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The Leeuwenhoek Lecture, 1997: Marek's disease herpesvirus: oncogenesis and prevention

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THE LEEUWENHOEK LECTURE, 1997
Marek's disease herpesvirus: oncogenesis and prevention

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CONTENTS

SUMMARY

There are a number of neoplasias for which a herpesvirus is an essential part of the aetiology. Of these, Marek's disease is the most common and provides excellent opportunities for the study of a herpesvirus-induced tumour both experimentally and under natural conditions in the field. Marek's disease is caused by an alpha herpesvirus; it differs from the other oncogenic herpesviruses which are gamma herpesviruses. It is a ubiquitous virus in poultry populations of the world and is highly cell-associated and contagious, yet only a proportion of infected fowl develop tumours.

Evidence is presented to suggest that at least one of the reasons for a wide variation in the incidence of the disease is a temporal interplay between virulent viruses and viruses of low or no virulence. The viral genes associated with the oncogenicity of Marek's disease virus (MDV) are discussed and it is concluded that it is likely that several genes are involved. Finally, a brief history of vaccination to control Marek's disease is given and mode of action discussed. It is concluded that the mechanism of protection is mainly through an antiviral cell mediated immune response, resulting in a lowered challenge virus burden. Marek's disease viruses over the past 40 years have been evolving greater oncogenicity, some of which are not adequately controlled by the vaccines that are currently available.

It is suggested that for MDV to produce tumours, there is a need for the cytolytic infection phase and that infection must be with an MDV which possesses a functional *gC*, *ICP4* for maintaining latency which allows the expression of at least the 1.8 kb family, pp38, *meq*, and possibly pp14 genes, for maintaining the tumour state and possibly initiating this state. Intervention in this process reduces the chance of tumour formation and incidence in a population which can occur through natural or man-mediated infection with non-pathogenic MDVs.

1. INTRODUCTION

The honour of presenting the Leeuwenhoek Lecture is self-evident. It is appropriate that these lectures are in the field of microbiology because it was Antony van Leeuwenhoek who first observed and described micro-organisms including protozoa and bacteria. However, it is not my intention to consider either of these groups of micro-organisms but to address some aspects of an important virus-induced tumour

condition of the domestic chicken which I believe has relevance to an understanding of virus disease and virus-induced neoplasia, particularly those caused by herpesviruses.

It is a characteristic of many tumour viruses that they are widespread infections, yet only a proportion of infected individuals develop tumours. This is particularly true of many oncogenic retroviruses and herpesviruses. This situation can most readily be explained by postulating that in these cases the development of tumours is the result of a multi-hit process or a low-probability event post-infection. However, it is clear from studies on both these groups of viruses that there are other factors that determine whether an individual develops a tumour post-infection or not. For example, considering oncogenic herpesviruses, there is the importance of co-existing malaria infection in the Epstein Barr herpesvirus (EBV) associated endemic Burkitt's lymphoma (Burkitt 1969) and the importance of climatic temperature in the development of the herpesvirus-induced Lucké renal carcinoma of frogs in the northern parts of the USA (Rafferty 1972; McKinnell & Ellis 1972). Studying these factors is difficult where experiments in the target species are impossible or difficult and/or where field studies are restricted because of the relative rarity of the condition. Of the well recognized herpesvirus induced tumours the one that is the most common and has neither of these restraints is the tumours found in Marek's disease. Marek's disease (MD) is also unusual because the causative virus is an alpha and not a gamma herpesvirus. I wish to address three points. (1) Why is there a wide range in incidence of MD despite its causative virus being ubiquitous in the host population. (2) Which virus genes are associated with oncogenicity. (3) Control of the disease by vaccination.

2. WHAT MAREK'S DISEASE IS

(*a*) *The disease*

Marek's disease is a lymphoproliferative disease of fowl caused by an alpha herpesvirus which has a predilection for peripheral nerve tissue. It is a ubiquitous virus in the fowl population and is highly contagious, yet the incidence of disease, prior to the use of vaccines, could range from a low percentage to, on rare occasions, as high as 60% and was quite commonly 20–30%. A characteristic of the disease was that the incidence could vary widely between houses on a farm and even between pens within a house.

The disease was first recognized and described by Marek (1907), but in the few cases he described the lesions were restricted to the nervous system. He called the condition a polyneuritis. It was not until 1926 that the disease was recognized as a neoplastic condition, when Pappenheimer *et al.* (1926, 1929) described lymphomatous tumour-like masses in 10% of a series of cases of the disease. It is now known that the disease can be manifest in a number of ways in a flock. The severity of the disease can vary from a mild form, in which lesions are mainly restricted to the nervous tissue, to severe disease in which there are lymphomas in a wide range of organs and tissues. The incidence in a flock tends to be correlated with the severity of the disease; it tends to be low for the mild type and increases with increasing proportion of individuals exhibiting lymphomas. Since the early recognition that lymphomas are part of the disease, forms have appeared with an increasing incidence of lymphomas. Today there are forms where the majority of birds with disease have rapidly growing lymphomas in a wide range of organs and tissues. In some cases the available vaccines are unable to control the disease. The incubation or latent period from infection to overt disease can vary from a few weeks to several months. Generally, the shorter the period the more severe the disease in a flock.

