ETIOLOGICAL STUDIES ON MAREK'S DISEASE VACCINE BREAKS
（マレック病ワクチンブレイクに関する病原学的研究）

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CONTENTS

PREFACE

Marek's disease (MD) was first described as polyneuritis in rooster by Marek (1907). MD is characterized by a mononuclear infiltration of various organs and tissues such as peripheral nerves, gonad, iris, visceral organs, muscle and skin. The etiology of MD was established during the late 1960's by the isolation of a herpesvirus from MD and the subsequent reproduction of the disease with the isolated virus (MD virus, MDV) (Churchill and Biggs, 1967; Nazerian et al., 1968; Solomon et al., 1968). MD was a subacute or chronic disease of chickens recognized mostly as fowl paralysis for many years since the first description of MD. However, outbreaks of so-called acute MD with unusually high mortality and incidence of visceral lymphomas occurred in the USA in the mid-1950s and subsequently became severe problems throughout the world until the development of vaccines.

Shortly after the first isolation of MDV, a herpesvirus was isolated from normal turkeys (herpesvirus of turkeys, HVT) (Kawamura et al., 1969; Witter et al., 1970). Subsequently, naturally avirulent MDV was isolated from normal chickens (Biggs and Milne, 1972; Zander et al., 1972; Schat and Calnek, 1978). At present, MDV and HVT strains are classified as Gallid herpesvirus 2 and Meleagrid herpesvirus 1 of the subfamily Gammaherpesvirinae, respectively. MDV strains are serologically divided into two serotypes using agar gel precipitation and indirect fluorescent antibody tests with conventional or type-specific monoclonal antibodies: serotype 1 includes virulent strains of MDV (vMDV) and their cell-culture attenuated strains; serotype 2 includes naturally avirulent strains of MDV (Bülow and Biggs, 1975a,b; Lee et al., 1983). HVT strains comprise the third serological group (serotype 3) since they are antigenically related to both MDV serotypes.

Vaccines from all three serotypes have been developed since the early 1970s. A cell-culture attenuated virulent serotype 1 MDV was first developed as a vaccine (Churchill et al., 1969a, b). Okazaki et al. (1970) reported the protective efficacy of HVT against MD, and HVT has been widely used as a live vaccine throughout the world including Japan. Rispens et al. (1972) developed a vaccine attenuated by serial tissue culture passages from CVI988 strain of a mildly virulent serotype 1 MDV. Avirulent serotype 2 MDVs were effective against MD (Biggs et al., 1972, 1973; Zander et al., 1972; Schat and Calnek, 1978). The SB-1 strain of serotype 2 MDV (Schat and Calnek, 1978) has been produced commercially as a vaccine. This vaccine strain is used in combination with HVT vaccine as a bivalent vaccine. In Japan CVI988 vaccine and a bivalent vaccine composed of HVT and SB-1 were recently licensed for a commercial use.

Vaccination has contributed to substantial reduction of MD losses of chickens. However, unacceptably excessive MD losses have been occasionally observed among vaccinated chicken flocks in several countries since the late 1970s (Eidson et al., 1981; Zanella, 1982; Powell and Lombardini, 1986; Reece et al., 1986; Shieh, 1988). Although it is difficult to retrospectively ascertain precise causes of excessive losses, several possible causes of the problems, commonly termed 'vaccine breaks', have been proposed (Witter, 1985). Breaks may be attributed to the factors such as inadequate vaccine production methods (Thornton et al., 1975), mismanagement of vaccine (Halvorson and Mitchell, 1979), early exposure to MDV before vaccinal immunity develops (Okazaki et al., 1973), delayed onset of vaccinal immunity owing to interference by homologous maternal antibodies (Calnek and Smith, 1972), the influence of genetic constitution of chickens (Spencer et al., 1974), depression of vaccinal immunity by other viruses such as infectious bursal disease virus (Giambrone et al.,

1976; Sharma, 1984) and reticuloendotheliosis virus (Witter et al., 1979). There have been reported that some vaccine strains induced MD lesions in MD-susceptible chickens (Bülow, 1977; Pol et al., 1986). The possibility may be hence exist that the pathogenicity of vaccine strains themselves is also involved in possible causes.

Although Okazaki et al. (1973) suspected the existence of MDV strains which were able to override HVT vaccinal immunity, they could not isolate such strains. The isolation of such MDV strains has been reported in the USA, Italy and Australia since 1978 (Eidson et al., 1978; Witter et al., 1980; Eidson et al., 1981; Schat et al., 1982; Witter, 1983; Powell and Lombardini, 1986; McKimm-Breschkin et al., 1990). Apparently, such strains were more virulent in HVT-vaccinated, susceptible chickens than conventional strains of vMDV, and they have been designated as very virulent MDV (vvMDV) to differentiate them from vMDV. VvMDVs have not yet been isolated in Japan.

VvMDVs were serologically indistinguishable from serotype 1 vMDVs (Lee et al., 1983). Also, no marked differences were observed between vvMDVs and vMDVs by restriction endonuclease analysis (Ross et al., 1984). Witter et al. (1980) reported that their isolates of vvMDV caused an early mortality syndrome characterized by early death, severer depression in body and bursal weight, bursal and thymic atrophy in absence of MD lymphomas, whereas those of vvMDV reported by other groups did not cause this syndrome (Schat et al., 1982; Powell and Lombardini, 1986).

The isolation of vvMDV was three times more frequent from vaccinated flocks with excessive losses than from vaccinated flocks without excessive losses (Witter, 1983), suggesting a close association of vvMDV with vaccine breaks.

Extensive studies to develop the vaccines for the control of vvMDVs

have been conducted. American groups showed that vvMDVs were better protected by a bivalent vaccine of HVT and serotype 2 MDV (Schat et al., 1982; Witter, 1987; Witter and Lee, 1984). A bivalent vaccine composed of HVT and attenuated serotype 1 MDV or attenuated serotype 1 MDV alone was also effective against challenge with vvMDV isolates (De Boer et al., 1986; Powell and Lombardini, 1986). Recently, however, bivalent vaccines composed of HVT and serotype 2 MDV did not provide good protection against vvMDV reported in Australia (McKimm-Breschkin et al., 1990).

In Japan, MD is largely well controlled by vaccination. However, unacceptably excessive MD losses have been occasionally observed in properly vaccinated chicken flocks similar to other countries (Kubo et al., 1982; Kazama and Oowaki, 1985; Sekiya et al., 1987). Many commercial flocks of Japanese quails are also kept for egg and meat production in Japan. Quail flocks have long had problems with recurring lymphoproliferative diseases (Kobayashi et al., 1986). Since the diseases were diagnosed as MD from pathological findings, vaccination of HVT has been conducted. Although MD in quail flocks has been largely well controlled by vaccination, excessive losses have been occasionally observed (Kobayashi et al., 1986). It is needed to make an inquiry into the cause of vaccine breaks; however, attempts have not been sufficiently performed.

Chicken anemia agent (CAA), which was first described by Yuasa et al.(1979) is a small, unclassified DNA virus (Goryo et al., 1987a; Gelderblom et al., 1989; McNulty et al., 1990; Todd et al., 1990). CAA is probably present worldwide in chicken flocks, and naturally occurring CAA-induced diseases have been reported (Yuasa et al., 1979, 1985, 1987; Bülow et al., 1983; Goryo et al., 1985, 1987b; Otaki et al., 1987; Engström, 1988; Vielitz and Landgraf, 1988; Chettle et al., 1989; Goodwin et al., 1989; McNulty et al., 1988, 1989; Rosenberger and Cloud, 1989; Firth and

Imai, 1990). The diseases induced by CAA were characterized by death, anemia due to severe atrophy of the hematopoietic tissues in bone marrow, hemorrhages, and severe atrophy of lymphoid organs. It is supposed that CAA infection results in immunosuppression in chickens by virture of damage of hematopoietic and lymphoid tissues. There was circumstantial evidence that CAA might cause opportunistic or secondary infections (Engström and Luthman, 1984; Goryo et al., 1987b; Vielitz and Landgraf, 1988). In addition, CAA influenced vaccinal immunity (Box et al., 1988; Otaki et al., 1988).

It was reported that the diseases caused by CAA were enhanced by dual infections with CAA and MDV or HVT, and similar to an early mortality syndrome (EMS) caused by some vvMDVs (Bülow et al., 1983; Otaki et al., 1987, 1988). However, the involvement of CAA infection in EMS has not been sufficiently examined. Two groups reported the isolation of CAA from vaccinated flocks with excessive MD losses (Otaki et al., 1988; De Boer et al., 1989). However, only one group described the association of CAA infection with MD vaccine breaks (Otaki et al., 1988). Thus there is a paucity of information on the association of CAA infection with vaccine breaks.

Genetic constitution of chicken lines used in MD experiments is a very important factor which influences the incidence of MD and vaccinal immunity (Calnek, 1985). It would be critical to use appropriate experimental chickens having definite susceptibility to MD in order to make an inquiry into the cause of MD vaccine breaks, particularly to demonstrate the existence of vvMDV. In Japan, however, such chickens have not been maintained. Therefore the author introduced genetically MD-susceptible P-2 chickens and commenced the studies on MD vaccine breaks.

During the present studies, the following results were obtained: MDVs

which were not adequately protected by HVT vaccine were isolated from MD-affected chickens. This is the first description of vvMDVs in Japan; the author demonstrated the effect of vaccines against challenge with these isolates of vvMDV; CAA infection impaired HVT vaccinal immunity against MD; the CVI988 strain used as a live vaccine induced MD in chickens, although the incidence was very low.

These studies are described in this thesis.

CHAPTER I Isolation and Characterization of Very Virulent Strain of Marek's Disease Virus

INTRODUCTION

Very virulent Marek's disease viruses (vvMDVs), which break through herpesvirus of turkeys (HVT) vaccinal immunity, have been isolated in the USA, Italy and Australia (Eidson et al., 1978, 1981; Witter et al., 1980; Schat et al., 1982; Powell and Lombardini, 1986; McKimm-Breschkin et al., 1990). They have been considered to be a primary cause of vaccine breaks.

Typing of vvMDV by a panel of monoclonal antibodies showed that vvMDVs are serologically indistinguishable from serotype 1 virulent MDVs (vMDVs) (Lee et al., 1983). However, American and Italian strains of vvMDV were more virulent in MD-susceptible chickens vaccinated with HVT than those of vMDV (Witter et al., 1980; Schat et al., 1982; Powell and Lombardini, 1986). VvMDVs have been also reported to induce more lymphomas (Witter et al., 1980; Schat et al., 1981, 1982; Powell and Lombardini, 1986) and more visceral and fewer neural lymphomas in unvaccinated chickens as compared with vMDVs (Witter, 1983; Powell and Lombardini, 1986). Some vvMDV strains also induced an early mortality syndrome (EMS) in unvaccinated chickens (Witter et al., 1980); others did not induce EMS (Schat et al., 1982; Powell and Lombardini, 1986).

American strains of vvMDV are known to be adequately protected by a bivalent vaccine composed of HVT and serotype 2 MDV as compared with HVT alone (Schat et al., 1982; Witter and Lee, 1984). Recently, however, it was reported in Australia that vvMDVs could not be adequately protected by a bivalent vaccine (McKimm-Breschkin et al., 1990). An attenuated serotype 1 MDV or a bivalent vaccine composed of serotype 1 MDV and HVT also gave

good protection against the vvMDV challenge (De Boer et al., 1986; Powell and Lombardini, 1986).

HVT vaccination successfully protects MD to a large extent in Japan. However, excessive losses of MD occasionally occurrs in vaccinated chicken and Japanese quail flocks (Kubo et al., 1982; Kazama and Oowaki, 1985; Kobayashi et al., 1986; Sekiya et al., 1987). The existence of vvMDVs has not been described in Japan, although the association of these problems with such very virulent strains has been suspected.

