with apathogenic properties of HVT allowed construction of recombinant HVT viruses that express heterologous antigens. It is obvious that new genes introduced to HVT genome can be "any" ones, not just those improving protection against MDV. Morgan *et al.* (1992) described isolation of recombinant HVT expressing the Newcastle disease virus fusion protein that protects chickens from both Newcastle disease and MD. The insertion site used in this case was HVT US10 homologue and its deletion did not alter significantly protective abilities of HVT. Ross *et al.* (1993*b*) demonstrated use of HVT TK gene as possible insertion site by insertion of MDV glycoprotein gB gene in this locus. When a low dose of vaccine was administered the protection against MDV has improved as compared to wild type HVT, demonstrating some advantage of insertion the gB gene from oncogenic MDV strain into HVT genome. However, the inactivation of the HVT TK gene expression resulted in a lower *in vivo* replication.

There are also other potential insertion sites in HVT genome. Despite an unusual genetic arrangement of recombinant short genome segment, it was demonstrated that a cluster of three HVT genes (US1, US10 and SORF3) is not essential for virus growth *in vitro* and *in vivo* (Zelník and Ross, unpublished data).

For easier and faster identification of non-essential genes or intergenic regions in HVT and MDV genomes, Marshall *et al.* (1993) developed method of positive selection of recombinant viruses using *E.coli* xanthine-guanine phosphoribosyltransferase gene expression cassette. Mapping of non-essential loci can be accomplished by retrovirus insertions after co-cultivation of MDV or HVT with reticuloendotheliosis virus (REV) or avian leukosis virus (ALV) (Isfort *et al.*, 1992). It is obvious that retroviral sequences integrate into herpesvirus genome in regions that would not interfere with virus growth. These insertions preferentially take place at junctions of unique and repeat (U_L/R_L and U_S/R_S) regions (Jones *et al.*, 1993). Interestingly, as another common site of such random integrations was identified the gD gene (Isfort *et al.*, 1994), providing thus further evidence of unusual properties of MDV and HVT gD genes.

Herpesviruses as vectors

One of the possible ways to improvement of recent MD vaccines might be the insertional mutagenesis of vaccine virus strains. Several aspects of construction of recombinant HVT viruses can be learned from other herpesviruses, where insertion/deletion mutagenesis is well described and used for analysis of gene function, tropism of viruses in infected organisms, attenuation of pathogenic viruses and gene expression regulation. Several approaches to insertion mutagenesis of herpesviruses were described. Most of them involve mutagenesis of cloned viral fragments followed by the introduction of modified fragments into viral genome by homologous recombination. The insertional mutagenesis using mini-phage and transposon sequences (Jenkins *et al.*, 1985; Weber *et al.*, 1987) and the linker insertion (De Wind *et al.*, 1990) offers a random introduction of foreign sequences into cloned virus genome fragments. On the other hand, site directed mutagenesis techniques utilize the insertion of reporter gene expression cassette at specific viral loci. Viral TK gene (Longnecker and Roizman, 1987), *E.coli* LacZ gene (Goldstein and Weller, 1988; MacLean *et al.*, 1991), *E.coli* ß-glucuronidase gene (Jones *et al.*, 1991) and firefly luciferase gene (Kovács and Mettenleiter, 1991) are those most widely used allowing further selection and characterization of recombinant viruses.

Although the work on use of HVT as a vector for expression of heterologous antigens has started only recently, recombinant HVT viruses might play an important role in control not only of MD but also in protection of commercial flocks from other avian pathogens. Especially in the case of MD, improved recombinant HVT viruses are expected to be more efficient than subgenomic vaccines based on other vectors.

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