(*b*) *The lesions*

The lesions in MD may be inflammatory, proliferative, and degenerative. The inflammatory lesions occur early in the pathogenesis of the disease and are in response to a cytolytic infection of B lymphocytes, or later in response to proliferating tumour cells. Some forms of the lesions in peripheral nerves are also considered to be inflammatory. The degenerative lesion is an arterial atherosclerosis (Fabricant *et al.* 1978). The proliferative lesion is a lymphoma in which the tumour cell is a T lymphocyte (Payne *et al.* 1976; Schat *et al.* 1991). This is unlike Burkitts lymphoma where the tumour cell is a B cell but similar to the tumours produced in non-human primates by herpesviruses saimiri and ateles. Lymphoblastoid cell lines can be established from MD lymphomas and the cells have T cell markers (Powell *et al.* 1974).

(*c*) *Pathogenesis*

The pathogenesis of MD has been detailed by Calnek & Witter (1997). Figure 1 summarizes the main steps in the disease process which has been derived from experimentally produced disease. There is no reason to believe that this process should differ in any fundamental way from that which occurs in the natural disease. The infection is believed to be via the respiratory route and shortly thereafter a cytolytic infection occurs of mainly B cells in the spleen, bursa of Fabricius, thymus and elsewhere. There is an inflammatory response to the results of the cytolytic infection which recruits a range of inflammatory cells, including macrophages, granulocytes and lymphocytes. Resting T cells are refractory to infection but the recruited activated T cells become infected and, as a result of the now developed immune response, the infection becomes latent. Early in the infection a cell-associated viraemia develops by which route infection is spread throughout the body. The virus is transported also to the feather follicle epithelium which is the only site where a fully productive infection occurs allowing the shedding of virus into the environment (Calnek *et al.* 1970*a*). In addition, these cells are desquamated in a keratinized form allowing protection to the intracellular virus. Infectivity

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of such material has been recorded to remain for up to a year (Hlozanek *et al.* 1973). This observation provides a reason for the highly contagious nature of the disease.

In latently infected lymphocytes there are less than five copies of viral DNA, and latency-associated transcripts are present in the form of a group of RNAs which map antisense to the homologue of the herpes simplex virus (HSV) *ICP4* gene (Li *et al.* 1994; Cantello *et al.* 1994).

Following this phase, there is a further cytolytic stage, resulting in damage to the primary lymphoid organs and immunosuppression. In those infected birds that develop tumours transformation of latently infected CD4+ T cells occurs (Schat *et al.* 1991). Transformed cells contain a greater number of viral DNA copies (at least 10–20) than latently infected cells (Ross 1985) and a number of viral genes are expressed which will be discussed later. The transformed cells form tumours and ultimately the death of the host.

(*d*) *The causative virus*

The causative virus of MD is a herpesvirus which was originally classified with EBV and the oncogenic herpesviruses of non-human primates, herpesvirus saimiri and ateles, as a gamma herpesvirus. However, the genomic structure and arrangement of the genes in the genome indicate a closer relationship with the alpha herpesviruses (Cebrian *et al.* 1982; Buckmaster *et al.* 1988). This classification, which is now generally accepted, indicates that an oncogenic herpesvirus does not have to be a gamma herpesvirus.

MDV is highly cell associated yet the infection is highly contagious. These two properties are the reason why it took so long for the causative virus to be identified. Attempts to transmit the disease were made by many groups since the 1920s but without convincing success. It was not until isolation facilities for experimental birds became available in the 1960s, and living infected cells were used, that transmission of the disease was convincingly successful and that further experiments in birds was made possible (Biggs & Payne 1963; Sevoian *et al.* 1962). The cell-associated nature of the virus, even in cell culture, slowed progress towards an understanding of the virus genomic structure and function until some of the more recent molecular biological techniques became available.

The MDV group consists of three subgroups, which can be distinguished serologically. First, there are the pathogenic and, in most cases, oncogenic viruses which vary in virulence and belong to serotype 1. Second, there are viruses which are non-oncogenic and belong to serotype 2 (MDV-2). Third, there is serotype 3, which contains the herpesvirus of turkeys (HVT) which is also non-oncogenic (table 1). For the sake of simplicity I shall refer to pathogenic/oncogenic serotype 1 MDV as MDV. Serological relationship between the three serotypes is close and, although they can be distinguished from one another serologically, they share many antigens (Bulow & Biggs 1975*a*, *b*). Serial passage in cell culture results in the attenuation of MDV strains (Churchill *et al.* 1969*a*). Such attenuated viruses provide protection against later challenge by virulent virus as do MDV-2 viruses and HVT (Churchill *et al.* 1969*b*; Biggs & Milne 1972; Zander *et al.* 1972; Okazaki *et al.* 1970). Both MDV and MDV-2 are widespread in poultry populations as is HVT in turkeys.

Table 1. *The Marek's disease virus group*

virus	serotype	oncogenicity
Marek's disease virus	1	+ strains vary in virulence
Marek's disease virus	2	–
Herpesvirus of turkeys	3	–

I have now provided the background information necessary to address the three areas I have already mentioned: causes of variability of incidence of MD; virus genes associated with oncogenicity; and control by vaccination.