The author directed his efforts to the isolation of vvMDVs from chicken and quail flocks with MD problems. This chapter describes the first isolation of vvMDVs and their characterization. In addition, the author evaluated protective ability of different vaccine types against the vvMDV isolates.

MATERIALS AND METHODS

Chickens and quails

Specific-pathogen-free (SPF) chickens were obtained from flocks of PDL-1 chickens (Furuta et al., 1980) and MD-susceptible P-2 chickens (Schat et al., 1981). The P-2 chickens were kindly provided by Dr. B.W. Calnek, Cornell University, the USA. The flocks of SPF chickens were monitored with post-mortem and periodical serological examinations against all known avian pathogens including MDV. The flocks were free of all MDV serotypes. The chickens used in the experiments were kept in isolators placed in rooms with a filtered air ventilation system.

Specimens for MDV isolation were obtained from chickens of twelve White leghorn flocks with MD problems in five prefectures of Japan. Fifty-nine chickens were collected from eleven flocks which had been vaccinated with HVT vaccine. The MD incidence in these flocks ranged from 2.4 to 17.8 %. These chickens were examined for MD gross lesions, and skin strips were collected from them. Thirteen-four of them had MD gross lesions, and the lesions were observed in a variety of visceral organs, skin, muscle or peripheral nerves. For MDV isolation a 20 % skin emulsion was prepared in sucrose, phosphate, glutamine and albumin (SPGA) buffer. Heparinized blood samples from two chickens of one unvaccinated flock with high mortality from MD were also received for virus isolation.

Sixty-one quails were obtained from eight flocks on seven farms which had experienced recurring outbreaks of lymphoproliferative disease in two prefectures and 17 out of them had MD gross lesions. The gross lesions were observed in the liver, spleen, lung, pancreas, proventriculus and small intestine (especially the duodenum). Microscopical examination revealed lymphoid proliferation in these tissues similar to that seen in MD-affected

chickens. No gross lesions in peripheral nerves were noticed. Quails from five out of eight flocks had been vaccinated with HVT.

Heparinized blood and skin samples were collected from these quails for MDV isolation. The blood samples from four flocks were pooled. A 20% skin emulsion in SPGA buffer was prepared. The kidney was pooled by flock and cultured directly for MDV isolation as described below.

Cell cultures

Chicken kidney cell (CKC) cultures were obtained from 8-week-old PDL-1 chickens by the modification of the method described by Kawamura and Tsubahara (1968). CKC suspension was grown in Dulbecco's modified Eagle medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% tryptose phosphate broth, 5% calf serum and 2% of 7.5% sodium bicarbonate ($NaHCO_3$) at 37 C in a humidified CO_2 incubator. CKC cultures were maintained in the same medium with 1% calf serum and 1% of 0.2 M tris (hydroxymethyl) aminomethane without CO_2. Quail kidney cell cultures were also conducted by the same method as CKC cultures.

Chicken embryo fibroblast (CEF) or duck embryo fibroblast (DEF) cultures were prepared from 10-day-old Line 15I (Hihara et al., 1980) or duck embryos as described previously (Witter et al., 1969). SPF duck embryonating eggs were kindly provided by Dr. S. Yamada, Kikuchi Laboratories of the Chemo-Sero-Therapeutic Research Institute, Kumamoto. The medium was similar to that of CKC cultures, except that $NaHCO_3$ was used to adjust pH in maintenance medium.

The QT35 cell line (Moscovici et al., 1977) was kindly provided by Dr. R.L. Witter, Agricultural Research Service, the USA. The media for QT35 cell line was similar to that of CEF cultures, except for an incorporation of fetal calf serum.

MDCC-MSB1 and LSCC-BK3 cells were cultured in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10 % fetal calf serum at 40 C in a humidified CO_2 incubator as described previously (Yamaguchi et al., 1981; Yuasa, 1983).

Assay for MDV and other known viruses

Duplicated cell cultures were inoculated with 0.2 ml of heparinized blood or skin emulsion samples per culture for MDV isolation. The cultures were observed for 14 days post-inoculation (dpi).

The same dose of the blood samples collected from quails was also intra-abdominally inoculated into one-day-old chicks for MDV isolation. Inoculated chickens were observed with uninoculated control or contact chickens for 43 to 60 dpi. Chickens which showed clinical signs and all the remainder of chickens at the end of the experiment were autopsied. Heparinized blood samples were collected for virus isolation.

The presence of MDV or HVT antigens in cell cultures was determined by an indirect immunofluorescent antibody (IFA) test using monoclonal antibodies to serotypes 1, 2 and 3 (Lee et al., 1983), and fluorescent isothiocyanate-conjugated anti-mouse IgG rabbit IgG (Zymed Laboratories, Inc., USA). The monoclonal antibodies were kindly provided by Dr. T. Mikami, Faculty of Agriculture, The University of Tokyo, Tokyo.

The very virulent strains, Md/5 (Witter et al., 1980) and RB-1B (Schat et al., 1981) and virulent strains, JM (Sevoian et al., 1962) and GA (Eidson and Schmittle, 1968) were used as reference strains.

CEF ($2x10^7$ cells/ml) infected with MDV isolates was freeze-thawed 3 times and centrifuged at 3,000 rpm for 10 min. The supernatant fluid was used as an inoculum to examine the presence of other viruses in MDV isolates. Assay for cytopathic viruses was carried out with CKC and CEF.

The inoculated cultures were observed for 14 dpi.

Isolation of chicken anemia agent (CAA) was performed using MSB1 cells according to the method of Yuasa (1983). Neutralizing antibodies to the A2 strain of CAA (Yuasa and Imai, 1986) were examined using a microtest system as described previously (Imai and Yuasa, 1990).

Assay for avian leukosis virus (ALV) subgroups A and B was performed by the resistance-inducing factor (RIF) test (Rubin, 1960). Antibodies to Rous associated virus (RAV-1) subgroup A were measured by the IFA test (Payne et al., 1966). Antibodies to subgroup B were measured by an enzyme linked immunosorbent assay (ELISA) (Tsukamoto et al., 1985).

Isolation of reticuloendotheliosis virus (REV) was conducted as described previously (Yuasa et al., 1976). Antibodies to REV-T strain (Robinson and Twiehaus, 1974) were measured by the IFA test.

Antibodies to infectious bursal disease virus (IBDV) were measured using BK3 cells infected with the J1 strain of IBDV by the IFA test as described previously (Yamaguchi et al., 1981).

Vaccines

The FC-126 strain of HVT (serotype 3) (Witter et al., 1970) and CVI988 strain of MDV (serotype 1) (Rispens et al., 1972) from commercial sources were used. A 2H strain of MDV (serotype 2) was kindly provided by Dr. H. Hihara, Poultry Disease Laboratory, National Institute of Animal Health, Gifu and used as a bivalent vaccine with HVT.

Pathogenicity tests

Chickens were inoculated intra-abdominally at one day of age with 2,000 or 5,000 plaque forming units (PFU) of MDV per chicken. Chickens were

observed for about 60 dpi. Birds that died or were killed at the end of the experiment were macroscopically examined for MD gross lesions. Dead birds which showed bursal and thymic atrophy without lymphomas were considered as EMS. Chickens which had MD gross lesions were divided into two categories, classical MD and acute MD. Classical MD was characterized by enlargement of peripheral nerves, while acute MD was characterized by lymphoma formation in the visceral organs.

To examine for effect in an early phase of infection, chickens were similarly inoculated at one day of age with 5,000 PFU of MDV per chicken. Controls were inoculated with uninfected CEF. Chickens were killed 11 dpi and examined for weights of body and bursa of Fabricius and microscopical lesions of lymphoid organs. Relative bursal weight was the bursal weight divided by the body weight times 100. The spleen, thymus and bursa were microscopically examined. Microscopical lesion scores of 0 (normal) to 3 (severe) were given.

Protection tests

PDL-1 or P-2 chickens were vaccinated subcutaneously with 2,000 PFU of HVT or CVI988 or a mixture of 1,000 PFU each of 2H and HVT per chicken at one day of age, and were challenged intra-abdominally with 2,000 or 5,000 PFU of vMDV or vvMDV at 7 or 10 days of age. Unvaccinated chickens were similarly challenged with vMDV or vvMDV. Chickens were observed for about 10 weeks after challenge. Birds that died or were killed at the end of the experiment were examined for MD gross lesions. Questionable lesions were microscopically confirmed. Five random serum samples were collected from each of the vaccinated, MDV-challenged groups to test the presence of antibodies to CAA, ALV, REV and IBDV. Protective index (PI) was calculated by the following formula.

PI=(% MD in unvaccinated, MDV-challenged control - % MD in vaccinated, MDV-challenged control) / % MD in unvaccinated, MDV-challenged controls x 100.

Histology

After birds were necropsied, tissues were collected, fixed in 10 % buffered formalin, embedded in paraffin, cut and stained with hematoxylin and eosin.

Statistics

Data were analyzed by Fisher's exact test or by analysis of variance.

RESULTS

Isolation of MDV from chickens and quails

Thirteen MDV-like viruses were isolated from vaccinated or unvaccinated chickens by inoculation of skin emulsion or blood samples into the cell cultures such as CKC, CEF or DEF. These viruses were identified as vMDVs on the basis of reactivity with serotype 1-specific monoclonal antibodies, the morphology of plaques on CEF cultures and oncogenicity in chickens. These isolates were designated as AM11, AM14, AM15, EM11, EM12K, EM12E, EM15, SM13, SM15, SM21, SM26, MS1 and MS2, respectively (Tables 1 and 2).

No MDV was isolated from quails by inoculation of blood or skin emulsion samples into CKC and/or CEF cultures, and by direct cultivation of quail kidney cells but HVT-like viruses were isolated from two out of the five flocks with vaccinated with HVT. These viruses were identified as HVT on the basis of reactivity with serotype 3-specific monoclonal antibodies, the morphology of plaques on CEF cultures and a susceptibility to QT35 cell cultures as described previously (Cho, 1981). However, four MDV-like viruses were recovered from SPF chickens inoculated with the quail blood samples or their contact chickens. These viruses were identified as vMDVs on the basis of reactivity with serotype 1 specific-monoclonal antibodies, the morphology of plaques on CEF cultures and oncogenicity in chickens, and they were designated as QM1, QM2, QM3 and QM4, respectively (Tables 1 and 2).

Virulence of MDV

Virulence of seventeen MDV strains isolated from chickens or quails was examined in PDL-1 and P-2 chickens. Virulence of the strains varied. The mortality rate from lymphomas caused by MDV strains in PDL-1 chickens

Table 1. Virulence of MDV strains in PDL-1 chickens[a]

Strain	No. of chickens	% Early death from EMS[b]	% Mortality from MD	% Total gross lesions	% with MD	
					Classical	Acute
AM11	20	0	20 (37.4)[c]	90	72	28
AM14	7	0	43 (55.3)	86	17	83
AM15	18	0	44 (45.0)	100	43	57
EM11	8	0	50 (51.3)	100	13	87
EM12K	19	0	0	16	33	67
EM15	8	0	38 (55.3)	100	50	50
SM13	19	0	37 (41.5)	63	42	58
SM15	15	0	40 (39.3)	100	33	67
SM21	17	6 (9.0)[c]	38 (39.0)	81	46	54
SM26	9	0	67 (49.0)	100	56	44
MS1	18	10 (24.0)	72 (37.5)	100	39	61
MS2	16	0	93 (28.7)	100	13	87
QM1	10	0	80 (44.2)	90	56	44
QM2	12	0	91 (39.0)	100	100	0
QM3	20	0	95 (42.8)	100	35	65
JM	18	0	83 (26.6)	83	87	13
Md/5	10	10 (27.0)	89 (37.4)	100	0	100
RB-1B	20	0	90 (35.7)	100	20	80

[a]One-day-old chickens were intra-abdominally inoculated with 5,000 PFU of MDV per chicken. Experiment was terminated about 60 dpi.

