REVIEW

Pathogenesis of Marek's Disease (MD) and Possible Mechanisms of Immunity Induced by MD Vaccine

Toshifumi MORIMURA, Kazuhiko OHASHI, Chihiro SUGIMOTO and Misao ONUMA

Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

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ABSTRACT. Marek's disease (MD) is a lymphoproliferative disease of chicken, which is characterized by malignant T cell-lymphoma formation. This disease can be effectively prevented by vaccination with attenuated MD virus (MDV), apathogenic MDV or herpesvirus of turkey. MD vaccines are ones of a few vaccines which can prevent virus-induced tumor among mammalian and avian species. To determine the roles of T cell subsets in the protection mechanism, chickens vaccinated with an attenuated MDV (CVI988) were depleted of either CD4⁺ or CD8⁺T cells by neonatal thymectomy and injections of monoclonal antibodies against chicken CD4 or CD8 molecules and then challenged with an oncogenic MDV. These birds were effectively protected from MDV-induced tumors. However, virus titers in CD4⁺T cells, which are the main target cells for MDV-latent infection and subsequent transformation, were much higher in CD8-deficient vaccinated chickens than in untreated vaccinated chickens at the early stage of the latent phase. These results suggested that CD8⁺T cell responses induced by the MD vaccine are essential for anti-virus but not anti-tumor effects. Here, we will discuss how the attenuated vaccine prevents chickens from lymphoma-formation by an oncogenic MDV. — **KEY WORDS:** apoptosis, Marek's disease, Marek's disease virus, vaccine.

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Marek's disease (MD) is a highly contagious malignant lymphoma of chicken caused by Marek's disease virus (MDV). Infected chickens are depressed, and many clinical signs including dullness, squat down and inverse feathers with progression of lymphoma development and paralysis or ataxia if lymphoma/lymphoblast cells infiltrate in peripheral nerves are observed [53]. The outcome of the disease depends on several host resistance factors and MDV virulence [8].

Three serotypes of MDV are recognized; serotype 1: oncogenic MDV such as RB1B, Md5 and GA strains, serotype 2: non-oncogenic MDV such as SB-1, and serotype 3: herpesvirus of turkeys (HVT) [83, 84]. Virulence or oncogenicity is associated only with serotype 1 MDV, and serotypes 2 and 3 viruses are not recognized as pathogens in avian hosts. Although distinguishable by serologic tests, the three serotypes share many common antigens, suggesting that these antigens could contribute to induction of antiviral immunity by MD vaccines. MDV is a highly cell-associated virus, and cell-free virus is produced only in the feather follicle epithelium of infected birds [9]. Cell-free oncogenic MDV naturally enters chicken via the respiratory tract, and travels to the lymphoid organs with englobing macrophages. In the lymphoid organs, MDV primarily causes lytic infection of B cells [75], which leads to suppression of humoral immunity [55]. After the early cytotoxic phase, MDV establishes the latent infection in lymphocytes, mainly T cells which are activated to respond against lytically infected B cells [11]. Schwann cells and satellite cells in spinal ganglia are also alternative target

cells for latent infection [56]. During the latent phase, MDV causes suppression of cellular immunity, which may be important for subsequent lymphoma development. Recently, several viruses including human immunodeficiency virus type 1 have been reported to accelerate apoptosis in lymphoid cells, and the apoptosis is highly correlated with the virus-induced immunosuppression (Table 1). This immunosuppression was also induced by modulation of expression level of cellular functional molecules by viruses [2, 65, 91]. It has been recently demonstrated that apoptosis of peripheral CD4⁺T cells and CD4⁺CD8⁺ thymocytes and down-regulation of the expression level of CD8 molecules occurred during the latent phase of MDV-infection [43, 44]. These immuno-modulations are assumed to be crucial for the suppression of cellular immunity. At the same time, secondary cytolytic infection starts in the feather follicle epithelium, where cell-free MDV continues to be produced for life of infected chickens [9]. Sequentially, lymphoma can be developed in several visceral organs, nerves, muscles and skins in chicken as early as 3 weeks after infection [66]. Phenotypic analysis of MD lymphoblast cell lines from tumor lesions revealed that MDV can mainly transform CD3⁺CD4⁺CD8⁺T cells *in vivo* [68].