3. CAUSES OF VARIABILITY IN THE INCIDENCE OF MAREK'S DISEASE

Several factors affect the susceptibility of fowl to the development of disease after infection with MDV. These can be broadly classified as those associated with the host and those associated with the virus. I wish to concentrate on those associated with the virus since this is a Leeuwenhoek lecture. Suffice it to say on the host side that genetic constitution plays a part, with genes at the major histocompatibility locus playing an important part, but other genes are also involved (Calnek 1985). The immune status also affects susceptibility to the disease as does age at infection (Biggs 1985). With respect to the virus, the strain is of importance; the more virulent strains produce severer disease at a higher incidence within a flock than less virulent strains (Schat 1985). However, it is clear that these are not the only important factors. Chickens derived from the same flock of the same genetic stock, and reared and grown on the same farm and in different pens in a single house, can vary in the incidence of Marek's disease by as much as 17-fold (Biggs *et al.* 1972).

We undertook further studies in the field, prior to the wide use of vaccines, in an attempt to determine the factors responsible for this wide variation of incidence in disease (Biggs *et al.* 1973). A flock was chosen where it was possible to impose a design that allowed the effect of the flock supplying the chicks, the rearing house and pen, and the production house and pen on mortality from MD to be examined. The flock was derived from two supply flocks, hatched in the same hatchery and reared in a number of pens

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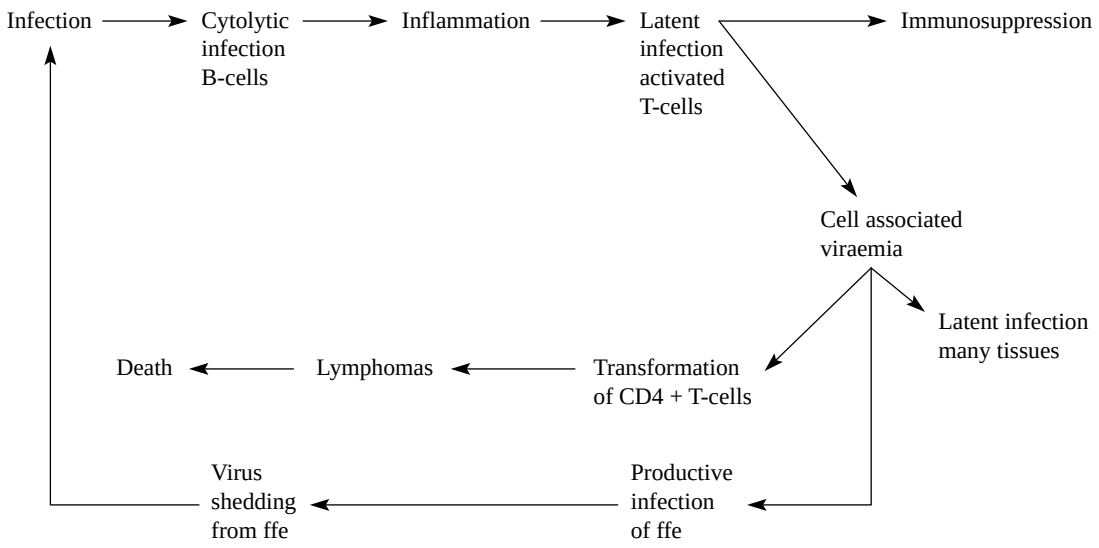

Figure 1. Diagrammatic representation of the pathogenesis of Marek's disease.

in each of three houses on a single site, with chicks from each supply flock kept in separate pens. At eight weeks of age the birds were moved to a production site on another farm with the birds in each rearing pen being divided between a pen in each of two production houses.

Statistical analysis of the percentage mortality from Marek's disease indicated that supply flock, hatch, production house and pen had no effect on the incidence of mortality from MD. The mortality from MD in the rearing houses and rearing house pens varied from 16.2–32.9% and 4.3–40%, respectively. Analysis of variance to test the effect of rearing house and pen showed that there was a significant difference between the rearing house pens but not between the rearing houses. This result raised the following question: what was happening during the rearing period (the first 8–9 weeks of life), which was responsible for the large variation in mortality from MD in pens within a single house? Because MD occurred in chickens derived from each rearing house pen within 18 days of being moved to the production site, a period shorter than the incubation period, infection must have taken place on the rearing site. Therefore the incidence of mortality must have been determined by events at the time of primary infection. Genetic constitution and presence of maternally derived antibody could not have had an effect because the supply flock and hatch had no effect on the incidence of MD. It is possible that the age at infection differed in each pen because there were 17 days between the disease appearing in chickens from the different rearing pens, but that spread of time is unlikely to have been responsible for the large differences in incidence of the disease. Although the virulence of the strain of virus with which a group of birds is infected will affect the severity and incidence of MD, it has been shown that single pens of birds can be infected with more than one strain of MDV (Biggs *et al.* 1972). It has also been shown that avirulant serotype 2 MDV offers protection against later challenge with virulent virus (Biggs & Milne 1972; Zander *et al.* 1972). It was therefore postulated that the variation in incidence of the disease was due to a differing interrelationship between virulent and non-virulent viruses in the pens.