[b]EMS=an early mortality syndrome.

[c]Median time to death (days) in parenthesis.

ranged from 0 to 95%, while the respective mortality rates by the reference strains, JM, Md/5 and RB-1B, were 83, 89 and 90, respectively (Table 1). The high mortality rate was observed in chickens inoculated with QM1 or QM2. However, these strains induced more classical MD than acute MD. QM1 could not induce MD gross lesions in all chickens inoculated. Among these strains, three strains (MS1, MS2 and QM3) seemed to cause a high incidence of death and gross lesions and be viscerotropic rather than neurotropic, which resembled the results of Md/5 and RB-1B of vvMDV. The early mortality syndrome was observed in chickens inoculated with SM21, MS1 or Md/5; however, the incidence of EMS was low.

Virulence of the strains in P-2 chickens also varied (Table 2). The mortality rate from lymphomas caused by the strains ranged from 31 to 100 %. Among these strains, three strains (MS1, MS2 and QM3) also induced a high mortality rate from MD and were highly viscerotropic as well as RB-1B of vvMDV. The EM11 and SM15 strains killed more than 80% of the chickens inoculated, although the incidence of death caused by these strains was not very high in PDL-1 chickens. When compared with the incidence of MD in the two lines of chickens inoculated with the same viruses, no marked differences were observed. In contrast, the incidence of EMS increased in P-2 chickens. No anemic bone marrows were observed in chickens with EMS.

Eventually, since three strains (MS1, MS2 and QM3) appeared to be virulent and induce acute MD rather than classical MD as well as the reference strain of vvMDV in both lines of chickens used, protective efficacy of HVT vaccine against these strains was examined as described in protection test. As a result, since MS1 and MS2 appeared to break through HVT vaccinal immunity, the further pathogenic characteristics of these strains were examined in comparison with reference strains of vMDV and vvMDV (Tables 3, 4 and 5). P-2 chickens inoculated with MS1 or MS2 were

Table 2. Virulence of MDV strains in P-2 chickens[a]

Strain	No. of chickens	% Early death from EMS[b]	% Mortality from MD	% Total gross lesions	% with MD	
					Classical	Acute
AM11	18	28 (9.4)[c]	46 (47.3)[c]	100	15	85
AM15	15	7 (12.0)	43 (51.2)	100	21	79
EM11	16	38 (13.1)	80 (54.7)	100	0	100
EM12E	14	21 (10.3)	73 (45.3)	100	18	82
SM15	20	15 (14.3)	94 (43.3)	100	35	65
MS1	24	33 (14.6)	100 (41.6)	100	6	94
MS2	16	18 (18.3)	100 (29.2)	100	8	92
QM3	26	0	96 (40.8)	100	15	85
QM4	16	0	31 (455)	100	33	67
JM	11	0	64 (33.3)	100	36	64
RB-1B	18	17 (10.6)	100 (30.6)	100	0	100

[a]One-day-old chickens were intra-abdominally inoculated with 5,000 PFU of MDV per chicken. Experiment was terminated about 60 dpi.

[b]EMS= an early mortality syndrome.

[c]Median time to death (days) in parenthesis.

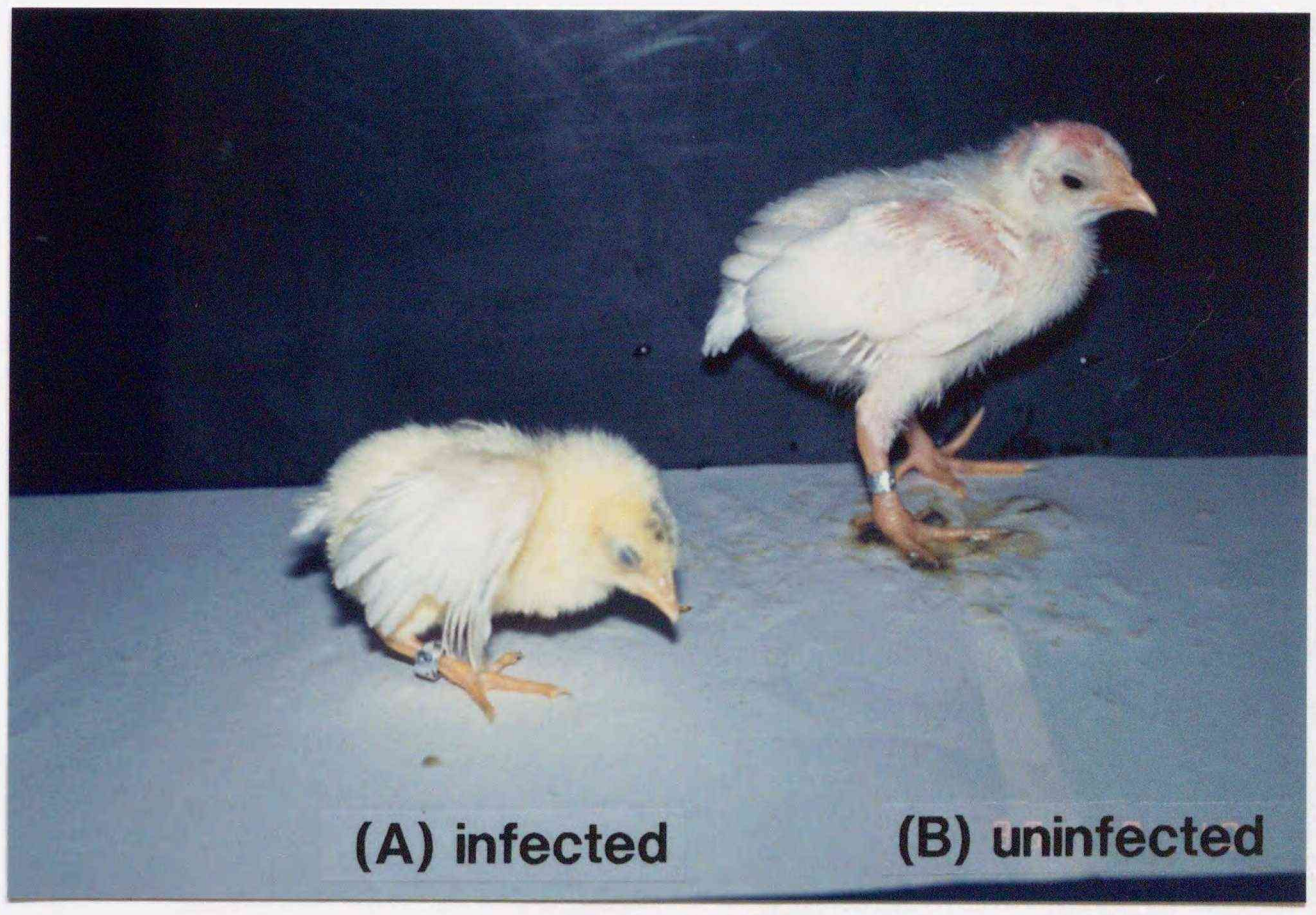

Fig. 1. Clinical signs. A) A chicken 11 days after inoculation with the MS2 strain of MDV. The affected chicken shows a marked loss in body weight and depression. B) A control chicken is inoculated with uninfected CEF.

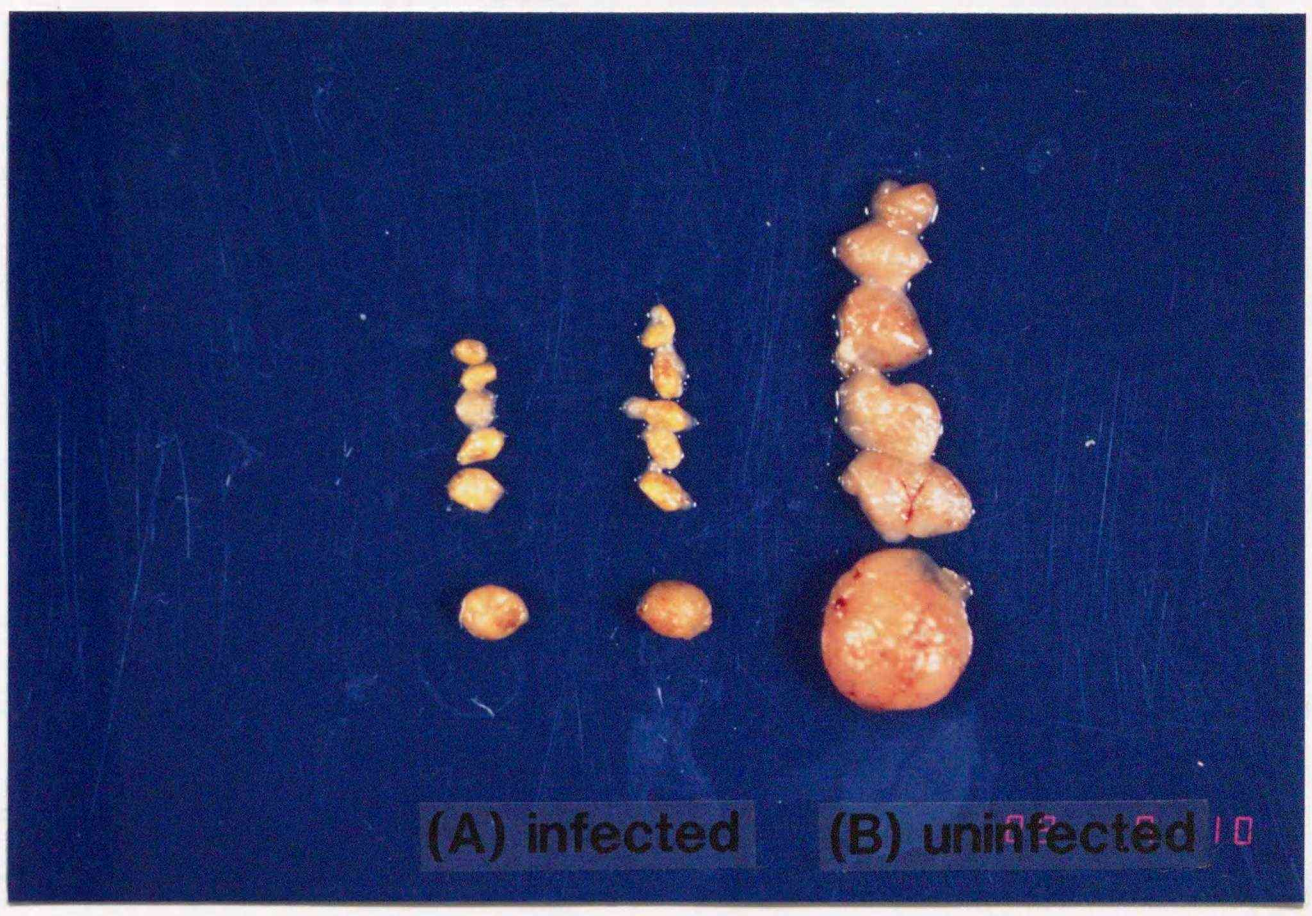

Fig. 2. Thymic lobes and bursa of Fabricius from a chicken 11 days after inoculation with the MS2 strain of MDV (A) or uninfected CEF (B). Severe atrophy of the thymus and bursa of Fabricius is observed (A).

