MDV GLYCOPROTEIN D IS EXPRESSED IN THE FEATHER FOLLICLE EPITHELIUM OF INFECTED CHICKENS

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Summary. – Glycoprotein D (gD) and its homologues are essential for many alphaherpesvirus life cycles. A gene encoding a homologue of gD was recently found in the Marek's disease virus (MDV) genome. Interestingly, gD-negative MDV mutants apparently replicate unimpaired in both cell culture and chickens. In this study, we show the expression of the gD homologue of MDV in feather follicle epithelium in infected birds. The gD homologue was detected in a few feather follicles even when most of the follicles were expressing pp38 or gB, other MDV-specific proteins. These observations indicate that the MDV gD homologue is expressed in a very limited set of cells and may be differently regulated. Since feather follicle epithelium of infected birds are the only place where the infectious cell-free MDV virions can be recovered, analysis of the transcriptional regulation of gD may lead to the understanding of the cell-associated nature of MDV.

Key words: Marek's disease; glycoprotein; herpesvirus; chicken; nonessential gene; replication

Introduction

MDV is an avian alphaherpesvirus causing a lymphoproliferative disease in chickens, characterized by lymphocytic infiltration of various organs (Calnek and Witter, 1997). MDV replication is strongly cell-associated, spreading through cell cultures in a cell-associated form by direct cell-to-cell contact.

In vivo, MDV spreads via infectious cell-free virus that has only been observed in the feather follicle epithelium of infected birds (Nazerian, 1969).

Several herpesviral glycoprotein genes play an essential role in the attachment and penetration of virus into cells. The herpes simplex virus (HSV) gD is a glycoprotein that is involved in both the attachment and penetration process and is required for both cell-free and direct cell-to-cell spread (Roizman and Sears, 1991). HSV gD has homologous counterparts in many α-herpesviruses (Audonnet *et al.*, 1990; Whittaker *et al.*, 1992; Spatz *et al.*, 1994). Although pseudorabies virus (PRV) also contains a gD gene homologue, gp50 gene, it is not essential (Hanssens *et al.*, 1995). Nevertheless, deletion of gp50 gene seriously inhibits infectivity, limiting virus infectivity to direct cell-to-cell spread (Mulder *et al.*, 1996). However, upon repeated cell passaging, high titers of cell-free gD⁻ PRV are eventually produced in culture (Schmidt *et al.*, 1997). Possibly other PRV gene products can eventually compensate for the loss of gD. Varicella zoster virus (VZV) is an exception in that no gD homologue has been detected in VZV (Davison and Scott, 1986).

Although MDV has a gD gene homologue (Ross and Binns, 1991; Brunovskis and Velicer, 1995), its gene product is either not expressed in infected tissue culture cells (Tan and Velicer, 1996) or is expressed only in a very few infected tissue culture cells (Ono *et al.*, 1996). Due to the unavailability of highly specific antibodies it is not clear whether MDV gD is expressed *in vivo*. Similarly to PRV, the MDV gD gene is non-essential for cell-to-cell infection. MDV mutants with retroviral insertions into the gD gene replicate both in tissue culture and in chickens (Isfort *et al.*, 1994). Recently, Morgan's group deleted the gD gene and demonstrated that the mutant MDV was still able to replicate both *in vivo* and *in vitro* (Parcells *et al.*, 1994; Morgan *et al.*, 1996; Anderson *et al.*, 1998). However, the highly conserved nature of the MDV gD gene suggests that gD is