The next study (Jackson *et al.* 1976) was designed to test this hypothesis. This study used chicks of a layer strain which were equally divided between five pens of a single house for the first 16 weeks of life. The birds were then transferred to layer cages and kept to 72 weeks of age. All cases of MD were recorded. Blood samples were taken at intervals from a sample of birds, and virus isolates made. The virus isolates were typed as pathogenic or non-pathogenic according to the results of tests in chicks or plaque characteristics in cell culture (Biggs & Milne 1972). The results are summarized in figure 2 and show that pens 2 and 3, which had the highest incidence of MD, also had the highest proportion of pathogenic to non-pathogenic virus isolates early in life and over the 72-week period. Only 2 out of 45 isolates made during the first 11 weeks were non-pathogenic. In contrast, in pens 8 and 10, which had the lowest incidence of MD, 20 out of 32 isolates were non-pathogenic. In pen 5, which had an intermediate incidence of the disease, 7 out of 24 were non-pathogenic. The proportion of pathogenic to non-pathogenic isolates made over the whole 72-week period correlates with the incidence of MD.

These results show that the sequence of infection and frequency of isolation of viruses of differing virulence can vary from pen to pen within a house, and that the mortality from MD can be closely associated with these differences. The early predominance of non-pathogenic virus in pens with a relatively low incidence of MD suggests that such virus provides protection against the challenge of pathogenic virus. Whether this protection is through immune mechanisms, i.e. natural vaccination, or some other mechanism such as viral blocking or interference cannot be answered from this study.

This series of studies suggests that the widespread presence of viruses of differing virulence is common.

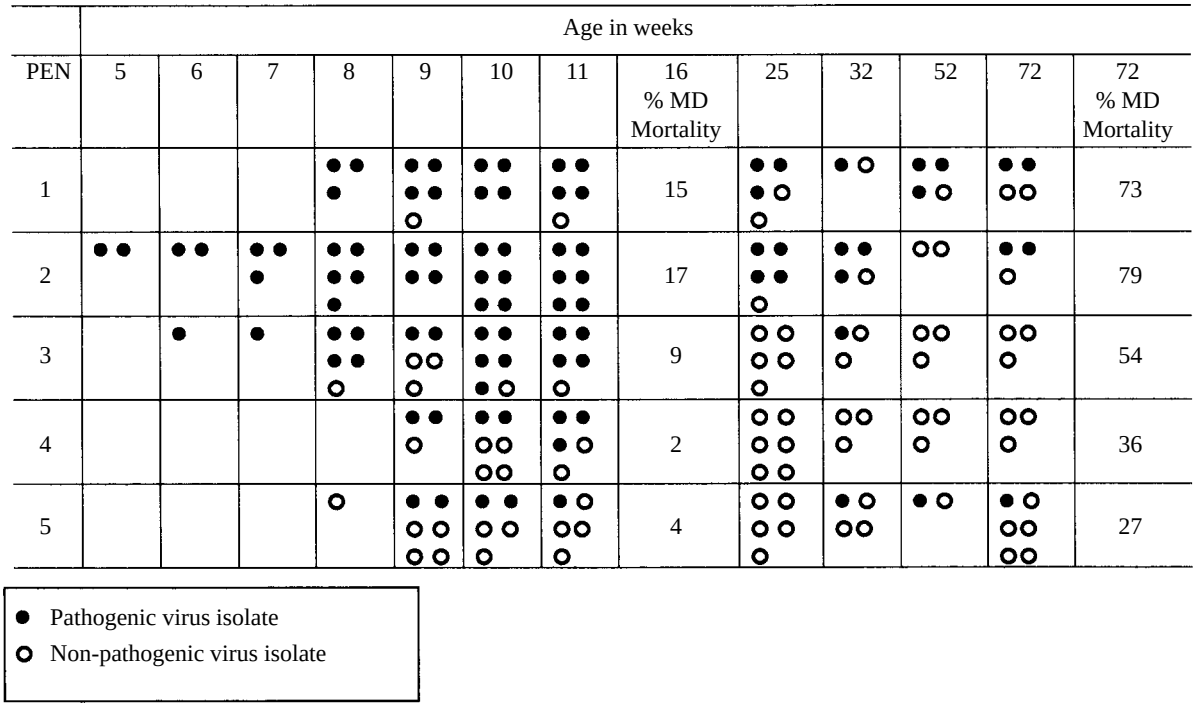

Figure 2. Distribution of isolates of pathogenic and non-pathogenic Marek's disease virus and incidence of Marek's disease in five pens of a single house. Adapted from Jackson *et al.* 1976.

It also appears likely that interplay of viruses of differing pathogenicity is important in determining the outcome of infection in populations and probably individuals.

4. VIRUS GENES ASSOCIATED WITH ONCOGENICITY

The MD system provides a number of approaches to identifying viral gene(s) that are responsible for, or contribute to, the transformation of T cells and the maintenance of that state. First, the genomes of virulent serotype 1 viruses can be compared with there attenuated derivatives. Second, genes present in virulent serotype 1 viruses but absent in serotype 2 viruses and HVT can be identified for further study. Third, genes that are expressed in lymphoblastoid cell lines derived from MD lymphomas and in lymphomas cells taken directly from the chicken can be examined. Using these approaches a number of genes and alterations in gene structure have been identified (figure 3). These include the deregulation of the *gC* gene, a number of genes in the inverted repeat regions including an expansion in a region of the internal and terminal inverted repeat regions flanking the unique long region, a '1.8 kb' gene family, pp38 gene, *meq* gene, and the *ICP4* gene. Finally, genes of interest can be deleted or neutralized by use of an antisense strategy, and the resulting virus tested for oncogenicity in chickens.