Table 3. Effect of MDV infection in P-2 chickens 11 days after inoculation

Strain	No. of chickens[a]	% Early death from EMS[b]	Body weight (g)	Relative bursal weight	Lesion score (mean score) Spleen	Bursa	Thymus
MS1	15	0	65.0*	0.09*	1.0	1.7*	2.4*
MS2	14	14	51.2*	0.10*	1.9*	2.2*	2.8*
Md/5	15	26	55.6*	0.09*	1.7*	2.2*	2.7*
GA	13	0	87.0	0.21	1.0	0.2	0.2
None (CEF)	14	0	98.7*	0.29*	0.0*	0.0	0.0

[a]One-day-old chickens were intra-abdominally inoculated with 5,000 PFU of MDV per chicken.

[b]EMS=an early mortality syndrome.

*Significantly ($P<0.05$) different from data in chickens inoculated with the GA strain.

examined for effect in an early phase of infections in comparison with reference strains, Md/5 of vvMDV or GA of vMDV (Table 3). MS2 and Md/5 killed 14% and 26% of infected chickens 11 dpi, respectively. Chickens inoculated with MS1 or MS2 as well as Md/5 showed severe depression or ataxia (Fig. 1A). Also, severe bursal and thymic atrophy were observed in chickens inoculated with MS1 or MS2 (Fig. 2). In chickens inoculated with MS1, MS2 or Md/5, a significant depression in body weight and relative bursal weight was observed compared to those inoculated with GA ($P<0.05$). Microscopical severe lymphocyte depletion of bursa and thymus was observed in chickens inoculated MS1, MS2 (Fig. 3) or Md5 and the lesion scores of bursa and thymus in chickens inoculated with these strains were significantly higher than those of bursa and thymus in chickens inoculated with GA ($P<0.05$). MS2 and Md/5 induced a significant severer spleen necrosis than GA ($P<0.05$), whereas MS1 did not induce the severe necrosis (Table 3).

In a long term experiment, virulence of the strains, MS1 and MS2 was compared to that of the reference strains of vMDV and vvMDV in P-2 and PDL-1 chickens (Tables 4 and 5). EMS was observed in 13-20% of P-2 chickens inoculated with MS1, MS2 or Md/5 (Table 4). The strains, MS1 and MS2 caused the high incidence rates of death and MD gross lesions in P-2 chickens as well as the reference strains. Among the strains, MS2 showed the shortest median time to death (MTD) from MD in P-2 chickens.

No EMS was observed in PDL-1 chickens inoculated with MDVs used (Table 5). The strains, MS1 and MS2 induced the high mortality rates from MD in PDL-1 chickens as well as Md/5 and JM. MS2 also showed the shortest MTD in PDL-1 chickens.

Although MS1 and MS2 induced the high incidence of visceral gross lesions in both chicken lines used, they also caused a similar high

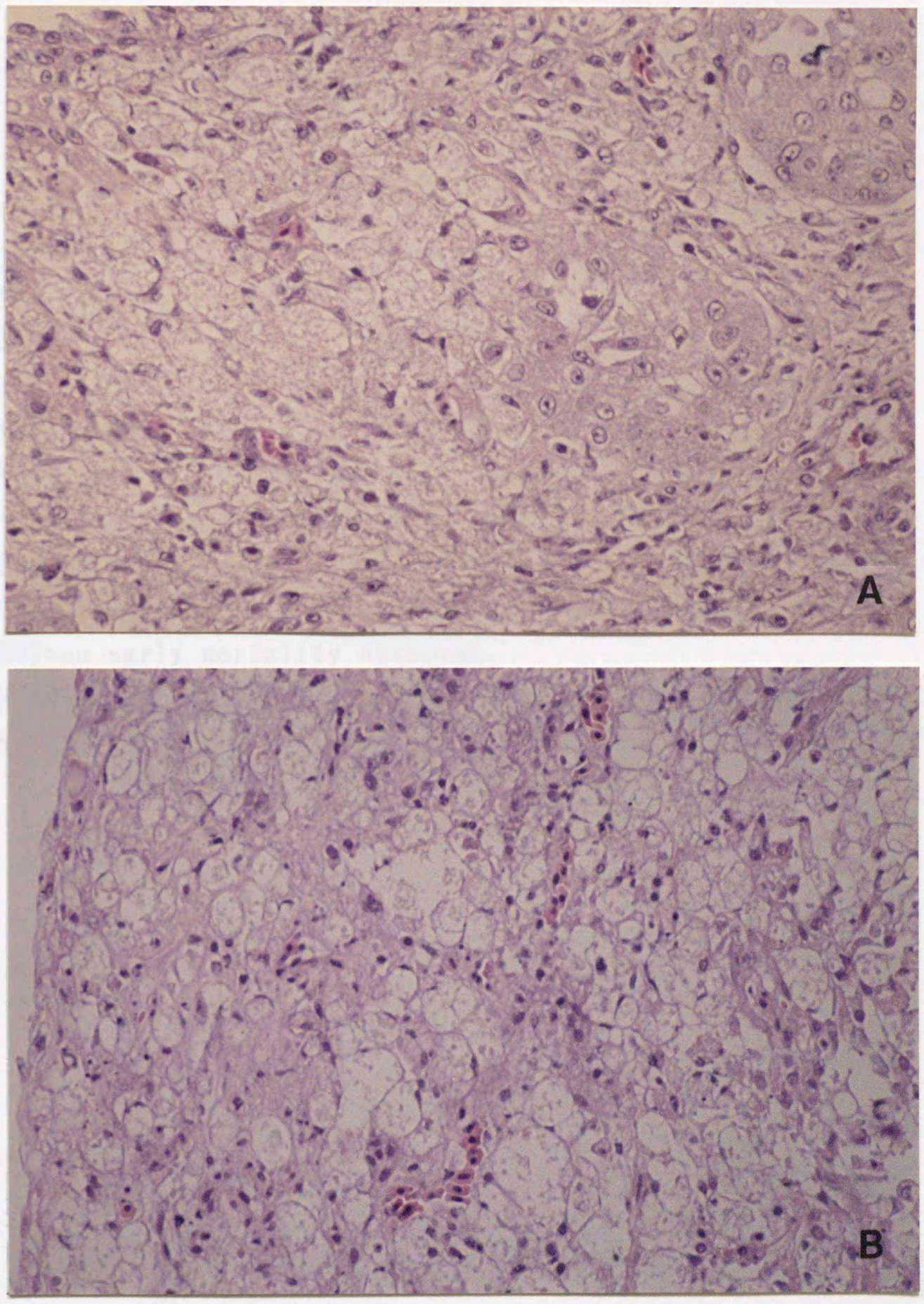

Fig. 3. Atrophy of lymphoid organs of a chicken 11 days after inoculation with the MS2 strain of MDV. A) Bursa showing severe depletion of lymphocytes. B) Thymus of the same chicken showing severe depletion of lymphocytes. Hematoxylin and eosin.

Table 4. Virulence of MDV strains in P-2 chickens[a]

Strain	Early death from EMS[b] (%)	MD lymphoma responses		
		Mortality (%)	MTD[c]	Total gross lesions (%)
MS1	2/16[d](13)	11/14 (79)	44	14/14 (100)
MS2	3/15 (20)	12/12 (100)	30	12/12 (100)
Md/5	3/15 (20)	12/12 (100)	35	12/12 (100)
GA	0/14 (0)	14/14 (100)	36	14/14 (100)
JM	0/14 (0)	12/14 (86)	39	14/14 (100)

[a]One-day-old chickens were intra-abdominally inoculated with 2,000 PFU of MDV per chicken and observed for 60 dpi.

[b]EMS=an early mortality syndrome.

[c]MTD=median time to death (days).

[d]Number of positive birds/total no. examined.

Table 5. Virulence of MDV strains in PDL-1 chickens[a]

Strain	Early death from EMS[b] (%)	MD lymphoma responses		
		Mortality (%)	MTD[c]	Total gross lesions (%)
MS1	0/17[d](0)	12/17 (71)	39	16/17 (94)
MS2	0/16 (0)	16/16 (100)	28	16/16 (100)
Md/5	0/16 (0)	15/16 (94)	43	16/16 (100)
GA	0/16 (0)	6/16 (38)	48	12/16 (100)
JM	0/15 (0)	14/15 (93)	32	14/15 (100)

[a]One-day-old chickens were intra-abdominally inoculated with 2,000 PFU of MDV per chicken and observed for 60 dpi.

[b]EMS=an early mortality syndrome.

[c]MTD=median time to death (days).

[d]Number of positive birds/total no. examined.

Table 6. Distribution of MD gross lesions[a]

Strain	Chicken line	% distribution of MD gross lesions								
		Liver	Kidney	Spleen	Provt.	Heart	Gonad	Lung	Total viscera	Nerves
MS1	P-2	42	53	7	14	21	7	7	64	92
	PDL-1	53	20	6	6	6	6	20	73	64
MS2	P-2	26	13	25	25	35	25	0	100	83
	PDL-1	62	43	12	43	50	6	0	94	81
Md/5	P-2	17	33	41	0	66	0	0	92	50
	PDL-1	75	43	31	0	37	0	0	100	43
GA	P-2	64	71	42	7	35	28	0	100	50
	PDL-1	31	12	18	16	12	6	6	67	31
JM	P-2	13	6	13	0	0	2	0	40	100
	PDL-1	14	0	0	0	0	0	0	14	100

[a]Data were obtained from Tables 5 and 6. Chickens which survived the early mortality syndrome were examined.

incidence of gross lesions in peripheral nerves to JM (Table 6). The incidence of gross lesions in nerves caused by MS1 was higher than that of gross lesions in visceral organs in P-2 chickens; however, visceral lymphomas induced by MS1 were much more widespread than those by JM.

Protection tests of chickens against MDV infection

To begin with, the protective efficacy of HVT against a reference of vvMDV strain, RB-1B in PDL-1 chickens and P-2 chickens was examined (Table 7). HVT provided adequate protection against challenge with RB-1B in PDL-1 chickens, but not in P-2 chickens. From this result, it proved that both PDL-1 and P-2 chickens were available for differentiation of vvMDVs from vMDVs.

Since three strains (MS1, MS2 and QM3) were highly pathogenic in unvaccinated chickens, the protective efficacy of HVT vaccine against challenge with these strains was examined in P-2 chickens (Table 8). Vaccination with HVT was not effective against challenge with MS1 or MS2, whereas this vaccine afforded good protection against challenge with QM3. HVT vaccine effectively prevented vaccinated chickens from EMS, although EMS occurred in 11 and 20 % of unvaccinated chickens challenged with MS1 and MS2, respectively.

Next, the protective efficacy of different type vaccines against challenge with MS1 or MS2 were further examined in both PDL-1 and P-2 chickens (Table 9). HVT vaccine afforded poor protection against challenge with MS1 or MS2 in P-2 chickens, whereas this vaccine was effective in protecting against challenge with GA. MS2 induced the higher incidence of MD (73%) than did MS1 (45%) in HVT-vaccinated P-2 chickens. CVI988 vaccine and a bivalent vaccine composed of HVT (serotype 3) and MDV (serotype 2) gave good protection against challenge with MS1 or MS2 in P-2 chickens.

Table 7. Protective efficacy of HVT vaccine against challenge with RB-1B of MDV[a]

Chicken line	Vaccine	Mortality+ gross lesions (%)		Protective index
PDL-1	None	36/37[b]	(97)	
	HVT	4/33	(12) *	87
P-2	None	38/38	(100)	
	HVT	15/37	(41) *	59

[a]One-day-old chickens were subcutaneously injected with 2,000 PFU of vaccine per bird, and challenged intra-abdominally with 5,000 PFU of RB-1B strain after 10 days.

[b]Number of positive bird/total no. examined.

* $P<0.05$.