MAREK'S DISEASE VACCINES

Marek's disease was first reported in 1907 in Hungary [39], and had been a major cause of economic losses of poultry industry until 1970s all over the world. In 1967, the first isolation of MDV was successfully achieved [13, 47],

Table 1. Accelerated apoptosis of lymphoid cells with several viral infections

Host	Viruses	Target cells	References
Human	human immunodeficiency virus-1	CD4 ⁺ and CD8 ⁺ T cells	4, 24, 25, 40
	measles virus	thymocytes	1, 6
	Epstein-Barr virus	thymocytes CD45RO ⁺ T cells	3 80
Mouse	murine cytomegalovirus	thymocytes	92
		peripheral T cells	38
	lymphocytic choriomeningitis virus	peripheral T cells	62
	influenza A virus	thymocytes, splenocytes	42
	LP-BM5 murine leukemia virus	peripheral T cells	32
Cattle	bovine herpesvirus-1	peripheral blood mononuclear cells	28
Pig	African swine fever virus	peripheral lymphocytes	23
Chicken	chicken infectious anemia virus	thymocytes	36
	infectious bursal disease virus	peripheral blood mononuclear cells	82

and enormous efforts for vaccine-developments were independently performed. In early 1970s, neonatal vaccination with live apathogenic MDV was found to prevent lymphoma formation, and since then, MD was effectively controlled by the vaccination. Currently, all three serotypes of MDV, serotype 1 (attenuated strains) [14, 64], serotype 2 (non-pathogenic strain) [67, 93] and serotype 3 (herpesvirus of turkey, HVT) [50, 60, 87] are used as vaccines.

Live MD vaccines, whether serotypes 1 (attenuated), 2 or 3, all induce anti-viral humoral and cell-mediated immunity. Virus-neutralizing antibodies can be detected within 1-2 weeks after infection, and persist throughout the life of the bird. A protective role for passively acquired antibody [7] or maternal antibody [54] has been observed in chicks. However, the effect of antibody is to reduce but not exclude level of infection by impeding spread, and evidence for a humoral immunity against tumor cells is lacking. One of the most striking characteristics of the MD vaccines is that these vaccines can prevent lymphoma formation without protecting chickens from MDV-infection [31, 60]. Therefore, it is generally accepted that the vaccination results in protection via anti-tumor rather than anti-viral effects. Cell-mediated rather than humoral immune responses are assumed to be more important mechanisms for protective effects of the MD vaccines, since bursectomy and X-radiation which can delete humoral immunity have no effect on protection in chickens vaccinated with attenuated MDV [17, 74]. It should be also noted that the similar treatment partially impaired vaccinal immunity induced by HVT [63].

All three serotypes of MD vaccines do not solely show 100% protection against the disease, and vaccination failures are sometimes seen in the field [21, 86]. As a monovalent vaccine, an attenuated serotype 1 strain, CVI988 [64], is the most effective vaccine among the three serotypes. Bivalent and trivalent vaccines combined with other serotypes

increase the protection efficacy against MD than monovalent vaccines [85]. These observations suggest that the anti-tumor mechanisms of vaccines derived from the 3 serotypes might be different from each other. MD vaccines can not completely prevent chickens from MDV-infection, and both vaccine and oncogenic strains are present in chickens for their life of the chicken [10]. It has been still unknown how the MD vaccine prevent chickens from lymphoma formation.

THE SIGNIFICANCE OF CD8⁺T CELLS IN THE PROTECTIVE EFFECTS OF MD VACCINE

Protective roles of T lymphocyte subsets against virus-infection and tumor progression have been examined by using lymphocyte-subset deficient animals, which are established by gene targeting technology or *in vivo* injections of monoclonal antibodies (MAbs) against marker molecules (Table 2). These studies have indicated that CD8⁺T cells play a central role in the clearance of pathogenic agents, inhibition of virus-induced diseases and tumors *in vivo*. The importance of CD8⁺T lymphocytes as effector cells was confirmed by following observations; 1) adaptive transfer of CD8⁺T cells can inhibit tumor development *in vivo* [57], 2) CD8⁺T cells can lyse the viral gene-expressing cells or the target cells for tumors *in vitro* [81, 90].

It has long been discussed whether CD8⁺T cells from MD-vaccinated chickens can effectively lyse target cells expressing viral antigens or MDV-transformed tumor cells. To examine the anti-viral effects of MD vaccines, Schat *et al.* [70] and Pratt *et al.* [58] developed a new assay system for cytotoxic responses against MDV by using reticuloendotheliosis virus-transformed cell lines which are stably transfected with MDV-specific genes. This experimental system showed that the vaccine can induce specific cytotoxic activity of CD8⁺T cells against several MDV antigens [51, 52, 69].