(*a*) *Glycoprotein C*

It was noted that during attenuation of an oncogenic MDV by passage in cell culture the protein termed the 'A' antigen disappeared (Churchill *et al.* 1969*a*). For this reason it was originally thought to be associated with pathogenicity. However, it was later shown that the protein was a glycoprotein (Ross *et al.* 1973) that was produced in small amounts by attenuated virus (van Zaane *et al.* 1982). The glycoprotein is the homologue of *gC* of HSV (Binns & Ross 1989). The defect in attenuated MDV is in the transcription of the *gC* gene, and therefore its regulation, rather than an alteration in its structural integrity (Wilson *et al.* 1994). The *gC* gene is present in MDV-2 and HVT (table 2) (Kitazawa *et al.* 1993). Because it is a late gene and is present in apathogenic MDV-2 viruses and HVT it might be thought unlikely to be associated with oncogenicity. However, a recent report (Morgan *et al.* 1996) suggests it may play a part because a mutation in the *gC* gene of an MDV greatly reduced its oncogenicity in chickens. Rescue experiments are awaited with interest. Studies of the pathogenesis of attenuated MDVs have suggested that attenuation reduces the efficiency of infection of, or replication in lymphocytes (Schat *et al.* 1985). It is possible that the change in the regulation of the *gC* gene is responsible, or partly responsible, for this and, if so, could be contributing to the reduction in oncogenicity.

(*b*) *132 base pair repeat and 1.8 gene family*

Other changes occurring with attenuation of oncogenic viruses by serial passage in cell culture have been recognized. An expansion occurs in the internal and terminal inverted repeat regions flanking the unique long region of the genome (Ross *et al.* 1983; Fukuchi *et al.* 1985). This expansion is due to a tan-

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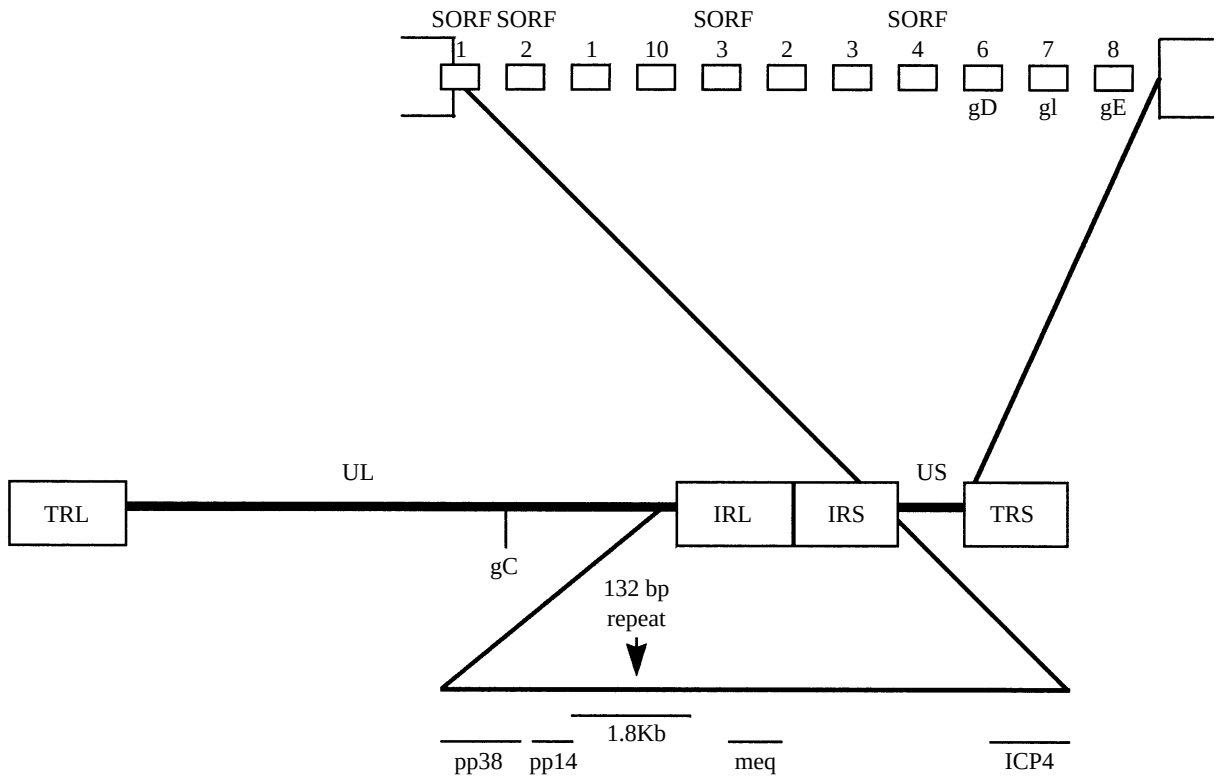

Figure 3. Diagrammatic representation of the genome of Marek's disease virus serotype 1 showing expansion of the unique short region and of the long and short internal repeats.