Table 8. Protective efficacy of HVT vaccine against challenge with MS1, MS2 or QM3 of MDV[a]

Challenge virus	Vaccine	Mortality+ gross lesions (%)		Protective index
MS1	None	17/17[b] (100)	*	
	HVT	9/16 (56)		44
MS2	None	20/20 (100)		
	HVT	13/16 (81)		19
QM3	None	16/16 (100)	*	
	HVT	3/17 (18)		82

[a]One-day-old P-2 chickens were subcutaneously injected with 2,000 PFU of vaccine per bird, and challenged intra-abdominally with 5,000 PFU of MDV after 7 days.

[b]Number of positive bird/total no. examined.

* $P<0.05$.

Table 9. Protective efficacy of vaccines of different types against challenge with MS1, MS2 or GA of MDV[a]

Trial	Challenge virus	Chicken line	Vaccine	Mortality+ gross lesions (%)		Protective index	
1	MS1	PDL-1	None	18/18[b] (100)	**		
			HVT	2/15 (13)		87	
		P-2	None	20/20 (100)	** ** **		
			HVT	10/22 (45)		55	**
			Bivalent	5/22 (23)	**	77	
			CVI988	2/21 (10)		90	
	GA	P-2	None	19/19 (100)	**		
			HVT	1/21 (5)		95	
2	MS2	PDL-1	None	15/15 (100)	**		
			HVT	4/20 (20)		80	
		P-2	None	20/20 (100)	* **		
			HVT	16/22 (73)		27	**
			CVI988	2/20 (10)	**	90	
	GA	P-2	None	15/18 (83)	**		
			HVT	1/17 (6)		94	

[a]One-day-old chickens were subcutaneously injected with 2,000 PFU of vaccine per bird, and challenged intra-abdominally with 5,000 PFU (Trial 1) and 2,000 PFU (Trial 2) of MDV after 10 days.

[b]Number of positive bird/total no. examined.

* $P<0.05$, ** $P<0.01$.

Differences in protective efficacy between HVT and CVI988 vaccines were significant ($P<0.05$). On the other hand, HVT gave good protection against challenge with MS1 or MS2 in PDL-1 chickens. All vaccines effectively prevented vaccinated chickens challenged with MS1 or MS2 from EMS .

Assay for other known viruses

No CAA, REV and ALV subgroup B were isolated from the lysates of CEF infected with MS1 or MS2. However, ALV subgroup A was detected only in CEF infected with MS1 by the RIF test.

No antibodies to CAA, REV, ALV subgroup B and IBDV were detectable in sera of the chickens inoculated with these isolates, whereas antibodies to ALV subgroup A were also present in sera of chickens inoculated with MS1.

Although CKC and CEF inoculated with the lysates of CEF infected with MS1 or MS2 were observed microscopically for 14 dpi, no cytopathic changes were detected.

DISCUSSION

In the present studies, a total of 17 MDV strains was isolated from field flocks of chickens and Japanese quails and classified as serotype 1 vMDV. Virulence of these strains varied, which confirmed previous findings that most chicken flocks were infected with MDV strains of varying virulence (Biggs, 1985), and similar tendency seemed to be exist in quail flocks since quail isolates also varied in virulence. In pathogenicity tests, it was demonstrated the existence of highly virulent strains, designated as MS1 and MS2, among the MDV strains isolated.

Witter (1989) proposed that the principal criteria for definition of vvMDVs was that vvMDV strains could induce MD lesions in HVT-vaccinated, susceptible chickens at a rate greater than prototype vMDV strains including JM or GA. Therefore, the Japanese strains (MS1 and MS2) should be classified as vvMDV on the basis of the proposed definition since they were more virulent than GA in HVT-vaccinated, susceptible P-2 chickens. This is a first description of vvMDV in Japan. It is generally believed that vvMDVs may contribute to excessive losses in HVT-vaccinated flocks since they were isolated more frequently from vaccinated flocks with excessive losses than vaccinated flocks without excessive losses (Witter, 1983). Therefore, the present results suggest that vvMDVs may cause the excessive losses in vaccinated flocks in Japan.

It has been reported that vvMDV strains seem to be more pathogenic than vMDV strains in unvaccinated chickens (Witter et al., 1980; Schat et al., 1982; Witter, 1983; Powell and Lombardini, 1986). In the present studies, however, there were no marked differences in virulence between the reference strains of vMDV used and the Japanese vvMDV strains. Also, vvMDVs have been reported to induce more visceral and fewer neural gross

lesions than did vMDVs (Eidson et al., 1981; Powell and Lombardini, 1986). In the present study, indeed, Md/5 of vvMDV caused the higher proportion of gross lesions in visceral organs than in nerves. However, the Japanese strains caused gross lesions in both visceral organs and nerves at similar rates. Since pathogenicity and distribution of gross lesions vary greately among different chicken strains, these characteristics may have only limited value for the identification of vvMDV strains.

Witter et al. (1980) reported that their vvMDV strains induced EMS. In the present study, the strains, MS1 and MS2 induced EMS. It was reported that another American (RB-1B) and Italian strains of vvMDV did not cause EMS (Schat et al., 1982; Powell and Lombardini, 1986). In the present study, however, RB-1B induced EMS. These discrepancies may be due to differences in experimental conditions like virus dose or chicken strains used.

Various strategies to improve the efficacy of HVT vaccine against vvMDVs, such as increasing the dose, delaying the age at challenge and using the different serotype vaccines in parent stock to avoid interference with HVT by homologous maternal antibody have been attempted; however, little effect was noted (Witter et al., 1980; Sharma and Witter, 1983). American groups showed that a bivalent vaccine composed of HVT and serotype 2 MDV afforded better protection against their strains of vvMDV than did HVT or serotype 2 MDV alone (Schat et al., 1982; Witter and Lee, 1984; Witter, 1987). They advocate that the bivalent vaccine should be used in situation where HVT vaccine does not provide adequate immunity, particularly in suspected cases where chickens are exposed to vvMDVs. Indeed, field trials with the commercial bivalent vaccine (HVT plus SB-1) had considerable success in the USA. Field trials showed that mortality in laying chickens receiving bivalent vaccines was reduced remarkably

compared with chickens vaccinated with HVT alone (Calnek et al., 1983). Similar reduction in MD condemnation of broiler flocks vaccinated with bivalent vaccines has been reported (Witter, 1984).

The enhanced protective effect obtained by combining two vaccine strains has been termed 'protective synergism' (Witter and Lee, 1984). The mechanism of this synergism is not yet understood. Since synergism is observed between HVT and various serotype 2 MDVs, it is generally thought to be probably a general property of serotype 3 and serotype 2 viruses (Witter, 1987). On the other hand, it has been reported that synergism was less obvious in vaccines containing mixtures of serotypes 1 and 3, or 1 and 2 than bivalent vaccines of serotypes 2 and 3 (Witter, 1987). However, another group found synergism in the bivalent vaccine containing attenuated serotype 1 (HPRS-16/att) and serotyp 3 viruses (Powell and Lombardini, 1986). These findings may show that effects of synergism are influenced by virus strains used. In the present studies, the author confirmed the results of American groups that the bivalent vaccine offered good protection against vvMDV challenge. The CVI988 vaccine was reported to offer better protection than HVT vaccine alone against vvMDV challenge (De Boer et al., 1986). The author indicated that the CVI988 vaccine was highly effective in protecting against Japanese vvMDV strains compared with HVT vaccine alone. Therefore, the present data obtained suggest that the use of CVI988 or bivalent vaccines may be also beneficial to prevent against MD, particularly in situation where HVT vaccine does not provide adequate protection against MD.

It was reported that HVT vaccine fully protected moderately or highly resistant chickens against challenge with vvMDV (RB-1B) (Schat et al., 1982). Therefore the enhancement of resistance to MD in commercial chickens can be a beneficial strategy for control of vvMDV-inducing diseases.

Susceptibility of PDL-1 chickens to MD has not been sufficiently clarified. In the present study, the incidence of EMS caused by MDV infection in P-2 chickens was apparently high as compared with that in PDL-1 chickens. In addition, HVT vaccine was effective in protecting against vvMDV strains in PDL-1 chickens. Genetically resistant chickens were known to be protected by vaccination to a greater extent than were susceptible ones (Spencer et al., 1974). From these findings, it seemed that PDL-1 chickens were less susceptible than P-2 chickens.

It is unknown whether vvMDVs existed originally in the field or whether they have newly emerged. Witter (1983) indicated that vvMDVs had recently become prevalent in the USA because none were present among 10 isolates before 1975. It is generally supported that vvMDVs have arisen by mutation although there is no direct evidence (Witter et al., 1980; Powell and Lombardini, 1986; Witter, 1989). It was unclear whether the Japanese strains of vvMDV arose from a single mutant strain from the USA or whether they independently arose by some selection pressure in Japan. Characteristics of the Japanese vvMDV strains appeared to be very similar to those of the strains in the USA. On the other hand, the author's strains appeared to be different from Australian strains which the bivalent vaccine failed to protect. Although Australian strains are temporarily classified as vvMDV, they may be a new pathotype in future by virtue of high pathogenicity in bivalent-vaccinated chickens, and we may need further vaccination strategies against such strains.

Immunosuppressive viruses such as CAA, IBDV and REV have been reported to interfere with the induction of MD vaccinal immunity (Giambrone et al., 1976; Otaki et al., 1988; Sharmer, 1984; Witter et al., 1979). In the present study, however, contamination of these viruses in the vvMDV strains was not demonstrated by the virological and serological tests.

Contamination of MS1 with ALV subgroup A was found. There has not been any evidence that dual infection of ALV subgroup A enhances MD lymphoma development in unvaccinated and vaccinalted chickens (Calnek, 1980). The author's preliminary study indicated that dual infection of subgroup A and vMDV did not enhance the MD incidence (data not shown). In the present study, since HVT vaccine provided good protection against challenge with MS1 in PDL-1 chickens, the presence of ALV subgroup A in MS1 did not appear to influence HVT vaccinal immunity.

It has been known that MD occurs in Japanese quails (Wight, 1963; Pradhan et al., 1985; Kobayashi et al., 1986); however, the etiology of the diseases has not been sufficiently elucidated. In the present study, MDVs were first isolated from affected quails in Japan. These MDVs caused MD in quails (data not shown). MDVs of quail origin exhibited the same characteristics as MDVs of chicken origin. Since the author failed to show the existence of vvMDVs in quail flocks, it remains unclear whether vvMDV strains are etiologically involved in excessive losses of quail flocks.

SUMMARY

Virulence of 17 MDV strains obtained from chickens and Japanese quails was examined in two genetically different lines of chickens, PDL-1 and P-2 chickens. These strains varied in virulence, and they were classified as vMDV (serotype 1 MDV) on the basis of reactivity with serotype 1-specific monoclonal antibody, the morphology of plaques on the cell cultures and oncogenicity. Among the strains examined, two strains (MS1 and MS2) from chickens and one (QM3) from Japanese quails were highly virulent in both chicken lines used. HVT vaccine did not provide good protection against the challenge with MS1 or MS2 in MD-susceptible P-2 chickens, whereas this vaccine was effective against the challenge with GA of vMDV. On the other hand, HVT vaccine was effective against the challenge with MS1 or MS2 in less susceptible PDL-1 chickens. These results indicate that MS1 and MS2 could be classified as vvMDV. This is the first isolation of vvMDV in Japan, suggesting that some of excessive MD losses in the vaccinated chicken flocks may be associated with such vvMDV strains. A bivalent vaccine composed of HVT and serotype 2 MDV, and CVI988 vaccine alone gave good protection against the challenge with the vvMDV strains in P-2 chickens. The use of these vaccines in the field may be beneficial in situation where HVT vaccine offers poor protection.

CHAPTER II Efficacy of Marek's Disease Vaccine Provided by Herpesvirus of Turkeys in Chickens Infected with Chicken Anemia Agent

INTRODUCTION

Vaccination of Marek's disease (MD) vaccine has been drastically reduced the economic losses from MD since the early 1970's. Vaccine provided by herpesvirus of turkeys (HVT) has been used most widely throughout the world, although several vaccine types have been developed. However, occasional failures of vaccines to provide expected protection levels against MD have been reported. Several possible factors are presumed to be involved in this problem; one of which is probably immunosuppression against the vaccine.