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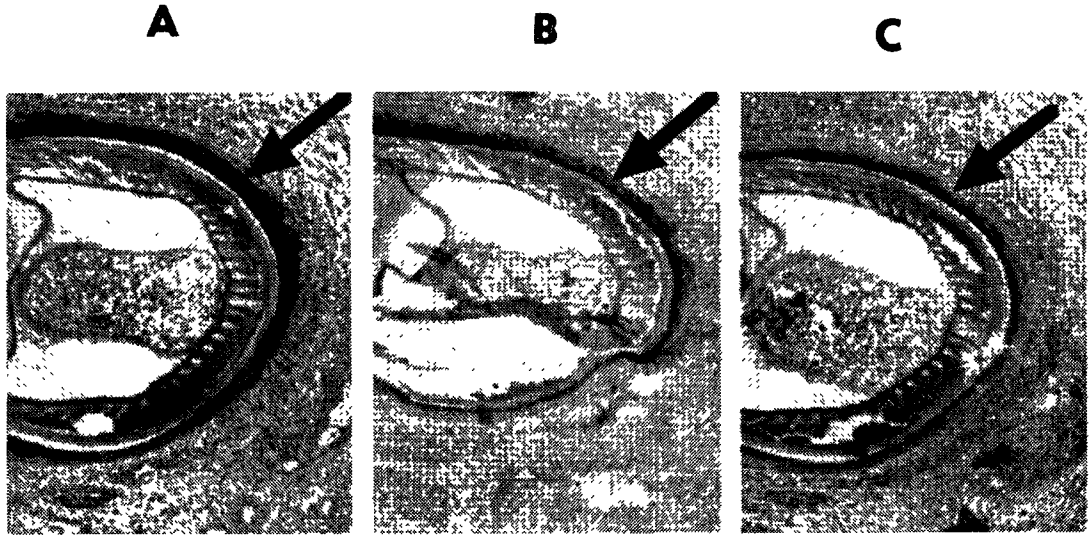

Fig. 1
Immunoperoxidase staining of serial sections taken from a feather follicle of a JM/102W-infected chicken
The arrows point to immunoperoxidase-stained cells. A. MAb to pp38, B. MAb to gB; C. MAb to gD.

probably expressed under certain conditions and plays an important role in MDV replication and/or spread.

Recently, we reported the production of monoclonal antibodies (MAbs) to recombinant gD of MDV expressed by a baculovirus expression system (Ono *et al.*, 1995). The specificity of these MAbs was confirmed by the specific reaction with another recombinant gD expressed by a retrovirus expression system. Using one of these MAbs, we could now demonstrate the expression of MDV gD in feather follicle epithelium in the infected birds. Further, we discuss the unique expression pattern of gD in the feather follicle epithelium in comparison to other MDV-specific proteins, pp38 and gB.

Materials and Methods

Cells, viruses and antibodies. Chicken embryo fibroblasts (CEFs) were prepared from line 0 chickens and maintained in Leibovitz's L-15 medium plus McCoy 5A medium (1:1) supplemented with 4% calf serum and antibiotics. CEF cells were used after one passage. MAb 6F11 specific for MDV-1 gD (Ono *et al.*, 1995) was used in this study. MAbs specific for gB (M51) (Ikuta *et al.*, 1984) and pp38 (Silva and Lee, 1984; Cui *et al.*, 1990) (kindly provided by L. Lee) were used as controls. Chickens were infected with pathogenic MDV-1 strains JM/102W, GA, RB1B, Md5, and 648B. For the immunofluorescent assays, CEFs were infected with pathogenic and attenuated JM/102W and Md11 strains. As a control, CEFs were infected with MDV-2 SB1strain and MDV-3 HVT strain.

Infection of chickens. Chickens (line $15I_5 \times 7_1$) maintained in our facility were infected with 50 or 1000 PFU of MDV per bird at the day of hatch. They were sacrificed on the indicated days after infection.

Immunohistochemistry. Skin samples were taken from the infected birds and embedded in OCT compound (Sakura Finetek, CA). Frozen serial sections were prepared from these samples and fixed in ethanol. Slides were stained with the appropriate MAbs and developed with peroxidase conjugate (Vectorstain, Vector Lab., CA). The immunofluorescent assay with infected CEFs was performed as described previously (Niikura *et al.*, 1991).

Results and Discussion

When we examined the expression of gD in secondary CEFs infected with MDV-1 strains JM/102W, Md5, Md11 and GA by immunofluorescent assay, a few cells were positive with any of these strains (data not shown). Also, secondary CEFs infected with either MDV-2 or MDV-3 were negative. These results were consistent with previous reports (Ono *et al.*, 1996; Tan and Velicer, 1996).