Table 2. T cell-subset deficient animals

Animals	Methods for depletion	Target molecules	Pathological agents	References
Mouse	antibody-injection	CD4 and CD8	murine cytomegalovirus	16
	antibody-injection	CD4, CD8 and INF- γ	<i>Toxoplasma gondii</i>	22
	antibody-injection	CD8	tumor cell lines	45
	gene targeting	CD8	lymphocytic choriomeningitis virus	18
	gene targeting	β_2 -microglobulin	lymphocytic choriomeningitis virus	61
Cattle	antibody-injection	CD4 and CD8	respiratory syncytial virus	77
Horse	antibody-injection	CD5	equine infectious anemia virus	79

Early studies showed that HVT did not protect neonatally thymectomized chickens or T cell-deficient chickens which were prepared by the pretreatment with immunosuppressive drug, cyclophosphamide, from the tumor progression by MDV [26, 59]. These reports indicated that the protective effects of HVT vaccine were mediated through T cell responses. Glycoprotein B (gB) is one of the candidates which induces the protective effects by the vaccine, because this antigen is shared by all three serotypes of MDV. Recently, it has been reported that recombinant fowlpox virus expressing glycoprotein B (gB) significantly reduced the level of cell-associated viremia and induced protective immunity against MD [46, 48, 88]. This recombinant fowlpox virus induces both humoral and cellular immunity via CD8⁺T cells immediately after the vaccination, indicating that immune responses against gB might be essential for the protective effects induced by the vaccines. Interestingly, a series of MD vaccines can induce specific cytotoxicity of lymphocytes against MHC-restricted allogenic but not syngenic tumor cell lines [71].

Recently, it has been reported that chicken T cell subsets can be depleted *in vivo* by neonatal thymectomy (NTx) and injections of MAbs against CD4 or CD8 molecules [49, 89]. By using these T cell-subset deficient chickens, we attempted to demonstrate the protective mechanisms by an attenuated MDV.

Chickens vaccinated with an attenuated MDV (strain CVI988) were depleted of either CD4⁺ or CD8⁺T cells by NTx and two repeated injections of anti-CD4 or CD8 MAb, and then challenged with a highly oncogenic MDV (strain Md5). These birds were observed daily for clinical signs up to 50 days after the challenge, and then subjected to histopathological examinations. Eight out of the 12 untreated non-vaccinated chickens showed MD-related clinical signs and died by 32 days after the challenge, 3 out of the 4 surviving birds were clinically ill. Histologically, mild to severe tumor lesions in visceral organs and nerves were observed. On the other hand, most of chickens from untreated vaccinated (n=8), CD4-deficient vaccinated (n=8) and CD8-deficient vaccinated (n=8) groups did not show any clinical signs associated with MD (Fig. 1). Tumor development was also not observed in chickens depleted of both CD4⁺ and CD8⁺T cells after the vaccination. These chickens were histologically examined to observe MD-

Fig. 1. Survival rates of untreated non-vaccinated (solid line, n=12), untreated vaccinated (broken line, n=8), CD4-deficient vaccinated (open circle, n=8) and CD8-deficient vaccinated chickens (closed circle, n=8). Schedules for vaccination and depletion of T cell subsets are also shown. These chickens were challenged with 20,000 PFU of strain Md5 at 5 days after hatching, and birds were observed daily for clinical signs up to 50 days after the challenge. NTx; thymectomy.

related tumor lesions in the visceral organs (especially liver, spleen and kidney) and peripheral nerves. In the untreated non-vaccinated group, most of the chickens showed mild to severe MD-lymphoma lesions in these organs. On the other hand, most of the chickens in the vaccinated three groups showed no MD-lymphoma lesions. These results indicated that CD4⁺ and CD8⁺T cell responses induced by vaccine strain CVI988 are not essential for anti-tumor immune mechanisms.

The neonatal vaccination with strain CVI988 decreased the number of MDV rescued from CD4⁺T cells, which are

Fig. 2. MDV-titers in CD4⁺T cells from non-treated vaccinated (n=7), non-treated unvaccinated (n=7) and CD8-deficient vaccinated (n=8) chickens. These chickens were challenged with 10,000 PFU of strain Md5, and MDV-titers were examined at 2 weeks after challenge. Splenocytes from two chickens of each group were pooled as one set, and then CD4⁺T cells were isolated from these pooled splenocytes. Each dot represents MDV titer of CD4⁺T cells of each set. CD4⁺T cells from CD8-deficient chickens inoculated only with strain CVI988 were also subjected to plaque assays (n=3). These data were obtained from two separated experiments and the statistical significance was determined by Student's *t*-test.