Chicken anemia agent (CAA) is a ubiquitous virus among chicken flocks and it produces a disease in young susceptible chickens characterized by early death, anemia associated with dysplasia of the bone marrow, and lymphoid depletion in the thymus and bursa of Fabricius (Yuasa et al., 1979; Taniguchi et al., 1982; Bülow et al., 1983; Yuasa et al., 1987; Bülow, 1988; Engström, 1988; McNulty et al., 1988; Vielitz and Landgraf, 1988; Chettle et al., 1989; Goodwin et al., 1989; McNulty et al., 1989; Rosenberger and Cloud, 1989; Firth and Imai, 1990; Lucio et al., 1990).

Since hematopoietic and lymphoid tissues in CAA-infected chickens are severely damaged, it is supposed that CAA infection results in deterioration of the immune systems in chickens.

The present chapter deals with the influence of CAA infection on the efficacy of HVT vaccine.

MATERIALS AND METHODS

Viruses

The FC-126 strain of HVT (Witter et al., 1970) which was provided by Dr. Burmester, Agriculture Research Service, the USA was propagated in chicken embryo fibroblast (CEF) cultures and used as MD vaccine. Commercial MD vaccines, CVI988 (Rispens et al., 1972) and HVT, were also used.

The Gifu-1 (G1) and A2 strains of CAA (Yuasa and Imai, 1986) were used. The inoculum, with a titer of $10^{6.5}TCID_{50}$/ml, was a centrifugal supernatant of the liver homogenate from the CAA inoculated chickens.

The JM (Sevoian et al., 1962), GA (Eidson and Schmittle, 1968), Md/5 (Witter et al., 1980) and SM15 strains of MDV were used as virulent MDVs for the challenge inoculation. Pathogenic characteristics of SM15 isolated in Japan were described in the previous chapter. Other strains of MDV were isolated in the USA. They were propagated in CEF cultures and used as inocula.

Viremia of HVT and MDV in the chickens was tested weekly by using chicken kidney cell (CKC) cultures. Heparinized blood samples (0.2 ml) collected from the chickens were inoculated onto CKC cultures for plaque assay in a humidified CO_2 incubator at 37 C for 7 days. The plaques produced by MDV and HVT were differentiated morphologically. Viremia of CAA was also tested by using MDCC-MSB1 cells according to the method of Yuasa (1983).

Chickens

Specific-pathogen-free chickens, PDL-1 (Furuta et al., 1980) and P-2 (Schat et al., 1981), maintained at the author's laboratory were used. The

chickens employed for the experiments were reared in isolators placed in rooms with a filtered air ventilation system.

Experimental designs

PDL-1 chickens (experiment 1) or P-2 chickens (experiment 2) were inoculated subcutaneously with 2,000 plaque forming units (PFU) of HVT at one day of age and intramuscularly with 0.1 ml of G1 of CAA at 4 days of age, and then challenged with 5,000 PFU of MDV at 8 days of age. In experiment 3, PDL-1 chickens were similarly inoculated with HVT and CAA except that the chickens were challenged with MDV at 18 days of age. The experimentally infected chickens, which died within 24 days after CAA inoculation with bone marrow dysplasia, were regarded as having died of CAA. The chickens which showed clinical signs after 3 weeks of age were autopsied to observe MD gross lesions. The chickens with MD gross lesions in visceral organs and/or peripheral nerves macroscopically were diagnosed as MD. In experiment 2, the P-2 chickens which died 15 to 21 days after MDV inoculation without MD lesions were thought to have suffered an early mortality by MDV (Witter et al., 1980).

In experiments 4 and 5, day-old chickens were inoculated with 10 doses of the commercial vaccine per chicken, and then A2 of CAA at one day of age (experiment 4) and at 3 days of age (experiment 5). Chickens were observed up to 3 weeks of age.

Protective index (PI) of HVT vaccine was calculated by the following formula.

PI= (% MD in unvaccinated, MDV-challenged controls - % MD in vaccinated, MDV-challenged controls) / % MD in unvaccinated, MDV-challenged controls x 100.

Statistics

The Tukey's t-analysis was used for the statistical analysis.

RESULTS

Influence of CAA infection on the efficacy of HVT vaccine

Efficacy of HVT vaccine against MD was examined in chickens inoculated with HVT at 1 day of age and CAA at 4 days of age, and then challenged with MDV at 8 days of age. In experiment 1, PDL-1 chickens were challenged with JM of MDV (Table 1). In experiment 2, P-2 chickens were challenged with SM15 of MDV (Table 2).

PI of HVT vaccine against MD was 93 in experiment 1 and 88 in experiment 2. On the other hand, PI in vaccinated chickens inoculated with CAA was 41 (experiment 1) and 46 (experiment 2), respectively. The efficacy of HVT vaccine was significantly depressed in chickens inoculated with CAA when compared with those not inoculated with CAA ($P<0.05$). However, even in chickens inoculated with CAA, HVT vaccine was still effective against MD since vaccinated chickens were more protected than unvaccinated ones, although the results were not statistically significant in experiment 1.

HVT vaccine was effective to prevent the early death caused by MDV infection (Table 2). CAA infection did not enhance the incidence of early death.

Efficacy of HVT vaccine in chickens challenged with MDV at 18 days of age

The efficacy of HVT vaccine was examined in PDL-1 chickens inoculated with HVT at 1 day of age and CAA at 4 days of age, and in those challenged with Md/5, GA or JM of MDV at 18 days of age (experiment 3). As shown in Table 3, HVT vaccine reduced MD even in chickens inoculated with CAA and challenged with any strain of MDV. The incidence of MD did not increase by inoculation with CAA. Depressed efficacy of HVT vaccine was not recognized even when chickens were challenged with a very virulent strain

Table 1. Efficacy of HVT vaccine in PDL-1 chickens inoculated with CAA and challenged with JM strain of MDV (Expt. 1)

Group	Inoculation Age in days 1	4	8	Mean[a] Ht value	Chickens[b] that died of anemia (%)	Clinical signs[c] MD gross lesions - (%)[e]	+ (%)	Protective[d] index	
1	HVT	CAA	MDV	18.7	3 (15)	3/17[f] (18)	8/17 (47)	*	41
2	HVT	-	MDV	34.0	0	0/18	1/18 (6)	ns	93
3	-	CAA	MDV	20.8	1 (10)	3/9 (33)	3/9 (33)	**	
4	-	-	MDV	33.2	0	0/10	8/10 (80)		
5	-	CAA	-	21.8	1 (10)	0/9	0/9		
6	-	-	-	34.4	0	0/9	0/9		

[a]Mean hematocrit value (Ht) of 10 to 20 chickens was measured at 14 days after inoculation with G1 strain of CAA.

[b]Chickens were observed up to 28 days of age.

[c]Chickens were observed between 29 to 51 days of age.

[d]Protective index against the incidence of MD gross lesions was compared with group 4.

[e]Chickens showed clinical signs without MD gross lesions.

[f]Number of positive birds/total no. examined.

* $P<0.05$, ** $P<0.01$.

Table 2. Efficacy of HVT vaccine in P-2 chickens inoculated with CAA and challenged with SM15 strain of MDV (Expt. 2)

Group	Inoculation Age in days 1	4	8	Mean[a] Ht value	Early[b] death by MDV (%)	MD gross lesions (%)	Total MD incidence (%)	Protective[c] index
1	HVT	CAA	MDV	11.8	0/20[d]	10/20 (50)	10/20 (50)	46
2	HVT	-	MDV	31.6	0/19	2/19 (11)	2/19 (11)	88
3	-	CAA	MDV	22.5	7/19 (37)	11/12 (92)	18/19 (95)	
4	-	-	MDV	27.1	4/17 (24)	12/13 (92)	16/17 (94)	
5	-	CAA	-	27.9	0/8	0/8	0/8	
6	-	-	-	31.9	0/9	0/9	0/9	

[a]Mean hematocrit value (Ht) was measured at 13 days after inoculation with G1 strain of CAA.

[b]Chickens died without MD gross lesions and anemia at 15 to 21 days of age.

[c]Protective index against the total incidence of MD was compared with group 4.

[d]Number of positive birds/total no. examined.

* $P<0.05$, ** $P<0.01$.

Table 3. Efficacy of HVT vaccine in PDL-1 chickens inoculated with CAA at 4 days of age and challenged with various strains of MDV at 18 days of age (Expt. 3)

Group	Inoculation Age in days 1	4	18	No. of chickens observed	No. of[a] anemic chickens (%)	Chickens that died of anemia (%)	Incidence[b] of MD (%)	Protective[c] index
1	HVT	CAA	MDV(Md/5)	13	12 (92)	2 (15)	1 (9)	89
2	-	CAA	MDV(Md/5)	11	8 (73)	0	9 (82)	
3	-	-	MDV(Md/5)	12	0	0	10 (83)	
4	HVT	CAA	MDV(JM)	14	11 (79)	5 (36)	0	100
5	-	CAA	MDV(JM)	12	9 (75)	1 (8)	7 (64)	
6	-	-	MDV(JM)	12	0	0	7 (58)	
7	HVT	CAA	MDV(GA)	11	10 (91)	0	0	100
8	-	CAA	MDV(GA)	12	10 (83)	0	2 (17)	
9	-	-	MDV(GA)	12	0	0	1 (8)	
10	-	-	-	12	0	0	0	

[a]Chickens had a hematocrit value below 27 % at 12 days after inoculation with G1 strain of CAA.

[b]Chickens were observed up to 13 weeks of age.

[c]Protective index against MDV-inoculated group.

of MDV (Md/5).

Detection of viremia of HVT, CAA and MDV from infected chickens

The recovery of HVT and MDV from peripheral blood, and CAA from serum was carried out in chickens inoculated with HVT at 1 day of age, CAA at 4 days of age and MDV at 8 days of age (Table 4). No significant difference in titers of recovered HVT and MDV between group 1 and group 2 was observed. The titer of recovered MDV in groups 1 and 2 was lower than that of recovered MDV in group 3 when chickens were examined at 2, 3 and 5 weeks old. In chickens of group 3, the titre of recovered MDV markedly decreased at 6 weeks old as compared with that of recovered MDV before 5 weeks old. CAA was detectable in serum of chickens inoculated with CAA and examined at 1 week of age.

Virulence of CAA in chickens inoculated with MD vaccines

The occurrence of anemia in chickens dually inoculated with HVT or CVI988 vaccine and CAA was examined. As shown in Table 5, mortality of chickens caused by CAA infection was significantly higher in dually inoculated chickens than in those inoculated with CAA alone. MD vaccines enhanced the pathogenicity of CAA.

Table 4. Recovery of HVT and MDV from blood of chickens inoculated with HVT, CAA and JM of MDV

	Inoculation			Mean PFU/ml of blood from 3 chickens											
	Age in days			Age in weeks											
				1		2		3		4		5		6	
Group	1	4	8	HVT	MDV	HVT	MDV	HVT	MDV	HVT	MDV	HVT	MDV	HVT	MDV
1	HVT	CAA	MDV	33	0	3	5	180	304	45	179	0	410	5	910
2	HVT	-	MDV	5	0	144	24	23	59	24	557	3	15	9	65
3	-	-	MDV	nd[a]	nd	0	1725	0	2598	0	435	0	6062	0	1

[a]nd=not done.