In order to investigate the gD expression *in vivo*, serial skin sections of the JM/102W-infected birds at 22 days post-inoculation (pi) were stained with MAbs to gD, pp38 and gB. Some of the feather follicles on the section were clearly stained positive with MAb to gD (Fig. 1C), indicating the expression of gD. The positive staining was observed similarly in the birds infected with other MDV-1 strains GA, RB1B, Md5 and 648A (data not shown), indicating no strain variation in the expression of gD in feather follicle cells.

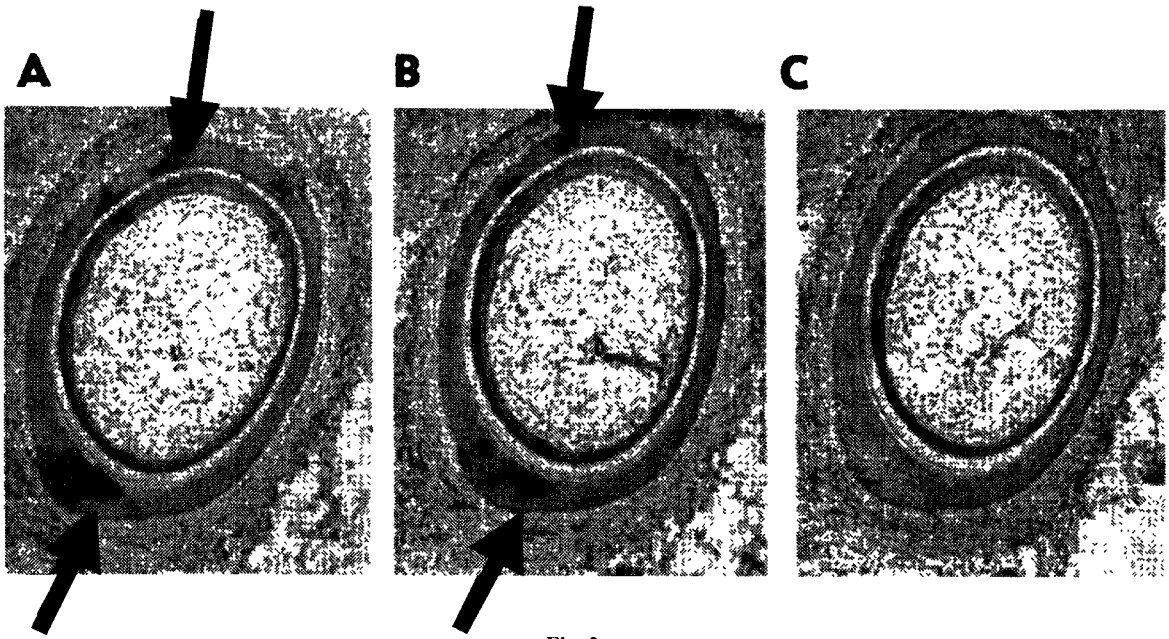

Fig. 2
Immunoperoxidase staining of serial sections taken from a feather follicle of a JM/102W-infected chicken
The arrows point to immunoperoxidase-stained cells. A. MAb to pp38; B. MAb to gB; C. MAb to gD.

Table 1. Distribution of antigens in individual follicles

Antigen status of pp38-positive follicles		No. of follicles examined		
gB	gD	Exp.1	Exp.2	Total
+	+	23	11	34
+	–	11	9	20
–	+	0	0	0
–	–	5	8	13

In two separate experiments, serial sections of feather follicle epithelium from MDV-infected chickens were stained with MAbs against pp38, gB and gD, respectively.

Table 2. Time course of MDV proteins expression in feather follicles

Days pi	Positive/total follicles		
	gD	pp38	gB
5	0/7	0/9	0/9
14	0/17	13/20	3/16
22	5/23	21/26	15/24
29	5/16	18/21	14/20

By comparing the neighboring serial sections stained with these three MAbs, two characteristics of gD expression were noticed. The first was that more feather follicles were positive with gB and pp38 than with gD. When we examined 67 pp38-positive feather follicles, 54 follicles were positive with gB as well, while only 34 follicles were positive for gD (Table 1). Fig. 2 illustrates one of such feather follicles showing positive reaction with pp38 but negative one with gD. Also, no follicle was positive with gD without also being positive for both pp38 and gB (Table 1). Fig. 1 shows a feather follicle positive with all three MAbs. Thirteen feather follicles were positive only for pp38 but not for gB or gD.