Table 2. *Presence of genes in Marek's disease viruses (MDV) and the herpesvirus of turkeys (HVT) and gene expression in MDV transformed cells and cytolytically infected cells*

	presence of genes in MDVs and HVT				Gene expression in MDV transformed cells and in cytolytic infection		
	MDV-1	MDV-1/att	MDV-2	HVT	lymphoma cells	lymphoblastoid cell line	cytolytic infection
gC	+	+ not transcribed	+	+	—	—	—
132 bp repeat	2–3 copies	multiple copies	?	?	—	—	—
1.8 kb family	+	truncated	?	?	—	—	—
pp38	+	+	+ partial homology	+ partial homology	+	+	+
pp14	+	+	?	?	?	+	+
meq	+	+	?	?	+	+	+
ICP4	—	—	—	—	+ antisense	+ antisense	+ sense

dem accumulation of 132 base pair direct repeats (figure 3 and table 2) (Maotani *et al.* 1986). In one oncogenic strain two repeats were found, whereas eight were present in its attenuated derivative (Ross *et al.* 1993*a*). In this respect it is interesting that only 2–3 132 bp repeats in MDV transcripts were found in the two lymphoblastoid cell lines derived from lymphomas which were examined (Kopacek *et al.* 1993).

The repeats are within the 1.8 kb gene family (figure 3), and it is possible that the expansion produces a functional change in the structure and function of the DNA of this region. This view is supported by the finding that pathogenic viruses produce a 1.8 kb family of transcripts whereas attenuated viruses do not; in attenuated viruses the 1.8 kb transcripts are replaced by truncated transcripts (table 2) (Bradley *et al.* 1989*a*, *b*; Kopacek *et al.* 1993). Whether the 1.8 kb gene family is present in strains of MDV-2 and HVT is not known. The findings with MDV and their attenuated derivatives have led to the suggestion that the 1.8 kb gene family is associated with the oncogenic potential of MDV (Bradley *et al.* 1989*a*, *b*). Supportive evidence for this view comes also from studies using an antisense strategy for examining the requirement of the 1.8 kb gene family for oncogenicity of MDV. An oligonucleotide complementary to the splice donor sequence of the 1.8 kb gene family inhibited the proliferation of an MDV-derived lymphoblastoid cell line but not an avian retrovirus-derived lymphoblastoid cell line. The complementary oligonucleotide also inhibited colony formation in soft agar of cells of the MDV-derived lymphoblastoid cell line (Kawamura *et al.* 1991).

(*c*) *Phosphorylated protein 38 (pp38)*

An MDV-specific phosphorylated protein was identified in a lymphoblastoid cell line and in lymphoma cells in MD tumours (table 2) (Naito *et al.* 1986; Nakajima *et al.* 1987). The complete sequence has been determined of a gene coding for a phosphorylated protein of 38 kDa which is expressed in lymphoblastoid cell lines and 20% of MD lymphoma cells. The gene spans the junction of the unique long region and the adjacent internal repeat (figure 3), and its promoter overlaps that of the 1.8 kb family gene (Cui *et al.* 1991; Chen *et al.* 1992). The finding of this protein in lymphoblastoid cell lines and lymphoma cells suggests that it might have a role in transformation of lymphoid cells. However, a similar sequence to the pp38 gene has been found in an attenuated virus (Ross *et al.* 1993*a*), and homologous genes have been described for MDV-2 and HVT but in each case it is only a partial homology (table 2) (Ono *et al.* 1994, 1995; Smith *et al.* 1995). In the case of MDV-2 the N-terminal 130 amino acids of the protein differ from the MDV-1 pp38 and they share no epitopes. The HVT protein is truncated at the N-terminal end. The fact that the pp38 gene is expressed in productive infections and is found in attenuated virus suggests that it does not play a direct role in oncogenicity. Nevertheless, the genes in MDV-2 and HVT do not produce the same product, and the pp38 gene is expressed in lymphoma cells and in lymphoblastoid cell lines. As yet, it cannot be ignored, particularly because the use of antisense oligodeoxynucleotide complementary to the translation initiation region of the pp38 gene greatly slowed the proliferation of a lymphoblastoid cell line and reduced colony formation in soft agar (Xie *et al.* 1996).

(*d*) *Phosphorylated protein 14 (pp14)*

A phosphorylated protein of 14 kDa specific to MDV-1 has been described in cells lytically infected with MDV and its attenuated derivative and in lymphoblastoid cells (table 2). The gene coding for this protein is located in the long internal repeat region next to the pp38 gene (figure 3) with which it shares a bidirectional promoter-enhancer (Hong & Coussens 1994; Hong *et al.* 1995). This is another gene found expressed in transformed cells and is located in the region that is associated with oncogenicity.