Table 5. Incidence of anemia in chickens inoculated with MD vaccines and CAA

Expt.	Chicken lines used	Group	Inoculation vaccine[a]	Inoculation CAA[b]	No. of chickens inoculated	No. of chickens that died of anemia (%)		Mean days to death
4	PDL-1	1	HVT	+	19	18 (95)	**	15.2
		2	HVT	-	22	0		
		3	CVI988	+	21	20 (95)	**	16.8
		4	CVI988	-	22	0		
		5	-	+	19	9 (47)		17.9
5	P-2	1	HVT	+	19	17 (89)	**	15.3
		2	HVT	-	20	0		
		3	CVI988	+	19	17 (89)	**	14.4
		4	CVI988	-	20	0		
		5	-	+	20	9 (45)		17.0

[a]Ten doses of commercial MD vaccine were inoculated into a day-old chicken.

[b]The A2 strain of CAA was inoculated orally at 1 day of age in expt.4 and 3 days of age in expt. 5. Chickens were observed up to 3 weeks of age.

** $P<0.01$.

DISCUSSION

It has been reported that several viral infections may influence the efficacy of MD vaccination. Chickens exposed naturally from the time of hatching to infectious bursal disease virus (IBDV) were not as well protected by HVT vaccine as chickens not exposed to IBDV against challenge with virulent MDV at 2 weeks of age (Giambrone et al., 1976). However, Sharma (1984) reported that IBDV interfered with vaccinal immunity only when virulent IBDV and HVT vaccine were inoculated at hatch to chickens without maternal antibody to IBDV and challenged at 7 days of age with vMDV. Thus, IBDV infection may rarely cause problems with MD vaccine in the field. Contamination of MD vaccines with reticuloendotheliosis virus has been shown to interfere with protection by HVT vaccine against challenge with MDV (Witter et al., 1979). However, since vaccine productions are now well controlled, such contamination does not occur at present.

Depressed effect of HVT vaccine in CAA-infected chickens was proved experimentally in the present experiment, although the mechanisms of immunodepression by CAA infection have not been clarified as yet. Such immunodepression was observed irrespective of MD-susceptibility of chicken lines used. Otaki et al. (1988) described the enhancement of MD pathogenicity in unvaccinated chickens by CAA infection. In the present experiment, however, the CAA infection did not cause any increase of MD in unvaccinated chickens. It was reported that the titers of vaccine virus in chickens was correlated with the development of vaccinal immunity (Okazaki et al., 1973; Cho et al., 1976; Riddell et al., 1978). In the present experiment, the titers of HVT in chickens were not influenced by CAA infection. On the other hand, CAA infection caused damage to the lymphoid organs, and these pathological changes were enhanced by dual infection with

HVT and CAA. It is thought that lymphoid damage probably induces cellular and humoral immunodeficiency in chickens.

It is known that HVT vaccine lowers MDV viremia level. In the present study, this effect of HVT vaccine on MDV proliferation appeared not to be influenced by CAA infection. The titer of MDV recovered from MDV-inoculated chickens at 6 weeks old remarkably decreased. The reasons of the reduction of MDV titer were unclear.

Recently, Otaki et al. (1988) reported the depression of HVT-vaccinal immunity to MD by CAA infection and they suggested that the depressive effect may be due to a severe impairment of T cell-mediated immunity. Further studies should be necessary to clarify the mechanisms of immunodepression by CAA infection.

HVT vaccinal immunity against MD was depressed by CAA infection when chickens were inoculated with CAA at 1 to 14 days of age and challenged with MDV at 8 days of age (Otaki et al., 1988). In the present study, immunodepression of HVT by CAA infection was found only in the chickens inoculated with CAA at 4 days of age and challenged with MDV at 8 days of age. Otaki et al. (1988) reported that the response of splenic lymphocytes to phytohemagglutinin stimulation was depressed in chicks dually inoculated with HVT and CAA at hatching when the chicks were examined at 13 days old but not 21 days old, suggesting that the depressive effect of CAA infection on vaccinal immunity was transient. In the present study, when MDV challenge was delayed to 18 days of age, HVT vaccine provided good protection against MD. Young susceptible chickens are known to be prevented from CAA-induced diseases by maternal antibody. Therefore, although CAA may be responsible for some of the problems that occur with MD vaccination, the incidence of such problems associated with CAA infection is thought to occur under limited conditions in the field.

Various pathological changes in the experimental chickens were observed depending on the chicken lines and MDV used. The early death caused by MDV infection was observed only when P-2 chickens were challenged with SM15 of MDV. Bülow et al. (1983) and Otaki et al. (1987) discussed the involvement of CAA infection in the early mortality syndrome by MDV (Witter et al., 1980). In the present experiment, the incidence of early death by MDV was not larger in chickens inoculated with both CAA and MDV than in those inoculated with MDV alone. Furthermore, no early death occurred even by dual inoculation with CAA and MDV when PDL-1 chickens were used and challenged with JM of MDV. It was reported that MDV infection enhanced the virulence of CAA and induced severe lymphoid damages such as those observed in chickens with the early mortality syndrome by MDV when MDV and CAA were inoculated simultaneously into chickens (Bülow et al., 1983; Otaki et al., 1987); however, such pathological changes may have been produced mainly by CAA but not MDV.

The PDL-1 chickens which were inoculated with HVT, CAA and JM of MDV and showed clinical signs including depression and emaciation were autopsied (experiment 1). Some of the chickens had no specific MD gross lesions or CAA lesions. The pathological changes of these chickens were not examined microscopically but were speculated to have been caused by concurrent infections with these three viruses. Because these abnormalities were not observed in chickens inoculated with CAA or MDV alone. These clinical manifestations produced by concurrent infection probably cause confusion in the diagnosis of the diseases in the field.

SUMMARY

Influence of CAA infection on the efficacy of MD vaccine, HVT, was examined. Significant depression of the efficacy of HVT vaccine by CAA infection was observed. Protective index against MD in chickens inoculated with HVT at 1 day, and CAA at 4 days, and challenged with MDV at 8 days of age was 41 to 46, whereas that in vaccinated but not CAA-infected chickens was 88 to 93. HVT vaccine showed good protection against MD in chickens dually infected with HVT and CAA, and challenged with MDV at 18 days of age but not at 8 days of age. No interference with HVT viremia was observed in CAA-infected chickens. CAA did not enhance the virulence of MDV. Thus, CAA infection may be involved in MD vaccine breaks.

CHAPTER III Virulence of Three Strains of Marek's Disease Vaccine for Chickens

INTRODUCTION

Marek's disease (MD) have been well controlled to a great extent by vaccination since the early 1970s. Herpesvirus of turkeys (HVT) vaccine (serotype 3) has been most widely used because relative large amounts of infectious virus are obtained to produce vaccines, and both cell-associated and cell-free vaccines are available. In addition to HVT vaccine, CVI988 (serotype 1) vaccine and a bivalent vaccine composed of HVT and SB-1 (serotype 2) are also used. However, excessive losses of MD in vaccinated flocks occasionally occurs.

Bülow (1977) described that CVI988 vaccine virus caused paralysis and nerve lesions in genetically MD-susceptible Rhode Island Red (RIR) chickens. It was also described that CVI988 and some clone viruses derived from this strain, and HVT produced similar signs and lesions in RIR chickens (Pol et al., 1986). Pol et al. (1985) reported that HPRS-24 which belonged to serotype 2 caused endoneural and visceral lymphomas in susceptible chickens. Thus, vaccine viruses may be pathogenic under certain circumstances. Thus, in this chapter, the virulence of commercial MD vaccines (serotypes 1 to 3) was tested.

MATERIALS AND METHODS

Chickens

Specific-pathogen-free chickens were obtained from flocks of PDL-1 (Furuta et al., 1980), P-2 (Schat et al., 1981) and Line 15I (Hihara et al., 1980) maintained at the author's laboratory. The chickens used in the experiments were reared in isolators placed in rooms with a filtered air ventilation system.

Viruses

The CVI988 strain of MDV (Rispens et al., 1972) was provided by Dr. I. Yoshida, Central Laboratory of Kyoritsu Shyoji, Ibaraki. The SB-1 strain of MDV (Schat and Calnek, 1978) was provided by Dr. Y. Sekiya, Ghen Corporation, Tochigi. These viruses were passaged twice in chicken embryo fibroblast (CEF) cultures in the author's laboratory. The FC-126 strain of HVT (Witter et al., 1970) which was provided by Dr. Burmester, Agriculture Research Station, USA, was passaged eight times in chicken kidney cell cultures and five times in CEF cultures, respectively.

Experimental designs

In experiment 1, four groups of P-2 chickens (21 to 25 chickens per group) were intramuscularly inoculated with 10,000 plaque forming units (PFU) of each vaccine strain or uninfected CEF per chicken at one day of age and observed for 8 weeks post-inoculation. Five chickens from each inoculated group were killed 13 days post-inoculation and examined for weights of body, bursa of Fabricius, thymus and spleen to know the influence of infection in the lymphoid organs. Relative organ weight was expressed as the organ weight divided by body weight times 100.

In experiment 2, three genetically different lines of chickens (PDL-1, P-2 and Line 15I) were inoculated 10,000 PFU of CVI988 per chicken at one day of age and observed for 8 weeks post-inoculation. The number of chickens used is shown in Table 3.

Histology

Chickens showing clinical signs were necropsied. The collected tissues were fixed in 10 % buffered formalin, embedded in paraffin, cut and stained with hematoxylin and eosin.

RESULTS

Virulence of three different serotypes of MD vaccines for chickens

In experiment 1, virulence of three different serotypes of MD vaccine strains (CVI988, SB-1 and FC-126) for P-2 chickens was examined. As shown in Table 1, no chickens inoculated with each vaccine strain showed any clinical signs (paralysis) and MD gross lesions during a 8-week period of observation.

As shown in Table 2, chickens inoculated with each vaccine strain were examined for body weights, and relative weight of bursa, thymus and spleen to know the effect of infection in the lymphoid organs. Significantly lower weights of bursa in chickens inoculated with CVI988 were observed as compared with that in control chickens inoculated with uninfected CEF. Slight splenomegaly was observed in vaccinated chickens; there were significant differences between chickens vaccinated with SB-1 and control chickens.

Susceptibility of three genetically different lines of chickens to CVI988 vaccine strain

In experiment 2, susceptibility of three lines of chickens (PDL-1, P-2 and Line 15I) to CVI988 strain was examined. As shown in Table 3, one of 24 PDL-1 chickens inoculated with CVI988 showed leg paralysis 36 days post-inoculation. Slight enlargement of sacral plexus was observed macroscopically in this chicken; however, no gross lesions were noted in other organs and tissues. Lymphoproliferative infiltrations were observed microscopically in peripheral nerves such as ischiatic, brachial and celiac nerves including sacral plexus. In such nerves, infiltration of large and

small lymphoid cells among nerve fibers, and swelling of Schwann's cells were observed (Fig. 1). From these findings, the chicken showing paralysis was diagnosed as MD. On the other hand, no clinical signs were observed in P-2 or Line 15I chickens.

Table 1. Virulence of three MD vaccine strains in P-2 chickens[a]

Group	Virus	Incidence of MD	
		Observation period	
		up to 5 weeks of age	5-8 weeks of age
1	CVI988	0/21[b]	0/16
2	SB-1	0/25	0/16
3	HVT	0/25	0/16
4	None (CEF)	0/22	0/16

[a]One-day-old chickens were intramuscularly inoculated with 10,000 PFU of each virus or uninfected CEF per chicken.

[b]Number of positive birds/total no. examined.

Table 2. Effect of infection of three MD vaccine strains on weights of body and lymphoid organs[a]

Group	Virus	Body weight	Relative organ weight (g)[b] Thymus	Bursa	spleen	
1	CVI988	85.5±7.0	0.248±0.06	0.238±0.02	0.152±0.01	
2	SB-1	105.6±2.3	0.268±0.03	0.352±0.06	0.166±0.03	
3	HVT	95.1±12.8	0.439±0.09	0.430±0.05	0.156±0.03	*
4	None (CEF)	96.3±6.9	0.366±0.09	0.444±0.09	0.112±0.03	

(** between groups 1 and 2 vs. groups 3 and 4)

[a]One-day-old P-2 chickens were intramuscularly inoculated with 10,000 PFU of each virus or uninfected CEF per chicken.