The second characteristic was the limited localization of gD-expressing cells in each positive feather follicle. For gD, only a couple of cell layers on the surface of epithelium were positive, but never the cells in deeper layers. In contrast, pp38 and gB expression occurred in cells located in deeper layers of the epithelium (Figs. 1A, B and Figs. 2A, B). We first detected gD expression at 22 days pi in feather follicle epithelial cells and expression continued until the end of the experiment period (29 days pi). Both pp38 and gB could be detected as early as 14 days pi (Table 2). Thus gD was expressed later in the course of the MDV infection than pp38 or gB. Furthermore, at the end of the experiment, less than one third of the follicles were positive for gD while more than 80% of follicles were positive for pp38.

With our recently developed MAb to MDV gD, we were able to demonstrate the expression of gD in the feather follicle epithelium of MDV-infected birds. All examined strains of MDV expressed gD. Thus, gD expression is not a strain-

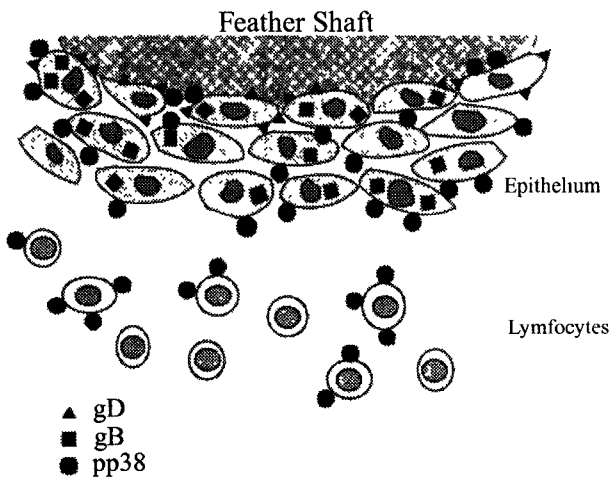

Fig. 3
Schematic representation of the expression of viral antigens in feather follicle epithelium cells
Infected cells are expressing pp38 (circles), gB (squares), or gD (triangles).

specific phenomenon and suggests that gD may be essential or at least advantageous for the life cycle of MDV in a natural environment. This idea is supported by the observation that the gD gene is highly conserved among all MDV serotypes (Ross *et al.*, 1991; Zelník *et al.*, 1993; Jang *et al.*, 1996).

Serial sections revealed that gD expression occurred in a subset of the feather follicles expressing pp38 and gB. In addition, gD expression was limited to the cells closest to the feather shaft. The pattern of gD expression suggests that the feather follicle epithelium was infected from the basal layers through infected lymphocytes, and that gD expression was turned on in accordance with the maturation of the epithelial cells (Fig. 3). This hypothesis may reasonably explain the delay of the detection of gD compared to gB in the infected birds (Table 2).

Under the control of the Rous sarcoma virus LTR, the MDV gD gene can be transcribed and translated (Ono *et al.*, 1995). However, in MDV-infected CEFs, it appears that the expression of gD is suppressed at the transcription level (Tan and Velicer, 1996). These observations together with our present findings suggest that gD may require additional transcriptional factor(s) to those required for the usual late gene transcription such as for gB. This factor(s) seems to exist specifically in the superficial layers of feather follicle epithelium but not in CEFs. This hypothesis is attractive in the light of our knowledge that the feather follicle epithelium is the only location where infectious cell-free MDV is produced. It is not known if the expression of gD is sufficient or even necessary to produce cell-free MDV. However, it can be speculated that there may be a set of genes, which are necessary to produce mature MDV virions and are expressed in very limited cells in the same manner as gD. Also we cannot rule out the possibility that MDV gD itself might play important roles not only in the early stage of infection (penetration) but also in a very late stage of MDV cell-free virion production such as assembling or budding. Thus, analysis of transcriptional regulation of gD and its involvement in cell-free MDV production may lead to the understanding of the cell-associated nature of MDV.

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