the main target cells for latent infection in lymphoid cells [31, Lee *et al.*, submitted for publication]. Next, we examined whether the anti-viral immunity induced by the MD vaccine was due to CD8⁺T cell responses. MDV titers in CD4⁺T cells were much higher in CD8-deficient vaccinated chickens as compared to untreated vaccinated chickens at the early stage of the latent phase, 2 weeks after the challenge. Surprisingly, MDV titers in CD4⁺T cells showed no differences between CD8-deficient vaccinated and untreated non-vaccinated chickens as shown in Fig. 2. Taken together, CD8⁺T cells play a crucial role for the anti-viral effects of the vaccine, but may not be essential for the prevention of lymphoma formation by an oncogenic MDV.

POSSIBLE MECHANISMS OF ANTI-TUMOR EFFECTS OF THE MD VACCINE

There are several possible mechanisms of anti-tumor effects of the MD vaccine, but the exact mechanisms still remain unknown. Probably, several other T cell populations may be involved in the anti-tumor effects. $\gamma\delta$ T cell population is one of the major peripheral T cells in chicken [12, 78]. The function of $\gamma\delta$ T cells is largely uncertain among avian and mammalian species. In mice and several other animals, it is assumed that $\gamma\delta$ T cells have a crucial

role for mucosal barrier, including respiratory and gut epithelium, because these organs contain large numbers of $\gamma\delta$ T cells rather than $\alpha\beta$ T cells [27]. In contrast, there is growing evidence that $\gamma\delta$ T cells are important for early responses against persistent infectious agents [27, 37]. For instances, $\gamma\delta$ T cells secrete type 1 cytokines earlier than $\alpha\beta$ T cells, depletion of $\gamma\delta$ T cells by the administration of a specific antibody impaired host resistance to listerial infection [33]. The important roles of $\gamma\delta$ T cells have also been reported in the protection against other pathogens such as *Plasmodium* [35], *Toxoplasma* [15, 34] and several viruses [20, 34]. Recently, $\gamma\delta$ T cells have been shown to play an important role in murine herpes simplex virus infection [72]. Moreover, certain populations of $\gamma\delta$ T cells have been reported to show strong cytotoxic activities against tumor cells [5, 19, 41]. Thus, $\gamma\delta$ T cells in chicken may also be crucial for protective efficacy of the MD vaccine.

Recently, minor lymphocyte populations have been reported in mice and human, for examples, CD4⁺CD8⁺T cells, natural killer (NK) T cells and NK cells. Some populations of these cells have been reported to display a strong cytotoxicity against tumor cell lines [29, 76], and the counterparts of these T cell populations in chickens may have cytotoxic effects against tumor cells. In addition, NK activity is also important for innate resistance against MD and is increased after the vaccination [30, 73]. Further characterization of these subpopulations in chickens would be important for understanding the protection mechanism of the MD vaccine.

We previously reported that MDV induces apoptosis in peripheral CD4⁺T cells [43], and in our preliminary experiment, the vaccination did not rescue apoptosis of CD4⁺T cells. MDV can mainly transform CD4⁺T cells [68], although the other phenotypic cells, e.g. CD4⁺CD8⁺T cells, were also transformed under certain conditions [49]. An alternate possibility of the anti-tumor effects of the MD vaccine may be due to apoptosis of CD4⁺T cells, which could reduce the number of target cells for transformation *in vivo*.

Based on our results and previous reports, we summarize the possible protection mechanism(s) in Fig. 3. In vaccinated chicken, MDV-infected cells which express viral antigens could be eliminated mainly by CD8⁺T cells. Remaining infected cells and/or transformed cells might be killed by cell populations other than CD8⁺T cells. These cells include NK cells, $\gamma\delta$ T cells and NKT cells. In addition, apoptosis of CD4⁺T cells (both infected and uninfected), targets for transformation by MDV, induced by MD vaccines may also contribute to the protection mechanism since the chances for transformation can be significantly reduced.

MD vaccines have long been considered as a model of protective immunity against herpesvirus-induced tumor. The three serotypes of MD vaccines are ones of a few effective vaccine which can prevent infectious lymphomatous disorders among mammalian and avian species. The

Fig. 3. The possible protection mechanism(s) induced by MD vaccine. Details are described in the text. NK: natural killer, NKT cells: natural killer T cells, DNT cells: double negative T cells.

demonstration of anti-tumor effects by MD vaccines, including attenuated strains, apathogenic strains and HVT, can provide useful information to develop immunotherapy against not only herpesvirus but also other virus-induced tumors.

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