[b]Weights of body and lymphoid organs were measured at 13 days of age. Data are means (±SD) of 5 chickens per each group.

* $P<0.05$, ** $P<0.01$.

Table 3. Virulence of CVI988 of MDV in three lines of chickens[a]

Group	Chicken lines	Incidence of MD	
		Observation period	
		up to 5 weeks of age	5-8 weeks of age
1	P-2	0/25[b]	0/15
2	15I	0/24	0/19
3	PDL-1	1/24	0/23
Total		1/73	0/57

[a]One-day-old chickens were intramuscularly inoculated with 10,000 PFU of virus per chicken.

[b]Number of positive birds/total no. examined.

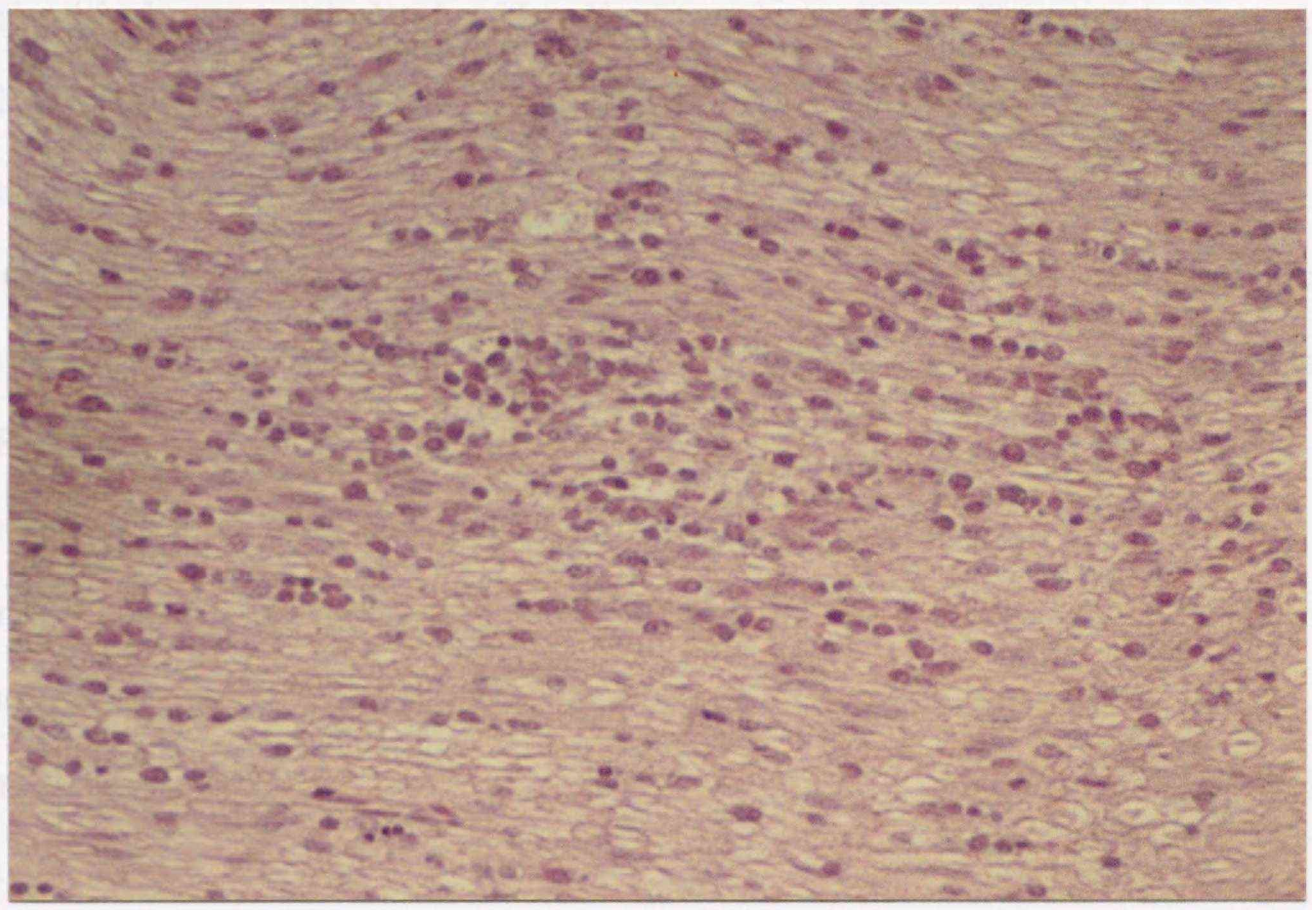

Fig. 1. A sacral plexus of a chicken 36 days after inoculation with the CVI988 strain of MDV. Infiltration of lymphoid cells among nerve fibers and swelling of Schwann's cells are observed. Hematoxylin and eosin.

DISCUSSION

One of 24 PDL-1 chickens (4.1%) inoculated with CVI988 showed paralysis and this bird was pathologically diagnosed as MD. This result confirmed that CVI988 possesses a potential inducing MD lesions in peripheral nerves as previously described by other workers (Bülow, 1977; Pol et al., 1986). However, it was unexpected that MD was observed in PDL-1 chickens inoculated with CVI988 since these chickens appeared to be less susceptible than P-2 chickens as described in Chapter I. It was unclear why CVI988 induced MD in less-susceptible PDL-1 chickens.

Bülow (1977) reported that CVI988 induced MD symptoms (paralysis) and lesions in up to 28 % of the inoculated RIR chickens. Pol et al. (1986) also reported that paralysis was observed in 88 % of RIR chickens inoculated with the same vaccine strain, and HVT FC-126 caused it in two of the 39 inoculated RIR chickens, indicating that RIR chickens were extremely susceptible to MD. In the present study, however, CVI988 did not induce any MD lesions in P-2 chickens. Therefore, these results may reflect differences of susceptibility between RIR and P-2 chickens.

On the other hand, the other two vaccine strains (SB-1 and FC-126) used did not produce any MD gross lesions and clinical signs in three lines of chickens used.

Although weights of lymphoid organs were examined in chickens inoculated with three vaccine strains to know the extent of damages to the lymphoid organs, there were no obvious differences among the vaccine strains.

It is known that marked differences of susceptibility to MD are observed within and among chicken strains (Calnek, 1985). The occurrence of paralysis based on CVI988 vaccination was suspected in some commercial

chicken flocks (personal communications). From the present and other workers' results, it is suggested possibility that the virulence of vaccine strain itself may be involved in MD vaccine breaks under limited conditions, since live vaccine used is not completely attenuated yet.

SUMMARY

Virulence of three different serotypes of vaccine strains, CVI988 of MDV (serotype 1), SB-1 of MDV (serotype 2) and FC-126 of HVT (serotype 3), was investigated in three genetically different lines of chickens (PDL-1, P-2 and Line 15I). No clinical signs were observed in MD-susceptible P-2 chickens inoculated with three vaccine strains during a 8-week period of observation. The CVI988 strain caused paralysis in one of the 24 PDL-1 chickens which was pathologically diagnosed as MD. Since CVI988 strain had a potential to produce MD lesions in peripheral nerves, it suggests that virulence of vaccine virus itself may be involved in MD vaccine breaks.

CONCLUSION

Unacceptably excessive MD losses, commonly termed vaccine breaks, are occasionally observed among vaccinated flocks. Studies are needed to make an inquiry into the causes of these problems and establish the preventive measures against them. Then this thesis was carried out to examine the possible causes of MD vaccine breaks etiologically and includes the following studies on 1) Virulence of MDVs isolated from chicken and quail flocks, especially characterization of vvMDV isolates. 2) Effect of vaccines against challenge with vvMDV isolates. 3) The influence of CAA infection on HVT vaccinal immunity. 4) Reevaluation of virulence of current vaccine strains.

The results obtained are summarized as follows.

1. A total of 13 MDV-like viruses was isolated from MD-affected chickens in vaccinated and unvaccinated flocks. Four viruses were also isolated from Japanese quails in vaccinated and unvaccinated flocks. This is the first isolation of MDV from quails. All isolates were identified as virulent MDV (serotype 1) on the basis of reactivity with serotype 1-specific monoclonal antibodies, the morphology of plaques on CEF cultures and oncogenicity in chickens.

2. Virulence of the MDV isolates in genetically different lines of chickens, PDL-1 and MD-susceptible P-2, varied. The mortality rate of the isolates ranged from 0 to 100%. Among all isolates examined, two isolates (MS1 and MS2) from chickens and one (QM3) from quails appeared to be highly virulent in both chicken lines, especially in P-2 chickens. HVT vaccine offered poor protection against challenge with chicken isolates (MS1 and MS2) in MD-susceptible P-2 chickens. This characteristic

was the same as that of vvMDV reported in America and Europe. However, HVT vaccine was well protective against MS1 or MS2 in PDL-1 chickens. CVI988 (serotype 1 MDV) vaccine and a bivalent vaccine composed of HVT and serotype 2 MDV offered better protection against challenge with MS1 or MS2 as compared with HVT vaccine alone.

3. CAA infection depressed HVT vaccinal immunity against MD when chickens vaccinated at one day of age were inoculated with CAA at 4 days of age, and then virulent MDV at 8 days of age. However, when chickens were challenged with MDV at 18 days of age, HVT vaccine was well protective against MD. The depression of vaccinal immunity caused by CAA infection was not influenced by susceptibility of chickens to MD. Virulence of CAA to chickens was enhanced by infection of MD vaccine strains. On the other hand, virulence of MDV was not enhanced by CAA infection. No interference with replication of HVT was observed in chickens infected with CAA.

4. Virulence of MD vaccines (CVI988, SB-1 and HVT) was tested and MD lesions could be observed in one of 24 PDL-1 chickens vaccinated with CVI988 but not other vaccines.

These results indicate the following: 1) The existence of vvMDVs, first demonstrated in Japan, suggests that some of excessive MD losses in HVT-vaccinated flocks may be associated with such vvMDVs. 2) The vaccines which are effective against challenge with vvMDVs may be beneficial in situation where HVT vaccine offers poor protection against MD. 3) CAA infection during the early stages of life may be involved in MD vaccine breaks. 4) It was suggested a possibility that virulence of vaccine virus itself may be involved in MD vaccine breaks under limited conditions.

ACKNOWLEDGEMENTS

The author is deeply indebted to Professor N. Hashimoto of the Faculty of Veterinary Medicine of Hokkaido University for his encouragement and critical review of the manuscript of this thesis; and to Professors C. Itakura and M. Onuma and Associate Professor I. Takashima of Hokkaido University for their critical review of the manuscript.

The author wishes to thank Dr. S. Matsuyama, Director General of the National Institute of Animal Health, and Dr. H. Hihara, Director of the Poultry Disease Laboratory of the National Institute of Animal Health for permission to perform this study.

The author wishes to thank Dr. M. Maeda, the National Institute of Animal Health for his guidance and help given during the course of this study.

The author is indebted to Dr. N. Yuasa, the National Institute of Animal Health for his invaluable discussions and constructive comments during the course of this study.

The author wishes to thank Drs. K. Nakamura and K. Tsukamoto, the Poultry Disease Laboratory of the National Institute of Aniaml Health for his assistance with the histopathological and serological examinations.

The author also wishes to thank Dr. K. Ohta, Nihon Pharmacy for his technical assistance.

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cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

Blue Cyan Green Yellow Red Magenta White 3/Color Black

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A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19