(*e*) *Meq gene*

A gene has been described that is present in the EcoR1 Q fragment that forms part of the long terminal and internal repeats called *meq*, an acronym for Marek's Eco Q (figure 3). This gene is of particular interest because its product is a protein which is highly expressed in MD lymphoblastoid cell lines and tumour samples (table 2) and not cell lines transformed by avian retroviruses, and has homology to the leucine-zipper class of nuclear oncogenes (Jones *et al.* 1992). This protein has 339 amino acids with a basic leucine-zipper domain near it's N-terminus, which shows homology with the *c-fos* and *c-jun* family of proteins, and a proline-rich domain near its C-terminus which resembles the Wilms tumour 1 supressor protein. This is an interesting protein which has been shown to have transactivation activity that resides in the C-terminal 130 amino acids with the last 33 amino acids being essential. It has also been shown that *meq* can dimerize not only with itself but also with *c-jun*, and that a *meq/c-jun* heterodimer can up regulate *meq* expression (Qian *et al.* 1995). It has also been shown that *c-fos* and p53 can interact with *meq* (Brunovskis *et al.* 1996). *Meq* has recently been shown to have transforming potential by studies that have demonstrated that it can morphologically transform cells, induce anchorage-independent growth, and inhibit apoptosis (Liu *et al.* 1996). It has also been shown that *meq* protein is present during lytic infection with a non-oncogenic attenuated MDV (Peng *et al.* 1995). This suggests that *meq* alone is not sufficient for oncogenicity and transformation. However, the use of antisense oligodeoxynucleotide complementary to the translation initiation region of the *meq* gene greatly slowed the proliferation of a lymphoblastoid cell line and reduced colony formation in soft agar (Xie *et al.* 1996).

(*f*) *Infected cell protein 4 (ICP4) gene*

An MDV gene homologous to the *ICP4* gene of HSV has been mapped to the inverted repeat flanking the unique short region of the MDV genome (figure 3) (Anderson *et al.* 1992). This gene is of interest because transcripts which map antisense to the *ICP4* homologue gene are found in lymphoblastoid cell lines and lymphomas and sense transcripts in lytic MDV infections (table 2) (Li *et al.* 1994; Cantello *et al.* 1994). It has been suggested that the antisense transcripts are latency-associated transcripts

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and therefore involved in the transformation process as latency is a prerequisite for oncogenesis and maintenance of the transformed state. Additional evidence for the *ICP4* gene playing a part in at least the maintenance of the transformed state comes from studies showing that oligodeoxynucleotide complementary to the predicted translation initiation region of the MDV *ICP4* gene greatly reduced proliferation of a lymphoblastoid cell line and colony formation in soft agar (Xie *et al.* 1996).

(*g*) *Us region*

The Us region of the MDV genome codes for four open reading frames for which homologues have not been found in other herpesviruses (figure 3) (Brunovskis & Velicer 1992, 1995) and are therefore candidates for association with the specific properties of MDV. A deletion in the unique short region which included three of the four MDV-specific open reading frames was found not to be essential for the transformation of chicken T cells or for the establishment and maintenance of latency (Parcells *et al.* 1995).

In summary, the information available suggests that more than one viral gene is involved in the oncogenesis of MD lymphomas. There is circumstantial and functional evidence suggesting that at least four genes, the 1.8 kb family of genes, pp38, *meq* and *ICP4* are involved in the maintenance of the transformed state of lymphoblastoid cell lines. Whether they are involved in initiation of tumour production or maintenance of tumours in the chicken is not known. To provide answers to those questions will require the development of deletion mutants for each gene and combinations of genes and testing of such mutants for oncogenic properties in the host animal. This has only been done for one gene, *gC*, which was found to reduce oncogenicity but its function in this respect is unknown although it could be related to a reduced ability to infect lymphocytes. Also, recently, a rescue experiment has shown that a cosmid clone spanning the internal repeats restores oncogenicity to an attenuated virus (Ross *et al.* 1996) indicating that at least some of the genes in this region are necessary for the oncogenicity of MDV.

5. VACCINATION

Vaccines have been used for the control of MD for over 25 years since it was shown that you can attenuate MDV by passage in cell culture and that such an attenuated MDV was protective under laboratory and field conditions (Churchill *et al.* 1969*b*; Biggs *et al.* 1970). Because of the nature of the virus it was not possible to produce a vaccine that could be lyophilized or stored at low temperature without special treatment. Because of the cell-associated nature of the virus the vaccine had to be in the form of infected cells. This in itself was novel but it was also the first vaccine to be effective against a neoplastic disease. The cell-associated nature of the vaccine required special techniques for freezing, storing, transporting and usage. Soon after, HVT was found to be an effective vaccine (Okazaki *et al.* 1970; Purchase *et al.* 1971) and that by using special procedures cell-free virus could be obtained from infected cell cultures (Calnek *et al.* 1970*b*). For several years HVT, in cell-associated and in cell-free lyophilized form, was the main vaccine used. The lyophilized HVT vaccine was popular at first for its ease of transport, storage and use. However, with time it gave way to the cell-associated form because this form was more effective in the face of maternally derived antibody, which is present in all chicks for about three weeks after hatching. The recognition that serotype 2 viruses could offer protection against later challenge with pathogenic viruses (Biggs & Milne 1972; Zander *et al.* 1972) led to such a virus being available as a vaccine (Calnek & Schat 1978*a*). Today, vaccines of all three serotypes are available, and widely used (table 3).

Table 3. *Vaccines available for the control of Marek's disease*

(MDV = Marek's disease virus, HVT = herpesvirus of turkeys.)

vaccine	form
attenuated serotype-1 MDV	cell associated
HVT (serotype-3)	cell associated cell free—lyophilized
serotype-2 MDV	cell associated

(*a*) *Use of vaccines*

All vaccines, with the exception of the cell-free lyophilized HVT, have to be administered parenterally in the form of living infected cells. Under field conditions, exposure to field virus occurs early in life requiring immunity in the young bird. For this reason vaccination takes place at one day of age. Protection develops by seven days of age (Ross & Biggs 1986) and is lifelong. However, vaccination does not prevent superinfection with field virus or the shedding of such virus. For this reason the widespread use of vaccine has not reduced the quantity of virulent virus in the field, which continues to be a constant challenge to its host. The consequence of this is that vaccination must continue to be used in all flocks. Over the years, increases in virulence of MDV have occurred, and these have continued since the widespread use of vaccination against the disease. It is likely that high-density population and the widespread immunity produced by vaccination play a part in these changes. The appearance of what is termed very virulent MDV occurred in the face of vaccination and was detected because HVT, the widely used vaccine at the time, no longer provided a satisfactory protection (Witter *et al.* 1980). This stimulated new vaccine strategies and a return to attenuated MDV and combinations of the three avail-

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able serotypes of vaccine. Although all combinations, including trivalent vaccines, are in use, synergism depends on the strain of virus of each serotype and has been demonstrated mainly between serotype 2 and 3 viruses (Witter 1992). Recent isolations of MDV have shown that evolution of MDV to greater virulence continues (Kross 1996; Venugopal *et al.* 1996; Witter *et al.* 1996).

These developments put a strain on control by vaccination. It is likely that the poultry industry will have to pay greater attention to genetically controlled resistance and management as adjuncts to vaccination. Further developments in vaccines could come from tailor-made recombinants and deletion mutants, but whether such vaccines will function better than those vaccines already available remains to be seen. Other strategies for control may come from a greater knowledge of the pathogenesis of the disease and of the changes in the virus responsible for increasing virulence.

(*b*) *Mechanism of vaccinal immunity to Marek's disease*

Vaccinal protection is immunological in nature because a degree of protection can be provided by virus-specific antigens and protection can be abrogated by immunosuppression (Lesnik & Ross 1975; Purchase & Sharma 1974). However, this does not exclude other mechanisms, such as viral interference, also being involved.

The immune response stimulated by vaccination could be antiviral and directed against virus antigens of cell-free virus and viral antigens expressed on the surface of infected cells. The immune response could also be directed at tumour cells. There is evidence for both these mechanisms. The evidence for the latter comes from studies that have shown that all types of vaccine can immunize against transplantable MD tumours (Mason & Jensen 1971; Calnek & Schat 1978*b*) and that tumour antigens can reduce the incidence of disease (Powell 1975; Murthy & Calnek 1979). To what antigens such a response is directed is not known, but the recently described tumour-associated antigen AV37 is a candidate (Ross *et al.* 1995). Marek's disease tumour-associated surface antigen (MATSA) (Powell *et al.* 1974) was believed to be tumour-specific, but later studies showed that it was present on activated T cells of uninfected chickens (McColl *et al.* 1987). Even so, it is interesting that anti-idiotype antibodies against MATSA were shown to immunize against challenge with a virulent MDV (Dandapat *et al.* 1994). However, it is likely that vaccinal immunity is largely antiviral because there is a major effect on the early pathogenesis of the disease. All vaccines protect against early cytolytic infection and lower the level of latent infection with field virus (Jackson *et al.* 1974; Smith & Calnek 1974; Calnek *et al.* 1980). If early cytolytic infection is responsible for recruiting the target cells for transformation as part of the inflammatory response then this function of a vaccine is important. Equally, the reduction in the number of latently infected cells and the virus load is just as important, if not more so, because there is a direct relationship between numbers of latently infected lymphocytes and lesion frequency (Witter *et al.* 1971; Jackson *et al.* 1974).

Little is known of the antigens to which antiviral immunity is directed. However, gB appears to be important because both purified gB from HVT and recombinants of fowl pox virus and of HVT with gB from MDV stimulate a protective immune response (Ono *et al.* 1985; Nazerian *et al.* 1992; Ross *et al.* 1993*b*).

Humoral immunity plays some part in vaccinal protection although it is not essential (Chubb & Churchill 1968; Ball *et al.* 1971; Else 1974; Payne *et al.* 1978). If humoral immunity is not essential then cell-mediated immunity is presumed to be important. This view is supported by studies which have shown that functional T cells are required for vaccinal immunity (Sharma *et al.* 1975; Payne *et al.* 1978).

In conclusion, the three aspects of MD I have discussed suggest that for MDV to produce tumours there is a need for the cytolytic infection phase, possibly, to recruit the target T cells. It is also suggested that infection must be with an MDV which possesses a functional *gC*, for an unknown reason, *ICP4* for maintaining latency which allows the expression of at least the 1.8 kb family, pp38, *meq*, and possibly pp14 genes, for maintaining the tumour state and possibly initiating this state. Intervention in this process reduces the chance of tumour formation and incidence in a population that can occur through natural or man-mediated infection with non-pathogenic MD viruses.

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